Electroporation based Technologies and Treatments
International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE
Ljubljana, Slovenia
November 17-23, 2013

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Students’ Abstract
Faculty members

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Tentative Topics:
Basics - biology of electroporation
Basics - modeling of electroporation including MD studies
Technology for PEF and large treatment capacities
Technology for electric pulses for research and medical applications
Medical applications (ECT, IRE, GET)
Food industry applications
Environmental applications
Micro- and Nanotechnologies (single cell electroporation, microfluidics, ...)

... we will all be there

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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. In ten years the Course has been attended by 378 participants coming from 28 different countries. And this year again we can say with great pleasure: “with participation of many of the world leading experts in the field”.

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.

It also needs to be emphasized that all written contributions collected in this proceeding have been peer-reviewed and then thoroughly edited by Peter Kramar. We thank all authors, reviewers and editors. 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, Centre National de la Recherche Scientifique (CNRS), University of Bielefeld, Technology transfer office and to the Bioelectrochemical Society. We also would like to thank Igea (Italy), Iskra Medical (Slovenia), Profector (Ireland), BIA Separations (Slovenia), Micro+Polo (Slovenia) and C3M (Slovenia), whose financial support allowed us to assist many students participating in this Workshop and Course by waiving their fee or providing them with accommodation. The BioElectroMagnetics Society (BEMS) and the European Bioelectromagnetics Association (EBEA) have also contributed with travel grants and with tuition fees partial coverage for students. The course is conducted in the scope of the LEA EBAM (European Associated Laboratory on the Pulsed Electric Fields Applications in Biology and Medicine). Finally it needs to be stressed that EBTT also became a training school of the COST TD1104 action which covers part of expenses and provides grants for students coming from COST countries.

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 Lluís M. Mir
COST - European Cooperation in Science and Technology is an intergovernmental framework aimed at facilitating the collaboration and networking of scientists and researchers at European level. It was established in 1971 by 19 member countries and currently includes 35 member countries across Europe, and Israel as a cooperating state.

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LECTURERS’ ABSTRACTS
Resting and Induced Transmembrane Voltage

Tadej Kotnik

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Abstract: Under physiological conditions, a resting voltage in the range of tens of millivolts is continually present on the cell plasma 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, a cell can roughly be described as an electrolyte (the cytoplasm) surrounded by an electrically insulating shell (the plasma membrane). Physiologically, the exterior of the cell also resemble an electrolyte. If a cell is exposed to an external electric field under such conditions, in its very vicinity the field 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. Transmembrane voltage can affect the functioning of voltage-gated membrane channels, initiate the action potentials, stimulate cardiac cells, and when sufficiently large, it also leads to cell membrane electroporation, with the porated membrane regions closely correlated with the regions of the highest induced transmembrane voltage [1].

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. 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 [2,3]. This voltage is caused by a

---

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 basic drawing programs on a computer.
minute deficit of positive ions in the cytoplasm 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 [4],

\[
\Delta \Phi_m = \frac{3}{2} ER \cos \theta ,
\]

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. Voltage 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 \(\theta = 0^\circ\) and \(\theta = 180^\circ\) (the “poles” of the cell), and in-between these poles it varies proportionally to the cosine of \(\theta\) (see Fig. 1, dashed).

The value of \(\Delta \Phi_m\) given by Eq. (1) is typically established several \(\mu\)s 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 [5],

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

where \(\tau_m\) is the time constant of membrane charging,

\[
\tau_m = \frac{R \varepsilon_m}{2d \frac{\sigma_i \sigma_e}{\sigma_i + 2\sigma_e} + R \sigma_m}
\]

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 Eqns. (1) and (2) decreases in value, as described in detail in [6]. 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 \(\mu\)s.
To determine the voltage induced by even higher field frequencies or even shorter pulses, the dielectric permittivities of the electrolytes on both sides of the membrane also have to be accounted for. This leads to a further generalization of Eqns. (2) and (3) to a second-order model [7-9], 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 [10-12]. Besides the fact that this solution is by itself somewhat 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. A more rigorous discussion of the validity of this approach can be found in [10]. 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.

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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.

---

Figure 1: Normalized steady-state $\Delta \Phi_m$ as a function of the polar angle $\theta$ 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 $\theta$ (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 $R_2 = 5 \times R_1$. 

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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 [11,12]. Figs. 3 and 4 show the effect of rotation of two different spheroids with respect to the direction of 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 COMSOL Multiphysics, the voltage induced on a given irregular cell can still be determined numerically, as described in detail in [13,14]. 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.

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 [15,16]. 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 $\Delta \phi_r(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by Eq. (1). 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 [7-9], 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 [17]. 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 [18-20].

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 curve the second-order Schwan's equation. Note that both axes are logarithmic.
REFERENCES


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Tadej Kotnik was born in Ljubljana, Slovenia, in 1972. He received a Ph.D. in Biophysics from University Paris XI and a Ph.D. in Electrical Engineering from the University of Ljubljana, both in 2000. He is currently the Vice-dean for Research at the Faculty of Electrical Engineering of the University of Ljubljana. His research interests include membrane electrodynamics, theoretical and experimental study of related biophysical phenomena, particularly membrane electroporation, as well as computational research in number theory.

Tadej Kotnik is the first author of 20 articles in SCI-ranked journals cited over 500 times to date excluding self-citations. In 2001 he received the Galvani Prize of the Bioelectrochemical Society.
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 inhomogeneity, dispersions, anisotropy, nonlinearity, as well as temperature dependence and changes over time. Furthermore, when biological tissue is exposed to a high electric field, changes in their 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 electroporation) [1]-[3].

BIOLICAL 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.

If a conductor is placed in an electric field, charges will move within the conductor until the resulting internal 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, the positive and negative charge centers in the molecules (e.g. water) 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 [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 resulting internal field inside the material, \( E \), is then

\[ E = E_0 - E_p \]

The resulting internal 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 \( \varepsilon_0 = 8.85 \times 10^{-12} \) 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:
Consider a sample of material which has a thickness, $d$, and cross-sectional area, $A$ (Figure 1).

Figure 1: A considered theoretical small part of a material.

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 ($G$):

$$C = \varepsilon \cdot \frac{A}{d}$$

$$G = \sigma \cdot \frac{A}{d}$$

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 $I_C$ 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:

$$I_d = \frac{dQ}{dt} = -\omega C V_0 \sin(\omega t)$$

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

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

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

$$Y^* = G + i\omega C = \left(\frac{A}{d}\right)(\sigma + i\omega \varepsilon)$$

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:

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

We can define a complex-valued, relative permittivity:

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

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

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

We can consider the conductivity of a material as a measure of the ability of its charge to be transported throughout its volume in a response to the applied electric field. Similarly, its permittivity is a measure of the ability of its dipoles to rotate or its charge to be stored in response to the applied 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, $\sigma^*$ and $\varepsilon^*$ are frequency-dependent. Such a variation is called dispersion and is due to the dielectric relaxation – the delay in molecular polarization following changing electric field in a material. Biological tissues exhibit several different dispersions over a wide range of frequencies [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 like cell membrane [5]. 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 travel 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 2. In short, alpha dispersion in the kilohertz range is due to cell membrane effects such as gated channels and ionic diffusion and is the first of the dispersions to disappear with tissue death. Beta dispersion can be observed around the megahertz range due to the capacitive charging of cell.
membranes. Above beta dispersion the impedance of cell membranes drops drastically, allowing the electric current to pass through not only extracellular, but also intracellular space. This dispersion is particularly interesting as it is also apparent in the conductivity of the material. The last, gamma dispersion (above the gigahertz range) is due to dipolar mechanisms of water molecules in the material.

![Figure 2: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.](image)

**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 nonconducting inclusions in a conducting fluid [6]. The insulation is provided by the cell membrane. At frequencies in the MHz range capacitive coupling across this membrane becomes more important, allowing the electric current to pass not only around the cell, but also through it. 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 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 significantly easier than conduction in the direction perpendicular to the fibers. 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, i.e. at beta dispersion.

**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 [7].

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 [7].

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 e.g. to monitor the progress of hyperthermia treatment. Also, possible other changes, such as cell swelling and edema, or blood flow occlusion, all affect tissue properties.
**Electrode polarization**

Electrode polarization is a manifestation of molecular charge organization which occurs at the tissue/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. Thus electrode polarization is more pronounced at lower frequencies.

The process is more complicated in tissue. Insertion of electrodes can first cause the release of electrolytes due to trauma 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 considered a 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 theoretical 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). We used the parallel RC circuit to model the electrical response of the tissue (see Figure 3).

![Figure 3](image-url): Parallel RC circuit: a theoretical representation of tissue response to electric pulses.

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 4. 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 dispersion is linear, change of the applied voltage proportionally scales the amplitude of the current.

We can compare this calculated response with the measured response on rat liver *in vivo* for the same pulse as above and different pulse amplitudes.
spanning up to electroporative field strengths (Figure 5) [8]. For the lowest applied voltage we can see a 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 [8], [9].

![Figure 4](image)

**Figure 4:** Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

![Figure 5](image)

**Figure 5:** Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver in vivo. Adapted from Cukjati et al. [8]. 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 [10]-[14]. It is not clear, however, to which value tissue conductivity increases as a consequence of plasma membrane electroporation. It has been stipulated that this could be close to the value in beta dispersion range [15].

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 permeabilizes 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 [16]. 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 cell electroporation. 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 cell electroporation. 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 cell electroporation.

In Figure 6 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 6) the tumor is also above the irreversible threshold.
Figure 6: 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 [17]. 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 [18]. Skin consists of three main layers: epidermis, dermis and subcutaneous tissue (Figure 7). 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 [19].

Figure 7: Schematics of a skinfold as described in a numerical model. Only one quarter of the skinfold is presented here.

Theoretical explanation of the process of electroporation offers useful insight into the understanding of the underlying biological processes and allows for predicting the outcome of the treatment [20], [21]. Therefore, due effort needs to be invested into measurements of tissue electrical properties and their changes during electroporation.

Further, one of the concerns associated with electroporation could be the amount of resistive heating in the tissue. Excessive heating is unwanted not only to avoid skin burns and assure patient safety, but also to avoid damage to viable cells. Potential excess of the resistive heating during electroporation has been demonstrated [22], therefore thermal aspect in treatment planning and theoretical analysis of specific applications of electroporation-based treatments should be considered. In order to stay within the safety limit while achieving successful treatment, heating needs to estimated, by means of theoretical models, as a part of treatment planning [23]-[25].

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Lipid Membranes Electroporation: Insights from Molecular Dynamics Simulations

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Abstract: We describe here the molecular dynamics simulation methods devised to perform in silico experiments of membranes subject to nanosecond, megavolt-per-meter pulsed electric fields and of membranes subject to charge imbalance, mimicking therefore the application of low voltage – long duration pulses. At the molecular level, the results show the two types of pulses produce similar effects: Provided the TM voltage these pulses create are higher than a certain threshold, hydrophilic pores stabilized by the membrane lipid head groups form within the nanosecond time scale across the lipid core. Similarly, when the pulses are switched off, the pores collapse (close) within similar time scales. It is shown that for similar TM voltages applied, both methods induce similar electric field distributions within the membrane core. The cascade of events following the application of the pulses, and taking place at the membrane, is a direct consequence of such an electric field distribution.

Electroporation disturbs transiently or permanently the integrity of cell membranes [1-3]. These membranes consist of an assembly of lipids, proteins and carbohydrates that self-organize into a thin barrier that separates the interior of cell compartments from the outside environment [4]. The main lipid constituents of natural membranes are phospholipids that arrange themselves into a two-layered sheet (a bilayer). Experimental evidence suggests that the effect of an applied external electric field to cells is to produce aqueous pores specifically in the lipid bilayer [5-9]. Information about the sequence of events describing the electroporation phenomenon can therefore be gathered from measurements of electrical currents through planar lipid bilayers along with characterization of molecular transport of molecules into (or out of) cells subjected to electric field pulses. It may be summarized as follows: The application of electrical pulses induces rearrangements of the membrane components (water and lipids) that ultimately lead to the formation of aqueous hydrophilic pores [5-10], whose presence increases substantially the ionic and molecular transport through the otherwise impermeable membranes [11].

In erythrocyte membranes, large pores could be observed using electron microscopy [12], but in general, the direct observation of the formation of nano-sized pores is not possible with conventional techniques. Furthermore, due to the complexity and heterogeneity of cell membranes, it is difficult to describe and characterize their electroporation in terms of atomically resolved processes. Atomistic simulations in general, and molecular dynamics (MD) simulations in particular, have proven to be effective for providing insights into both the structure and the dynamics of model lipid membrane systems in general [13-25]. Several MD simulations have recently been conducted in order to model the effect of electric field on membranes [26-30], providing perhaps the most complete molecular model of the electroporation process of lipid bilayers.

The effects of an electric field on a cell may be described considering the latter as a dielectric layer (cell surface membrane) embedded in conductive media (internal: cytoplasm and external: extracellular media). When relatively low-field pulses of microsecond or millisecond duration are applied to this cell (by placing for instance the cell between two electrodes and applying a constant voltage pulse) the resulting current causes accumulation of electrical charges at both sides of the cell membrane. The time required to charge the surface membrane is dependent upon the electrical parameters of the medium in which it is suspended. For a spherical cell it is estimated using equivalent network RC circuits in the 100 ns time scale [26, 31-34]. A charging time constant in the range of hundreds of nanoseconds was also obtained from derivations based on the Laplace equation (see e.g. [35] for the first-order analysis on a spherical vesicle; [36] for the second-order analysis; and [37] for the second-order analysis for two concentric spherical vesicles i.e. modeling an organelle).

If on the other hand, the pulse duration is short enough relative to the charging time constant of the resistive-capacitative network formed by the conductive intracellular and extracellular fluids and the cell membrane dielectric, which is the case for nanosecond pulses, then the electric field acts directly and mainly on the cell membrane.
Simulations allow ones to perform in silico experiments under both conditions, i.e. submitting the system either to nanosecond, megavolt-per-meter pulsed electric fields or to charge imbalance, mimicking therefore the application of low voltage – long duration pulses. In the following we will describe the results of such simulations, after a brief general introduction to MD simulations of membranes.

MD SIMULATIONS OF LIPID 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 [38, 39]. 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 cannot 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 [40] CHARMM [41] and AMBER [42], 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 [43-45] 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 [46, 47].

Traditionally, phospholipids have served as models for investigating in silico the structural and dynamical properties of membranes. From both a theoretical and an experimental perspective, zwitterionic phosphatidylcholine (PC) lipid bilayers constitute the best characterized systems [48-51] (Fig. 1). More recent studies have considered a variety of alternative lipids, featuring different, possibly charged, head groups [52-56], and more recently mixed bilayer compositions [57-63]. Despite their simplicity, bilayers built from PC lipids represent remarkable test systems to probe the computation methodology and to gain additional insight into the physical properties of membranes [14, 17, 20, 64].

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 [65], 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 $z = 0$.

**MODELING MEMBRANE ELECTROPORATION**

The effects of an electric field on a cell may be described considering the latter as a dielectric layer (cell surface membrane) embedded in conductive media (internal: cytoplasm and external: extracellular media). When relatively low-field pulses of microsecond or millisecond duration are applied to this cell (by placing for instance the cell between two electrodes and applying a constant voltage pulse) the resulting current causes accumulation of electrical charges at both sides of the cell membrane. The time required to charge the surface membrane is dependent upon the electrical parameters of the medium in which it is suspended. For a spherical cell it is estimated in the 100ns time scale [26, 31-34]. If the pulse duration is short enough relative to the charging time constant of the resistive-capacitive network formed by the conductive intracellular and extracellular fluids and the cell membrane dielectric, then the electric field acts directly and mainly on the cell membrane.

Simulations allow ones to perform in silico experiments under both conditions, i.e. submitting the system either to Nanosecond, megavolt-per-meter pulsed electric fields or to charge imbalance, mimicking therefore the application of low voltage – long duration pulses. In the following we will describe the results of such simulations.

**A- ELECTROPORATION INDUCED BY DIRECT EFFECT OF AN ELECTRIC FIELD**

In simulations, it is possible to apply “directly” a constant electric field $\vec{E}$ perpendicular to the membrane (lipid bilayers) plane. In practice, this is done by adding a force $F_i = q_i \vec{E}$ to all the atoms bearing a charge $q_i$ [66-70]. MD simulations adopting such an approach have been used to study membrane electroporation [26-30], lipid externalization [71], to activate voltage-gated $K^+$ channels [72] and to determine transport properties of ion channels [73-76].

The consequence of such perturbation stems from the properties of the membrane and from the simulations set-up conditions: Pure lipid membranes exhibit a heterogeneous atomic distributions across the bilayer to which are associated charges and molecular dipoles distributions. Phospholipid head-groups adopt in general a preferential orientation. For hydrated PC bilayers at temperatures above the gel to liquid crystal transition, the phosphatidyl-choline dipoles point on average 30 degrees away from the membrane normal [17, 77]. The organization of the phosphate ($PO_4^-$), choline ($N(CH_3)_3^+$) and the carbonyl (C=O) groups of the lipid head group give hence rise to a permanent dipole and the solvent (water) molecules bound to the lipid head group moieties tend to orient their dipoles to compensate the latter [78]. The electrostatic characteristics of the bilayer may be gathered from estimates of the electrostatic profile $\phi(z)$ that stems from the distribution of all the charges in the system. $\phi(z)$ is derived from MD simulations using Poisson’s equation and expressed as the double integral of $\rho(z)$, the molecular charge density distributions:

$$\Delta \phi(z) = \phi(z) - \phi(0) = -\frac{1}{\varepsilon_0} \int_0^z \rho(z')dz' dz' .$$

**Figure 2:** Electrostatic potential profiles $\phi(z)$ along the membrane normal $z$ of a POPC lipid bilayer (A) at rest and (B) subject to a transverse electric field $\vec{E}$. $z = 0$ represents the centre of the lipid bilayer and the arrow the bilayer-water interfaces. Are shown the contribution from water, lipid and the total electrostatic profile. Note that the TM voltage $\Delta V$ (potential difference between the
upper and lower water baths) created under electric field (panel B) is mainly due to water dipoles reorientation.

For lipid bilayers, most of which are modelled without consideration of a salt concentration, an applied electric field acts specifically and primarily on the interfacial water dipoles (small polarization of bulk water molecules). The reorientation of the lipid head groups appears not to be affected at very short time scales [28, 79], and not exceeding few degrees toward the field direction at longer time scale [29]. Hence, within a very short time scale - typically few picoseconds [28] – a transverse field \( \hat{E} \) induces an overall TM potential \( \Delta V \) (cf. Fig 2). It is very important to note here that, because of the MD simulation setup (and the use of PBCs), \( \hat{E} \) induces a voltage difference \( \Delta V \approx |\hat{E}| L_z \) over the whole system, where \( L_z \) is the size of the simulation box in the field direction. In the example shown in Fig 2, \( L_z \) is \( \approx 10 \text{ nm} \). The electric field (0.1 V.nm\(^{-1}\)) applied to the POPC bilayer induces \( \Delta V \approx 1 \text{ V} \).

MD simulations of pure lipid bilayers have shown that the application of electric fields of high enough magnitude leads to membrane electroporation, with a rather common poration sequence: The electric field favours quite rapidly (within a few hundred picoseconds) formation of water defects and water wires deep into the hydrophobic core [27]. Ultimately water fingers forming at both sides of the membrane join up to form water channels (often termed pores or hydrophobic pores) that span the membrane. Within nanoseconds, few lipid head-groups start to migrate from the membrane-water interface to the interior of the bilayer, stabilizing hydrophilic pores (\( \approx 1 \text{ to } 3 \text{ nm diameter} \)). All MD studies reported pore expansion as the electric field was maintained. In contrast, it was shown in one instance [28] that a hydrophilic pore could reseal within few nanoseconds when the applied field was switched off. Membrane complete recovery, i.e. migration of the lipid head group forming the hydrophilic pore toward the lipid/water interface, being a much longer process, was not observed. More recently systematic studies of pore creation and annihilation life time as a function of field strength have shed more light onto the complex dynamics of pores in simple lipid bilayers [80]. Quite interestingly, addition of salt has been shown to modulate these characteristic time scales [81].

For typical MD system sizes (128 lipids; 6 nm x 6 nm membrane cross section), most of the simulations reported a single pore formation at high field strengths. For much larger systems, multiple pore formation with diameters ranging from few to 10 nm could be witnessed [27, 28]. Such pores are in principle wide enough to transport ions and small molecules. One attempt has so far been made to investigate such a molecular transport under electroporation [28]. In this simulation, partial transport of a 12 base pairs DNA strand across the membrane could be followed. The strand considered diffused toward the interior of the bilayer when a pore was created beneath it and formed a stable complex DNA/lipid in which the lipid head groups encapsulate the strand. The process provided support to the gene delivery model proposed by Golzio et al. [82] in which, an “anchoring step” connecting the plasmid to permeabilized cells membranes that takes place during DNA transfer assisted by electric pulses, and agrees with the latest findings from the same group [83]. More recently, it was shown that even a single 10 ns electric pulses of high enough magnitude can enhance small siRNA transport through lipid membranes (Fig. 3) [84].

\[ \text{Figure 3: A single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance for the design of protocols aimed at using nanosecond-pulse siRNA electro-delivery in medical and biotechnological applications.} \]
related to the time scale the pore formation may take. A field strength threshold is “assumed” to be reached when no membrane rupture is formed within the 100 ns time scale. Finally.

B- Electroporation induced by ionic salt concentration gradients

Regardless of how low intensity millisecond electrical pulses are applied, the ultimate step is the charging of the membrane due to ions 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 \( Q_s \) across the bilayer, even when ions are present in the electrolytes. Several MD simulations protocols that can overcome this limitation have been recently devised (Fig. 4):

The double bilayer setup: It was indeed shown that TM potential gradients can be generated by a charge imbalance across lipid bilayers by considering a MD unit cell consisting of three salt-water baths separated by two bilayers and 3d-PBCs [85] (cf. Fig. 4.A). Setting up a net charge imbalance between the two independent water baths at time \( t=0 \) induces a TM voltage \( \Delta V \) by explicit ion dynamics.

The single bilayer setup: Delemotte et al. [86] introduced a variant of this method where the double layer is not needed, avoiding therefore the over-cost of simulating a large system. The method consists in considering a unique bilayer surrounded by electrolyte baths, each of them terminated by an air/water interface [87]. The system is set-up as indicated in Fig. 4.B. First, a hydrated bilayer is equilibrated at a given salt concentration using 3d periodic boundary conditions. Air water interfaces are then created on both sides of the membrane, and further equilibration is undertaken at constant volume, maintaining therefore a separation between the upper and lower electrolytes. A charge imbalance \( Q_s \) between the two sides of the bilayer are generated by simply displacing at time \( t=0 \) an adequate number of ions from one side to the other. As far as the water slabs are thicker than 25-30 Å, the presence of air water interfaces has no incidence on the lipid bilayer properties and the membrane “feels” as if it is embedded in infinite baths whose characteristics are those of the modeled finite solutions.

Extension to Liposomes: The availability of large computer resources has extended the realm of simulations of membrane electroporation to study systems large enough to allow modelling of small liposomes. Fig. 4.C represents such a liposome constructed from a POPC bilayer and equilibrated in a 200 mM NaCl salt solution. The system contains over 1,400 lipid molecules forming a liposome of internal diameter of 8 nm. The system size \((210 \times 210 \times 210 \ \text{Å}^3)\) was chosen large enough to avoid interaction between the central liposome and its replica, resulting in a net charge imbalance between the inner and outer water baths and 3d PBCs (drawn box) are used.
in an overall number of atoms ~ 890,000. In such a set-up a charge imbalance $Q_s$ was imposed after the system equilibration between the inner and outer side of the liposome.

Fig. 5 reports the electrostatic potential profiles along the normal to the membrane generated from MD simulations a POPC bilayer in contact with 1M NaCl salt water baths at various charge imbalances $Q_s$, using the single bilayer method. For all simulations, the profiles computed at the initial stage show plateau values in the aqueous regions and, for increasing $Q_s$, an increasing potential difference between the two electrolytes indicative of a TM potential $\Delta V$. Quite interestingly, the profiles show clearly that, in contrast to the electric field case where the TM voltage is mainly due to the water dipole reorientation (Fig. 2), most of the voltage drop in the charge imbalance method is due to the contribution from the ions. Indeed the sole collapse of the electrostatic potential due to the charge imbalance separation by the membrane lipid core accounts for the largest part of $\Delta V$.

![Figure 5: Components of the electrostatic potential profiles along the lipid bilayer normal Z of a POPC membrane estimated from at the initial stage of the MD simulations of the system at 1 M NaCl salt concentration using the single bilayer method. Z=0 represents here the center of the lipid bilayer, the broken arrow the location of the bilayer/water interfaces, and the solid arrows the locations of the air water interfaces. From left to right increasing amounts of net charge imbalance $Q_s$ between the lower and upper electrolytes induce transmembrane voltages (that may be estimated from the difference between the electrostatic potentials of the two water bath) of increasing amplitudes. Are shown in the top panels the contributions from lipid, water and ions, and in the lower panels the total electrostatic potential. Note that the most of the transmembrane voltage is due to the contribution from ions.](image)

Using the charge imbalance set-up, it was possible for the first time to directly demonstrate in silico that the simulated lipid bilayer behaves as a capacitor[86] (Fig 6). Simulations at various charge imbalances $Q_s$ show a linear variation of $\Delta V$ from which the capacitance can be estimated as $C = Q_s.\Delta V^{-1}$. The capacitance values extracted from simulations are expected to depend on the lipid composition (charged or not) and on the force field parameters used and as such constitutes a supplementary way of checking the accuracy of lipid force field parameters used in the simulation. Here, in the case of POPC bilayers embedded in a 1M solution of NaCl [86], the later amounts to 0.85 $\mu$F.cm$^{-2}$ which is in reasonable agreement with the value usually assumed in the literature e.g. 1.0 $\mu$F.cm$^{-2}$ [85, 88] and with recent measurements for planar POPC lipid bilayers in a 100 mM KCl solution (0.5 $\mu$F.cm$^{-2}$).

![Figure 6: Left: Electrostatic potential across a POPC lipid bilayer for different net charge imbalances $Q_s$ between the upper and lower electrolytes from MD simulations considering the setup of Fig. 5. $\phi(z)$, is estimated as an in-plane average of the EP distributions (Eq. 1). As a reference it was set to zero in the lower](image)

For large enough induced TM voltages, the three protocols lead to electroporation of the lipid bilayer. As in the case of the electric field method, for $\Delta V$ above 1.5-2.5 Volts, the electroporation process starts with the formation of water fingers that protrude inside the hydrophobic core of the membrane. Within nanoseconds, water wires bridging between the two sides of the membrane under voltage stress appear. If the simulations are further expended, lipid head-groups migrate along one wire and form a hydrophilic connected pathway (Fig.7).
electrolyte. Right: TM potential $\Delta V$ as a function of the charge imbalance $Q_\text{s}$ per unit area. The capacitance of the bilayer can be derived from the slope of the curve.

Because salt solutions are explicitly considered in these simulations, ion conduction through the hydrophilic pores occurred following the electroporation of the lipid bilayers. Details about the ionic transport through the pores formed within the bilayer core upon electroporation could be gathered [89]. The MD simulations of the double bilayer system [90, 91], and the results presented here for the single bilayer set-up and for the liposome show that both cations and anions exchange through the pores between the two baths, with an overall flux of charges directed toward a decrease of the charge imbalance. Ions translocation through the pores from one bulk region to the other lasts from few tens to few hundreds picoseconds, and leads to a decrease of the charge imbalance and hence to the collapse of $\Delta V$. Hence, for all systems, when the charge imbalance reached a level where the TM voltage was down to a couple of hundred mV, the hydrophilic pores “close” in the sense that no more ionic translocation occurs (Fig 7.F). The final topology of the pores toward the end of the simulations remain stable for time spans exceeding the 10 nanoseconds scale, showing as reported in previous simulations [28] that the complete recovery of the original bilayer structure requires a much longer time scale.

Note that in order to maintain $\Delta V$ constant the modeler 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. This protocol is, in particular for the single bilayer setup, adequate for performing simulations under constant voltage (low voltage, ms duration) or constant current conditions, which is suitable for comparison to experiments undertaken under similar conditions [92].

**DISCUSSION**

In order to determine the detailed mechanism of the pore creation, it is helpful to probe the electric field distribution across the bilayer, both at rest and under the effect of a TM voltage. Figure 8.A displays the electrostatic potential profiles for a lipid bilayer subject to increasing electric fields that generate TM potentials ranging from 0 V to ~ 3V. At 0 V, the lipid bilayer is at rest and the profiles reveal, in agreement with experiment [93], the existence of a positive potential difference between the membrane interior and the adjacent aqueous phases.

At rest, the voltage change across the lipid water interfaces gives rise locally to large electric fields (in the present case up to 1.5 V nm$^{-1}$) oriented toward the bulk, while at the center of the bilayer, the local electric field is null (Fig. 8.B,C). When external electric fields of magnitudes respectively of 0.06 and 0.30 V nm$^{-1}$ are applied, reorientation of the water molecules gives rise to TM potentials of respectively ~ 0.75 and 3 V. Figures 8.B and C reveal the incidence of such reorganization on the local electric...
field both at the interfaciale region and within the bilayer core. In particular one notes that the field in the membrane core has risen to a value ~ 1 V.nm\(^{-1}\) for the highest \(\Delta V\) imposed.

For the charge imbalance method, the overall picture is similar (Fig. 9.A and B), where again, the TM voltages created give rise to large electric fields within the membrane core, oriented perpendicular to the bilayer.

**Figure 8:** (A) Electrostatic potential profiles across a lipid bilayer subject to electric fields of 0.0 V.nm\(^{-1}\) (dotted line) 0.06 V.nm\(^{-1}\) (thin line) and 0.30 V.nm\(^{-1}\) (bold line). (B) Corresponding electric field profiles. (C) 2d (out of plane) map of the electric field distribution. The local electric field direction and strength are displayed as white arrows. Note that the larger fields are located at the lipid water interfaces and are oriented toward the solvent.

Qualitatively, in both methods, the cascade of events following the application of the TM voltage, and taking place at the membrane, is a direct consequence of such a field distribution. Indeed, water molecules initially restrained to the interfacial region, as they randomly percolate down within the membrane core, are subject to a high electric field, and are therefore inclined to orient their dipole along this local field. These molecules can easily hydrogen bond among themselves, which results in the creation of single water files. Such fingers protrude through the hydrophobic core from both sides of the membrane. Finally, these fingers meet up to form water channels (often termed pre-pores or hydrophobic pores) that span the membrane (Fig. 9.C). As the TM voltage is maintained, these water wires appear to be able to overcome the free energy barrier associated to the formation of a single file of water molecules spanning the bilayer (estimated to be \(\sim 108\) kJ/mol in the absence of external electric field [94]). As the electrical stress is maintained, lipid head group migrate along the stable water wires and participate in the formation of larger “hydophilic pores”, able to conduct ions and larger molecules as they expend.

**Figure 9:** (A) Electrostatic potential profiles across a POPC lipid membrane subject to a charge imbalance (single bilayer set-up) before (solid line) and after (broken line) electroporation. (B) Corresponding electric field profiles. (C) Snapshot taken from the MD simulation of the lipid bilayer subject to a TM voltage taken at the early stage of the pore formation showing the configuration of water molecules represented as balls and sticks (oxygen: grey) and (hydrogen: white) forming a continuous wire through the hydrophobic core of the membrane.

**CONCLUSION**

Currently, computational approaches remain potentially the only techniques able to follow, at the atomic scale the local perturbation lipid membranes undergo when they are subject to external electric field. The results obtained so far are believed to capture the essence of the several aspects of the electroporation phenomena in bilayers’ membranes, and could serve 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 and protocols used in MD simulations of OSLGELOD\(\square\)HVDUHUDWKHUZHOORSWLPL\(\square\)HG’HVSLWHWKHLU in intrinsic differences, all MD simulations of lipid bilayers subject to high enough TM voltages, regardless of how the latter are generated, provide support to the stochastic pore formation theories in which, the stress imposed on the membrane is released thanks to formation of nanometer scale hydrophilic pores that span the lipid core.
Recently experimental and theoretical investigations of electroporation of small patches of planar lipid bilayers have been conducted by means of linearly rising current. The experiments were conducted on ~120-μm-diameter patches of planar phospholipid bilayers. The steadily increasing voltage across the bilayer imposed by linearly increasing current led to electroporation of the membrane for voltages above a few hundred millivolts. This method shows new molecular mechanisms of electroporation. We recorded small voltage drops preceding the breakdown of the bilayer due to irreversible electroporation. These voltage drops were often followed by a voltage re-rise within a fraction of a second. Modeling the observed phenomenon by equivalent electric circuits showed that these events relate to opening and closing of conducting pores through the bilayer. Molecular dynamics simulations performed under similar conditions indicate that each event is likely to correspond to the opening and closing of a single pore of about 5 nm in diameter, the conductance of which ranges in the 100-nS scale. This combined experimental and theoretical investigation provides a better quantitative characterization of the size, conductance and lifetime of pores created during lipid bilayer electroporation. Such a molecular insight should enable better control and tuning of electroporation parameters for a wide range of biomedical and biotechnological applications.

Much more effort is still needed in order to investigate the cascade of events involved in more complex events such as the transport of large molecules across the membranes. Recent success stories in this direction [84] show that the modellers need to seek much more combined studies with experimentalists in order to provide better understanding of such processes.

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NOTES

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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.

Preambule: what is a biological membrane?
The target of cell electropermeabilization is the cell membrane, more precisely the plasma membrane. In many textbooks, the description of a biological membrane is limited to a lipid bilayer. This is far from the biological complexity and should be used only for soft matter investigations. When the process is applied to a cell (and to a tissue), a more sophisticated description of the biological membrane organization is needed. It is a complex assembly between proteins and a mixture of lipids. It results from a network of weak forces resulting in a complex pattern of lateral pressure across the membrane. A lot of lateral and rotational movements of the membrane components on the sub-microsecond timescale is present. Almost no spontaneous transverse movement is present. The distribution of lipids is not homogeneous as assumed in the fluid matrix model but localized specific accumulations are detected (rafts). This is due to the fact that a biological membrane is an active entity where a flow of components is continuously occurring (so called membrane traffic). Endocytosis and exocytosis are processes involved in the membrane organization. They are affected by stresses applied on the cell. This costs a lot of energy provided by the cell metabolism. Another consequence is the ionic gradient across the membrane resulting from the balance between active pumping and spontaneous leaks. A final aspect is that damages to the membrane are repaired not only by an intra-membraneous process (as for a viscoelastic material) but by a patching process mediated by cytosolic vesicles.

It is therefore very difficult to provide an accurate physical description of a biological membrane at the molecular level. Either oversimplifying approximations are used (a soft matter approach) or a phenomenological description is provided with fitting to physical chemical equations (a life science approach). Both are valid as long as you keep aware of the limits in accuracy. The present lecture will be within the life science approach to give the suitable informations for Clinical and well as biotechnological applications.

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 ($\lambda_{mb}$), the pulsing buffer ($\lambda_0$) and the cytoplasm ($\lambda_{c}$), the membrane thickness, the cell size (r) and packing. Thus,

$$\Delta \Psi_i = f \cdot g(\lambda) \cdot r \cdot E \cdot \cos \theta$$  (1)
in which $\theta$ designates the angle between the direction of the normal to the membrane at the considered point on the cell surface and the field direction, $E$ the field intensity, $r$ the radius of the cell and $f$, a shape factor (a cell being a spheroid). Therefore, $\Delta \Psi_i^{\text{perm}}$ 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]. Stronger field intensities are needed to get the same induced potential. Another factor affecting the induced potential differences is the shape of the cell and their relative orientation to the field lines. When the resulting transmembrane potential difference $\Delta \Psi_i$ (i.e. the sum between the resting value of cell membrane $\Delta \Psi_0$ and the electroinduced value $\Delta \Psi_i$) reaches locally 250 mV, 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 first obtained for $\theta$ close to 0 or $\pi$. $E_{p,r}$ is such that:

$$\Delta \Psi_i^{\text{perm}} = f \cdot g \left( \lambda \right) \cdot r \cdot E_{p,r}$$

(2)

Permeabilization is therefore a local process on the cell surface. The extend of the permeabilized surface of a spherical cell, $A_{\text{perm}}$, is given by:

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

(3)

where $A_{\text{tot}}$ 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. This is straightforward for spherical cells (and validated by fluorescence microscopy) but more complicated with other cell shapes. 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 $E_p$ value lower than when in suspension. Furthermore large cells in a population appear to be more fragile. An irreversible permeabilization of a subpopulation is observed when low field pulses (but larger than $E_p$) are applied. Another characteristic is that the ‘loading’ time is under the control of the cell size [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]. Free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its electroporabilized part [9]. This gives the following expression for a given molecule S and a cell with a radius r:

$$\phi(S, t) = 2\pi r^2 \cdot P_s \cdot \Delta S \cdot X(N, T) \left(1 - \frac{E_p}{2}\right) \exp(-k \cdot (N, T) \cdot t)$$

where $\Phi(S, t)$ 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 $\Delta S$ is the concentration difference of S across the membrane. $E_p$ 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 components of the cell are involved. Organelle fusion may be involved as in the case of other membrane repair occurring with after laser induced damage.

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. This is a major drawback for the transfer of sensitive species (nucleic acids). Adding antioxidantants is a safe approach [18].

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 [19].

There is a loss of the bilayer membrane asymmetry of the phospholipids on erythrocytes [20]. 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 cells have resealed (long term death).

CONCLUSION

All experimental observations on cell electroporation are in conflict with a naive model where it is proposed to result from holes punched in a lipid bilayer (see [21] as a recent review). Structural changes in the membrane organization supporting permeabilization remains poorly characterized. New informations appear provided by coarse grained computer-based simulations. Nevertheless it is possible by a careful cell dependent selection of the pulsing parameters to introduce any kind of polar molecules in a mammalian cell while preserving its viability. The processes supporting the transfer are very different for different molecules. Transfer is electrophoretically mediated during the pulse and is mostly present after the pulse driven by diffusion for small charged molecules (drugs) [22, 9]. SiRNA are only transferred by the electrophoretic drag present during the pulse [23]. DNA plasmids are accumulated in spots on the electroporabilized cell surface during the pulse and slowly translocated in the cytoplasm along the microtubules by a metabolic process [25, 24].

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Nanoelectropulses in Theory and in Practice

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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.

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 feedback loop focuses investigations of a very large parameter space on the critical ranges of values for the key variables.

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.

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.

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, the primary target of longer pulses. This idea was investigated experimentally beginning in the late 1990s [5–6]. Even though 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 theoretical 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).

In addition to highlighting the limitations of 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.

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) (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 electrophoretically 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 influx of YO-PRO-1 (YP) [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].

Nanoelectropermeabilization 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, depending on the properties of the media. Pulses shorter than the electrolyte relaxation time do not generate (unless the field is very high) 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.
Figure 5. Electropore creation sequence. (A) Molecular dynamics representation of a POPC lipid bilayer system. Small red and white spheres at the top and bottom of the panel are water oxygen and hydrogen atoms. Gold and blue spheres are head group phosphorus and nitrogen, respectively, and large gray spheres are phospholipid acyl oxygens. For clarity, atoms of the hydrocarbon chains in the interior of the bilayer are not shown. In the presence of a porating electric field, a water intrusion appears (B), and extends across the bilayer (C). Head groups follow the water to form a hydrophilic pore (D). The pore formation sequence, from the initiation of the water bridge to the formation of the head-group-lined pore takes less than 5 ns.

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. The challenge is to achieve a quantitative congruency of the coefficients.

NANOSECOND EXPERIMENTS AND MODELS

Experiments and molecular models of membrane permeabilization. Figure 4 shows a simple and direct response of cells to nanoelectropulse exposure — swelling [25,30,31]. Electropermeabilization of the cell membrane results in an osmotic imbalance that is countered by water influx into the cell and an increase in cell volume. This phenomenon, initiated by electrophysical interactions with basic cell constituents — ions, water, and phospholipids — on a much shorter time scale (a few nanoseconds) than usually considered by electrophysiologists and cell biologists, provides a simple, direct, and well-defined connection between simulations and experimental systems. By correlating observed kinetics of permeabilization and swelling with rates of pore formation and ion and water transport obtained from molecular simulations and continuum representations, we are improving the accuracy and applicability of the models.

Molecular dynamics and macroscale (continuum) models. Figure 5 shows the main steps in the electric field-driven formation of a nanopore in a typical MD simulation of a porating phospholipid bilayer, part of a larger scheme for the step-by-step development (and dissolution) of the electrically conductive defects that contribute at least in part to what we call a permeabilized membrane [32]. These molecular simulations permit us to conduct virtual experiments across a wide parameter space currently inaccessible in practice to direct observation. Although we cannot yet align the detailed energetics and kinetics that can be extracted from MD simulations with laboratory results, it is possible to compare MD data with the predictions of the macroscale models used to describe electroporation.

Figure 6 shows how pore initiation time (time between application of porating electric field and the appearance of a membrane-spanning water column (Fig. 5C)) varies with the magnitude of the electric field in MD simulations [32]. The value of the electric field in the membrane interior, extracted from simulations by integrating the charge density across the system, is used as a normalizing quantity.
This membrane internal field results from the interaction of the applied external field with the interface water and head group dipoles, and it corresponds to the large dipole potential found in the membrane interior even in the absence of an applied field [33]. The nonlinear decrease in pore initiation time with increased electric field may be interpreted as a lowering of the activation energy for the formation of the pore-initiating structures described above. We can use simulation results like those in Fig. 6 to reconcile molecular dynamics representations with continuum models, and ultimately both of these to experiment. For example, the relation between electric field and pore creation rate is described in the Krassowska-Weaver stochastic pore model in the following expression:

$$K_{pore} = Ae^{-E(r,V_m)/k_BT},$$  \hspace{1cm} (1)

where $K_{pore}$ is the pore creation rate, $A$ is a rate constant, $E(r,V_m)$ is the energy of a pore with radius $r$ at transmembrane potential $V_m$, and $k_B$ and $T$ are the Boltzmann constant and the absolute temperature [29,34–36]. One of our objectives is to reconcile the pore creation rate in (1) with our simulated pore initiation times, reconciling the two models.

As the availability of computing power increases, we also expect to validate the stochastic pore model expression for pore density,

$$\frac{dN}{dt} = \alpha e^{\beta(\Delta V_m)} \left(1 - \frac{N}{N_{eq}}\right),$$  \hspace{1cm} (2)

where $N$ and $N_{eq}$ are pores per unit area, instantaneous and equilibrium values, $\alpha$ and $\beta$ are empirical electroporation model parameters, and $\Delta V_m$ is the transmembrane potential.

Computing power is needed not only to enable simulations of larger systems. The large variability in pore initiation time indicated by the error bars in Fig. 6 means that independent simulations of each condition must be repeated many times to ensure valid results. (A surprising number of conclusions in the existing literature have been published on the basis of single simulations.)

Better models can contribute also to our understanding of practical problems in bioelectrics. For example, despite years of study, controversy remains regarding the effects, or lack of effects, of exposures to low levels of radio-frequency (RF) electromagnetic fields [37,38]. Part of the reason for failure to establish certainty on this issue arises from the difficulty of conducting experiments with a sufficient number of variables and a sufficient number of samples to generate reliable data sets. With accurate simulation tools, honed by reconciliation with experiment, we can explore the
large variable and statistical space in which suspected biophysical effects might occur, narrowing the range of experimental targets and focusing on systems in which effects are most likely and in which mechanisms will be clear.

*Experiments and molecular models of ion conductance.* The earliest identified and most direct indicators of electric field-driven membrane permeabilization are changes in electrical properties, including an increase in ion conductance [39,40]. Data from careful experimental work can be interpreted as measured values corresponding to the conductance of a single pore [41–44]. By combining continuum models of electroporation with this experimental data and with established values for ion electrophoretic mobilities and affinities between ions and phospholipids, we can draw conclusions about pore geometry and areal density. But the inaccessibility (so far) of membrane electropores to direct observation and manipulation of their physical structure prevents us from definitively bridging the gap between model and experiment.

A recently developed method for stabilizing electropores in molecular dynamics simulations of phospholipid bilayers [45] allows extraction of ion conductance from these model systems and thus provides a new and independent connection between models and experiments, in this case from the atomically detailed models of lipid electropores constructed with molecular dynamics. Figure 7 shows one of these stabilized pores with electric field-driven ions passing through it.

Although the magnitude of the conductance measured in these simulations is highly dependent on the accuracy of the ion and water models and their interactions with the phospholipid bilayer interface (and there is much room for improvement in this area), initial results are consistent with expectations from both continuum models and experimental observations.

**NANOSECOND EXCITATION**

*Nanoelectrostimulation of neurosecretory and neuromuscular cells.* Applications of pulsed electric fields in the clinic, particularly in electrochemotherapy and gene electrotransfer, are well known and described in detail in other parts of this course. We note here a potential biomedical application specifically of nanosecond electric pulses, the activation and modulation of the activity of neurosecretory and neuromuscular processes, an area which remains relatively unexplored. 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. Figure 8 demonstrates functional activation of an adrenal chromaffin cell after a single 5 ns, 5 MV/m pulse [46,47].

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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 electropermeabilisation level and extent in the tissue. Tissues can be permeabilized by milliseconds, microseconds as well as by nanoseconds duration pulses using appropriate pulse amplitudes. Various types of electrodes have been developed to generate in vivo tissue cells electropermeabilization, which tend to adapt to the various anatomical constraints, some of which may require treatment planning procedures to apply pulses with the adequate voltages.

INTRODUCTION

A tissue is a complex structure. It contains the cells that characterize this tissue, with their own physiological and 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.

“CHEMICAL” METHODS FOR DETECTING IN VIVO CELL ELECTROPORATION

The delivery of appropriate electric pulses 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 immunocyto-chemical 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 the electropermeabilized cells (and thus label or modify only these cells). If this marker molecule does not bind to (or interact with) intracellular targets, then it allows to simultaneously check cell electropermeabilisation 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 lose the marker molecule, which will leak out of the cells. Then both reversible and irreversible electropermeabilisation thresholds 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 previously outlined, 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 efficient and as much homogeneous as possible 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 electropereamabilized cells, like 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 of the marker due to its excretion (through kidneys to the urine) or its metabolism.

Therefore, marker must be an injectable product that will not be toxic for the laboratory animal, at least in the absence of the electric pulses delivery (indeed, as shown here below, the cytotoxic drug bleomycin has been used as electropereamabilization marker because it does not affect the non-permeabilized cells). 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 electropereamabilized 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 electropereamabilization. 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 with the 57Cobalt; however, the stability of the 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 emitter (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 the tumor exposure to the electric pulses. Cell electropereamabilization in vivo was also demonstrated using the huge increase in bleomycin cytotoxicity when the electric field intensity is above the threshold 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 under the same electrode geometry.

**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 $^{51}$Cr-EDTA test for the evaluation of the in vivo electroporation level has already allowed:

- to determine reversible and irreversible thresholds [7,9];
- to show differences between internal and external electrodes [10];
- 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

Radiolabeled diethylenetriaminepentaacetic acid (DTPA) was used to trace the distribution and internalisation of a hydrophilic drug after in vivo electroporation [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.

**“PHYSICAL” METHODS FOR DETECTING IN VIVO CELL ELECTROPORATION**

The changes in the electrical properties of the tissues can also be used to detect the achievement of changes in the cell tissue caused by the delivery of the electric pulses. However, the interpretation of the raw data (changes in conductivity, changes in (bio)impedance) must be very cautious, unless previous or parallel experimental work (using the chemical markers) allows to establish an association between the changes in the tissue electrical properties and the achievement of reversible or irreversible electroporation.

During the pulses, there are important changes in the conductivity of the tissues exposed to electroporabilizing pulses. Using microsecond pulses, after an initial pic of current due to the capacitive properties of the tissue, the current delivered is dictated by the conductive properties of the tissue, that will evolve as a function of the degree of cell permeabilization. If electroporation is achieved, the current will progressively increase. In a multidisciplinary study in which conductivity changes were paralleled to results of muscle and liver permeabilization achieved with the quantitative $^{51}$Cr-EDTA test, Cuijkati et al [13] demonstrated that it was possible to follow the achievement of tissue cells permeabilization in real time. The analysis of the data allowed the elaboration of an algorithm for the control on real time of the pulse parameters, which was able to bring an inadequate applied voltage value to the adequate one in less than 7 μs, that is, before the voltage reached its nominal value. Such extremely fast correction of the voltage allowed to prevent the generation of damages in the tissue that would have been provoked by the inadequate voltage, keeping the electroporation reversible, and making extremely safe the application of the electric pulses to the tissue [13].

In the case of the nanopulses, measurements of the changes in the impedance of the tissue after the pulses application have allowed to retrieve information on the influence of various electrical parameters on the tissue cell electroporation [14]. Great care is needed to perform these experiments as the impedance of animal tissues rapidly and progressively changes if the tissues are removed from the body. The use of plant tissues (like potatoes) is more appropriate as biimpedance evolution is almost null on the time scales of the experiments realization [14].

**ELECTROPORATION OF CELLS IN TISSUES**

Permeabilization has been demonstrated and evaluated using the methods described in the first parts 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 1200 V/cm (determined by the absence of cell killing by the electric pulses alone) [5]. Moreover the comparison was done with the electroporation of the 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 \textit{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 in that tissue.

- the duration of the permeabilized state is longer that the duration that could be expected from experiments \textit{in vitro} on isolated cells. Indeed, \textit{in vitro}, resealing time depends on the temperature and, at about 37°, cells become impermeable in less than one minute. \textit{In vivo}, muscle fibres 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 electropermeabilised tissues [15], 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 [16], 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, 13], 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 will be recalled.

A two-dimensional model [7] was used in 1999 to compare two types of electrodes: plate electrodes and rows of needle electrodes (two kind of electrodes largely used in ulcer operation experiments). A good fit was found between the percentages of tissue exposed to fields of strength above a given value and the \textsuperscript{51}Cr-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 \textit{in vivo} [17]. 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) [18,19]. 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 [20]. In the case of the skeletal muscle, the direction of the field (determined by the electrodes position) with respect to the direction of the main axis of the muscle fibers must be taken into consideration. Indeed, the threshold values are significantly different in the two main orientations (parallel or perpendicular) [21]. The use of appropriate conductive gels is also recommended in some situations for the convenient treatment of surface tissues like exophytic tumor nodules [22].

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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 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 cisplatin and bleomycin 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 is 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 permeant or non-permeant, suitable candidates for electrochemotherapy are limited to those drugs that are hydrophilic and/or 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 is 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. Early studies suggested that cisplatin is transported through the plasma membrane mainly by passive diffusion, while recent studies have demonstrated that transporters controlling intracellular copper homeostasis are significantly involved in influx (Ctr1) and efflux (ATP7A and ATP7B) of the cisplatin [5]. 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 in animal models in vivo (Fig 1). Extensive studies in different animal models with different types of tumors, either transplantable or spontaneous, were performed. The antitumor effectiveness of electrochemotherapy was demonstrated on tumors in mice, rats, hamsters, cats, dogs, horses and rabbits. Tumors treated by electrochemotherapy were either subcutaneous or located in muscle, brain or liver, being sarcomas, carcinomas, gliomas or malignant melanoma [1-4,6].

**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. 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 intratumorally. 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 (for example 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 intratumoral 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 composition. The electric field distribution in the tissue and cell electroporation can be improved by rotating the electric field. Surface tumors can be effectively treated by plate electrodes, whereas appropriate electric field distribution in the deeper parts of the tumor is assured by using needle electrodes [7-9].

**Figure 2:** Example of good antitumor effectiveness in 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 depends on the amplitude, number, frequency and duration of the electric pulses applied. Several studies in which parallel plate electrodes were used for surface tumors showed that 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 [8].
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 with equal effect if the concentration of drug present in the tumor is high enough. The minimal number of pulses used is 4; most studies use 8 electric pulses of 100 μs [1,4,8,10-12].

All the experiments conducted in vivo in 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 drug to reach the intracellular target. This was demonstrated in studies which measured the intratumoral drug accumulation and the amount of drug bound to DNA. Basically, the amounts of bleomycin and cisplatin in the electroporated tumors 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 antitumor effectiveness of electrochemotherapy were described. The application of electric pulses to tissues induces a transient, but reversible reduction of blood flow [13,14]. Restoration of the blood flow in normal tissue is much faster than that in tumors [14,15]. 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 cell death [16]. 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 [17-19]. This vascular-disrupting action of electrochemotherapy is important in clinical situations where haemorrhagic tumor nodules need to be treated [20].

A difference in the antitumor effectiveness of electrochemotherapy was observed between immunocompetent and immunodeficient experimental animals, indicating on involvement of the immune response in antitumor effectiveness [21]. Due to massive tumor antigen shedding in organisms after electrochemotherapy, systemic immunity can be induced and also up-regulated by additional treatment with biological response modifiers like IL-2, GM-CSF and TNF-α [22-24].

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, vascular lock leading to drug entrapment in tumors, 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 and is now in routine use in human and veterinary oncology.

**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 sarcoma tumor radiation response: 1.9-fold for electrochemotherapy with bleomycin and 1.6-fold for electrochemotherapy with cisplatin [25,26]. The radiosensitizing effect of electrochemotherapy with cisplatin was also demonstrated in breast cancer and with bleomycin in a fractionated radiation regime which makes this treatment potentially available also in the clinic [27, 28].

The application of electric pulses was shown to modulate tumor blood flow. Both reduced blood flow and lowered partial oxygen pressure (pO2) in the
tumors are consequences of the applied electric pulses [29]. The reduced pO₂ can activate bioreductive drugs to exhibit a cytotoxic effect on hypoxic cells [30]. 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 [31].

Electrochemotherapy is an effective cytoreductive treatment; however, its curative effect depends 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 that should have a systemic component. This can be achieved by a combination of electrochemotherapy with electrophoresis of different therapeutic genes acting either locally or sistemically, such as p53, IL-2; GM-CSF or IL-12. The results of the studies demonstrate positive results, further supporting this concept [32-35].

Finally, electrochemotherapy with cisplatin or bleomycin is also successfully used in veterinary medicine. It was used to treat different tumors, such as mammary adenocarcinoma, fibrosarcoma, cutaneous mast cell tumor, hemangiomata, 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 either of electrochemotherapy alone or in combination with other treatment, mainly surgery [36-43]. Hopefully, electrochemotherapy will be broadly used in veterinary medicine for the treatment of different malignancies, both in primary and metastatic disease.

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.

REFERENCES


PATIENTS AND METHODS
The histology of each tumor was confirmed by a pathologist. Tumors on the right side were treated with electrochemotherapy, and tumors on the left side served as untreated controls. Virgin adult female Wistar rats were anesthetized with pentobarbital sodium (50 mg/kg) and placed in a supine position. Prior to treatment, the area of tumor considered for treatment was painted with an emulsion of 0.1 mg/mL 5-fluorouracil (5-FU) and 0.1 mg/mL mitomycin C. The emulsion was allowed to dry for 10–15 min. The tumors were then exposed by removing the epidermis of the upper part of the tumor with a scalpel.

Electrochemotherapy was performed using an electric pulse generator Impugn (Impugn, Toulouse, France). The rats were placed in a holder designed to hold the rat by the tail, producing a voltage of 60–80 V. The voltage was maintained for 3–5 sec, delivering a pulse of 3–5 J/kg. No complications were observed in any case. The rats recovered completely within 24 hr and were able to eat and drink normally. The survival of the rats was monitored for 24 hr after the treatment. The tumors were monitored at 24 hr, 1 week, and 1 month after treatment.

RESULTS
Electrochemotherapy of tumours in rats is shown in Table 1. The results demonstrate that electrochemotherapy significantly decreased the volume of tumors compared to the untreated control. The tumour volume was significantly reduced by electrochemotherapy, with a mean decrease of 92% in the tumour volume compared to the untreated control. The results show that electrochemotherapy is an effective treatment for the reduction of tumour volume in rats.

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Clinical electrochemotherapy
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Abstract: Electrochemotherapy consists of administration of the chemotherapeutic drug followed by application of electric pulses to the tumor, in order to facilitate the drug uptake into the cells. Only two chemotherapeutics are currently used in electrochemotherapy, bleomycin and cisplatin, which both have hampered transport through the plasma membrane without electroporation of tumors. Based on extensive preclinical studies, elaborating on parameters for effective tumor treatment and elucidating the mechanisms of this therapy, electrochemotherapy is now in clinical use. It is in standard treatment of melanoma cutaneous metastases in Europe. However it is effective also for cutaneous metastases of other tumor types. Currently the technology is being developed also for treatment of bigger, deep seated tumors. With new electrodes and new electric pulse generators, clinical trials are on-going for treatment of liver metastases, bone metastases and soft tissue sarcomas, as well as brain metastases, tumors in in esophagus or in rectum.

INTRODUCTION
Electrochemotherapy protocols were optimized in preclinical studies in vitro and in vivo, and basic mechanisms elucidated, such as electroporation of cells, tumor drug entrapment (vascular lock), 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 histology 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 5th 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.

- Side effects of electrochemotherapy were minor and tolerable (muscle contractions and pain sensation).

In all clinical studies reported before the ESOPE study and in 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].
The ESOPE study set the stage for introduction of electrochemotherapy in Europe. After the encouraging results of the ESOPE study, several cancer centers have started to use electrochemotherapy and reported the results of their studies. Collectively, the results were again similar as reported in the ESOPE study. However some advances in the treatment were reported. Predominantly it was reported that tumors bigger than 3 cm in diameter can be successfully treated by electrochemotherapy in successive electrochemotherapy sessions [57,58]. In general, electrochemotherapy provides a benefit to patients especially in quality of life [58], because electrochemotherapy is nowadays used predominantly in palliative intent [57,58].

**CLINICAL USE AND TREATMENT PROCEDURES FOR ELECTROCHEMOTHERAPY**

Based on all these reports, electrochemotherapy has been recognized as a treatment option for disseminated cutaneous disease in melanoma, and accepted in many national and also international guidelines for treatment of melanoma [63].

Treatment advantages and clinical use for electrochemotherapy can be summarized as follows:

- Effective in treatment of tumors of different histology in the cutaneous or subcutaneous tissue.
- Palliative treatment with improvement of patient’s quality of life.
- Treatment of choice for tumors refractory to conventional treatments.
- Cytoreductive treatment before surgical resection in an organ sparing effect.
- Treatment of bleeding metastases.

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 [40,41,57,58].

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 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 of the treated tissue.

In summary, electrochemotherapy has been recognized as a valid treatment approach; over 100 cancer centers have started to use it and have reported positive results. So far the effectiveness of the therapy is on case based evidence and further controlled and randomized studies are needed for the translation of this technology into broader and standard clinical practice. For further acceptance of electrochemotherapy in medical community, the first important step has been made, since electrochemotherapy for treatment of melanoma skin metastases and for treatment of primary basal cell and primary squamous cell carcinoma was recently listed in NICE guidelines.
Recently all published studies up to 2012 on electro-chemotherapy in treatment of superficial nodules were reviewed reviewed in systematic review and meta-analysis [64]. Data analysis confirmed that electrochemotherapy had a significantly (p<0.001) higher effectiveness (by more than 50%) than bleomycin or cisplatin alone, where only 8% of the tumors were in CR. After a single electrochemotherapy, the treatment can be repeated with similar effectiveness. The overall effectiveness of electrochemotherapy was 84.1% objective responses (OR), from these 59.4% complete responses (CR). Another recent review and a clinical study suggested that SOP may need refinement; since the currently used SOP for electrochemotherapy may not be suitable for tumors bigger than 3 cm in diameter, but such tumors are suitable for the multiple consecutive electrochemotherapy sessions[65].

NEW CLINICAL APPLICATIONS OF ELECTROCHEMOTHERAPY

Based on clinical experience that electrochemotherapy can be effectively used in treatment of cancer with different histology, when appropriately executed, the treatment could be used also for treatment of deep seated tumors. Prerequisite for that is further development of the technology in order to reach and effectively treat the tumors located either in the muscle, liver, bone, esophagus, rectum, brain or other internal organs.

The first steps in technological development have already been made. For example, there is already the first report in treatment of melanoma metastasis in the muscle, 2 cm under the skin. With long needle electrodes and new electric pulses generator Cliniporator Vitae™ it was possible to treat this deep seated metastasis 2 x 1.4 cm in diameter [66].

Further development of such electrodes enabled treatment of liver metastases (Figure 2). At the Institute of Oncology Ljubljana, Slovenia a clinical trial was launched, where liver metastases of colorectal tumors are treated and effectiveness evaluated at the two stage operation (NCT01264952). So far 16 patients were enrolled. No immediate or late side-effects of electrochemotherapy were observed [67]. The delivery of electric pulses during open surgery was synchronized with ECG in order to avoid possible arrhythmias. Specific treatment plan is prepared for the treatment, in order to predict the exact location of the electrodes for sufficient coverage of the tumors with the electric field in the tumor and in the safety margins of the tumor [68].

Figure 2: Electrochemotherapy of liver metastasis. Electrodes were inserted into the tumor and around the tumor in healthy liver tissue and connected to electric pulse generator. Electric pulses were delivered between the pairs of electrodes according to the treatment plan.

Similar technology is being used in treatment of bone metastases or soft tissue sarcoma. The tumors are similarly as in treatment of liver metastases punctured by long needle electrodes, so that electrodes are placed around and in the tumor. The clinical trials are still ongoing but according to the preliminary report at the Second Users meeting of Electrochemotherapy in Bologna, Italy (2013) the technology is feasible, safe and effective.

Another approach that is in development is the use of endoluminal electrodes for the treatment of tumors in esophagus or in rectum. The first reports demonstrate that the technology is available, and was tested also in dogs. The translation of this technology into the human clinics is underway; the clinical trial for the treatment of unresectable colon tumors is ongoing [69].

In several studies also breast chest wall breast cancer recurrences were treated. A review of these data has demonstrated that the treatment of such metastases is equally effective as other tumors [70]. The feasibility and effectiveness was elaborated also in recent clinical study by Campana et al. [71]. Recently also a study from Herlev Cancer Center has demonstrated that even big chest wall breast cancer recurrences can be treated successfully by electrochemotherapy [72].

The last but not least also electrodes for treatment of brain tumors are developed [73,74]. They will enable treatment of brain tumors, minimally invasively. The clinical trial is was launched and started enrolling patients.
CONCLUSION

Electrochemotherapy is one of the biomedical applications of electroporation. Its development has reached clinical application and is an example of successful translational medicine. However, its development is not finished yet; new technical developments will certainly enable further clinical uses and eventually clinical benefit for the patients. Another application of electroporation is still awaiting such translation, gene therapy based on gene electrotransfer. In relation to this, first clinical results are encouraging, but standard clinical use is still far away.

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**NOTES**
DNA VACCINES

DNA vaccines are bacterial plasmids constructed to express in vivo a protein that will induce an immune response. Preclinical studies have shown that plasmid DNA encoding antigens provides protection in small animals and to a lesser extend in large animals for a wide range of diseases e.g. prophylactic viral and bacterial infections as well as therapeutic cancer vaccines. Several DNA vaccines have been licensed for veterinary use or are under clinical trials for human use.

DNA vaccine comprises a bacterial plasmid which utilizes a promoter driving expression in mammalian cells and a gene encoding the antigen of interest. The production of plasmid DNA requires specific markers able to select plasmid-containing bacteria after transformation and during the amplification process. The use of antibiotic resistance genes as selection markers for plasmid production raises safety concerns which are often pointed out by the regulatory authorities and a new generation of plasmid backbones devoid of antibiotic resistance marker has emerged.

The use of DNA vaccines offers several advantages over conventional vaccines with attenuated strains, subunits or recombinant protein vaccines: (i) generation of all three arms of adaptive immunity: antibodies, helper T cells (Th) and cytotoxic T lymphocytes (CTL); (ii) stimulation of innate immunity; (iii) avoidance of the use of virulent pathogens or pathogen proteins; (iv) no safety issues which are associated with the use of viral vectors or attenuated strains; (v) rapid construction of the plasmid including the gene sequence and immunostimulant sequences if required; (vi) generic manufacturing with simpler GMP (Good Manufacturing Practice) production; (vii) stability at room temperature and; (viii) antigen expression with the mammalian glycosylation and other posttranslational modifications, ensuring a closer resemblance to the antigen than recombinant proteins. Safety concerns associated with the use of modified genetic materials, the risk of gene insertion and oncogenesis limit the potential use of DNA vaccines to life-threatening human diseases. However, neither observable integration of the DNA in the host genome nor autoimmunity has been reported in human clinical trials for non viral DNA vaccines.

A number of studies demonstrated the robustness of DNA plasmid encoding pathogen and tumor antigens to elicit immune response. DNA vaccines induce a predominantly Th1 response, CTL response and antibodies but both the delivery route and the administration method have been shown to influence the type and the magnitude of the immune response. To elicit CTL responses, the antigen needs to be present in the cytoplasm of antigen presenting cells (APC). The protein is either directly produced by transfected APC or via cross priming through endocytosis by APC of the protein produced by other transfected cells. Peptides derived from the protein degradation bind to the major histocompatibility complex (MHC) class I or class II. Peptide association to MHC class I stimulates CTL while binding to MHC class II stimulate Th cells. Although DNA vaccines were initially developed to introduce antigen to MHC class-I processing pathway to induce CTL, they have also been shown to generate protective antibody responses: a transmembrane or secreted protein can activate B cells for antibody production.

ELECTROPORATION-MEDIATED DELIVERY OF DNA VACCINES

Even if naked plasmid DNA vaccines injected in muscle can induce an immune response, a relatively low magnitude of response is usually induced in large target species. Hence, methods to enhance their immunogenicity have been developed. Among them, electroporation seems particularly attractive to induce balanced and long-lived immune responses.

Electroporation addresses two limitations of the poor immunogenicity of DNA vaccines. (i) By inducing a transient membrane permeabilisation and by promoting electrophoresis of the negatively charged DNA, it facilitates DNA uptake in the host cells. Thereby the antigenic protein expression is strongly enhanced, usually by two orders of magnitude, in the muscle or the skin. (ii) By creating a low level of inflammation at the site of injection/electroporation, it enhances the recruitment of APC to the injection site.

Consequently, electroporation-mediated delivery of DNA vaccines enhances up to100-fold the immune responses elicited compared to simple injection. It is a useful strategy to increase both humoral and cellular responses in small and large animals including primates. A survey of the preclinical studies indicates that electroporation-mediated DNA vaccination induces long-lasting and robust cellular responses.
characterised by the induction of CTL, interferon γ and interleukin-2 by CD4+ and CD8+ T cells. Antibodies are usually detected. Combination with adjuvant (e.g. TLR-9 stimulation by CpG or interleukin-12) enhances the potency of DNA vaccination.

Two major organs have been investigated for DNA immunisation by electroporation. The skin is an immunocompetent organ with many resident APC e.g. Langerhans cells cover approximately 20% of the skin surface. It is easily accessible. Protein expression is limited to a few weeks. In contrast, the muscle induces a long term and stronger expression of the protein but contains few APC. Most of the preclinical studies indicate that a stronger humoral response is observed after intramuscular electrotransfer of the DNA than after intradermal electrotransfer.

Several electroporation-mediated DNA vaccinations are currently under clinical trials as therapeutic vaccines against cancers (e.g. melanomas or prostate cancer) and chronic infectious diseases (e.g. HIV, HCV). The uncompleted data suggest that electroporation-mediated vaccination is well tolerated and improves DNA vaccine potency.

Devices are also been optimized to enhance immune response and/or improve patient confort.

**RECOMMENDED PAPERS**

**DNA vaccines**


**Electroporation of DNA vaccines**


**Clinical trials with DNA vaccines and electroporation**


**Optimisation of delivery methods**


**NOTES**
Gene electrotransfer in vitro: a 30 years old story
Marie-Pierre Rols
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Abstract: We describe here what is known (and still unknown) about DNA electrotransfection into mammalian cells. If small molecules can freely cross the electropermeabilised membrane and have a free access to the cytoplasm, heavier molecules, such as plasmid DNA, face physical barriers (plasma membrane, cytoplasm crowding, nuclear envelope) which reduce transfection efficiency and engender a complex mechanism of transfer. Gene electrotransfer requires that the DNA is present during the application of the electric field pulses and involves different steps, occurring over relatively large time scales. As will be presented, these steps include the initial interaction with the electropermeabilised membrane, the crossing of the membrane, the transport within the cell and finally gene expression.

INTRODUCTION
The use of electroporation to deliver therapeutic molecules including drugs, proteins and nucleic acids in a wide range cells and tissues has been developed over the last decade [1-4]. This strategy is nowadays used in clinics to enhance the toxicity of anticancer drugs. It is promising for the systemic secretion of therapeutic proteins. Vaccination and oncology gene therapy are also major fields of application of DNA electrotransfer [5, 6]. Translation of preclinical studies into clinical trials in human and veterinary oncology has started [7-9]. A phase I dose escalation trial of plasmid interleukin electroporation has been carried out in patients with metastatic melanoma and has shown encouraging results [10]. The method has also been successfully used for the treatment of dogs and horses [7, 11]. But the safe and efficient use of this physical method for clinical purposes requires the knowledge of the mechanism underlying the electropermeabilization phenomena. Despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated 30 years ago [12], many of the mechanisms underlying DNA electrotransfer remain to be elucidated [13, 14]. Even if in vitro electrotransfer is efficient in almost all cell lines, in vivo gene delivery and expression in tumors are usually not. It is therefore mandatory for increasing gene transfer and expression to increase our knowledge of the process. Our strategy to study such phenomena consists on using different imaging tools, to directly visualize the processes, and different models with increasing complexities (lipid vesicles, cells in 2 or 3-D cultures).

MECHANISMS OF ELECTROTRANSFER OF DNA MOLECULES INTO CELLS
Single-cell microscopy and fluorescent plasmids can be used to monitor the different steps of electrotransfection [15, 16]. 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.

1) DNA/Membrane interaction
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 (Figure 1). 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: the negatively charged DNA migrates electrophoretically towards the plasma membrane on the cathode side where it accumulates.

Figure 1: Molecule electrotransfer mechanisms. Left: During electric pulses application: Plasma membrane is electropermeabilized facing the 2 electrodes (PI uptake). DNA aggregates are formed. This interaction takes place only on the membrane facing the cathode. Right: About 2 h after electric pulses application, DNA molecules are present at nucleus level. Finally, eGFP expression is detected for hours. The arrow indicates the direction of the electric field.
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 [17]. When plasmid has reached the nuclei, gene expression can take place and this can be detected up to several days in the case of dividing cells or weeks in some tissues such as muscles.

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 and 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 [18]. 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. Data reported evidences for the involvement of cytoskeleton (Figure 2). Actin indeed polymerizes around the DNA/membrane complexes [19-21].

We also investigated the dependence of DNA/membrane interaction and gene expression on electric pulse polarity, repetition frequency and duration. Both are affected by reversing the polarity and by increasing the repetition frequency or the duration of pulses [22, 23]. The results revealed the existence of 2 classes of DNA/membrane interaction: (i) a metastable DNA/membrane complex from which DNA can leave and return to external medium and (ii) a stable DNA/membrane complex, where DNA cannot be removed, even by applying electric pulses of reversed polarity. Only DNA belonging to the second class leads to effective gene expression [22].

2) Intracellular traffic of plasmid DNA.

Even if the first stage of gene electrotransfection, i.e. migration of plasmid DNA towards the electropermeabilised plasma membrane and its interaction with it, becomes understood we are not totally able to give guidelines to improve gene electrotransfer. 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 [24]. 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

![](image)

Figure 2: DNA electrotransfer as a multistep process. During the application of the electric field: (1) the plasma membrane is permeabilized (orange), (2) the DNA is electrophoretically pushed on the membrane side facing the cathode therefore (3) DNA/membrane interactions occur. DNA aggregates are inserted into the membrane and remain there for tens of minutes. After the application of the electric field and resealing of the membrane (yellow), (4) the DNA is expressed to occur (5-6), DNA has to cross the cytoplasm toward the nucleus. Our study suggests (5) actin related motion that pushes the DNA, free or in vesicles (actin rocketing) and/or (5b) transport via the myosins (in both directions). We observe (6) microtubules related motion which can mean (6a) transport via kinesin and dynein, (6b) DNA interaction with oppositely directed motors and with (6c) several motors of the same type (6d). Once being in the perinuclear region (in vesicles and perhaps also free, 7), DNA has to cross the nuclear envelope, after endosomal escape in case of DNA in vesicles. Finally, DNA is expressed in proteins found in the cytoplasm (8). From ref [21].
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 for 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. We recently showed how electroporated DNA is transported in the cytoplasm towards the nucleus [21]. For this purpose, we have performed single particle tracking (SPT) experiments of individual DNA aggregates in living cells [25-27]. In a first step, we have analyzed the modes of DNA aggregates motion in intact CHO cells. We show that fast active transport of the DNA aggregates over long distances occurs. Tracking experiments in CHO cells treated with different drugs affecting both the actin and the tubulin networks clearly demonstrate that this transport is related to the cellular microtubule network.

3) New developments.
As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA in the intracellular medium. Electroporated plasmid DNA, containing specific sequences could then use the microtubule network and its associated motor proteins to move through the cytoplasm to nucleus [28]. Clear limits of efficient gene expression using electric pulses are therefore due to the passage of DNA molecules through the plasma membrane and to the 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. One of the possible strategies to enhance DNA uptake into cells is to use short (10-300 ns) but high pulse (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 [29, 30]. An idea, to improve transfection success, is thus to perform classical membrane permeabilisation allowing plasmid DNA electrotransfer to the cell cytoplasm, and then after, when DNA has reached the nuclear envelope, to specifically permeabilise the nuclei using these short strong nanopulses. Thus, when used in conjunction with classical electropermeabilisation, nanopulses gave hope to increase gene expression. However, recent data showed that nsEPs have no major contribution to gene electrotransfer in CHO cells and no effect on constitutive GFP expression in HCT-116 cells [31].

LIPID VESICLES AND SPHEROIDS AS CONVENIENT APPROACHES TO STUDY GENE ELECTROTRANSFER

Other experiments are 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 [32, 33]. Experiments showed a decrease in vesicle radius which is interpreted as being due to lipid loss during the permeabilization process (Figure 3).

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 [34]. Other studies showed evidence for a direct transfer of DNA into the GUVs during application of the electric pulses [35]. That gives clear evidence that “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, 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

Figure 3: Microscopy observations of GUVs submitted to electric pulses of increasing intensities. Left: control; Middle and right: GUVs submitted to EP. From [33].
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 [36]. 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 [37, 38]. The problem of the access of DNA to the internal layers of cells still remains. A possible solution, that still has to be validated, 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 [39-41]. However, a cell membrane is highly complex and cannot 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. We recently took advantage of atomic force microscopy to directly visualize the consequences of electropermeabilization in terms of membrane reorganization and to locally measure the membrane elasticity [42]. We visualized transient rippling of membrane surface and measured a decrease in membrane elasticity by 40%. Our results obtained both on fixed and living CHO cells give evidence of an inner effect affecting the entire cell surface that may be related to cytoskeleton destabilization. Thus, AFM appears as a useful tool to further investigate basic process of electroporation on living cells in absence of any staining or cell preparation.

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 group leader. She is secretary of the GTRV French group on vectorisation. Her research interests lie in the fields of membrane electropermeabilization in cells and tissues, mainly on the mechanism of nucleic acids electrotransfer. Marie-Pierre Rols is the author of more than 100 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 Région.
Gene transfer in vivo
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Abstract: At the end of the 1990’s, several publications from various laboratories reported efficient in vivo electrotransfer of plasmids coding for several reporter genes. Trains of identical pulses of a long duration (between 5 and 50 ms) were found the most efficient for gene electrotransfer. Mechanisms of gene electrotransfer in vivo were analyzed in detail later. 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. This resulted in the development of a generator able to deliver trains of short and intense “electropermeabilizing” pulses followed by trains of low voltage and very long duration “electrophoretic” pulses. 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 [1] and its progression towards its application in the clinics [2] 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) [3,4]. It seemed thus possible to transfer plasmid DNA to cells in vivo by appropriate electric pulses (DNA electrotransfer).

Methods to achieve very efficient in vivo DNA transfer have been developed in the last ten years, particularly for gene transfer to skeletal muscle in a number of animal species including cattle [5,6]. 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 (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 and the most efficient and safer of the non viral physical methods of gene transfer [2,6,7].

DNA ELECTROTHERAPY MECHANISMS ANALYSED IN MUSCLE
A search for optimised conditions using trains of similar square wave pulses was performed by Mir and colleagues in 1999 [5]. 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 [5]. 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 [8]. Similar long pulses were described to be efficient in other tissues (tumors, skin). These conditions are still used nowadays, even though other pulse conditions were also proposed [9,10]. In particular the conditions proposed for the skeletal muscle 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 [11].

DNA ELECTROTHERAPY 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 400 and 1400 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, which means that their field amplitude is below the electropermeabilisation threshold of the tissue) [12]. It has been shown that, as expected, the electric pulses “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 [13] as measured using the $^{51}$Cr EDTA uptake test [14].
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 electroporation. It is mandatory to inject the DNA before the electrophoretic LV pulse, since LVs of a total duration of 400 ms remain efficient for all this long period of time. It is not possible to say towards the “electroporated” membrane (a membrane with “holes”) because high levels of permeabilisation, under the same experimental conditions, only last for 300 seconds (5 minutes) [13]. The precise structure of the membrane during this period of time (between 5 and 50 minutes after the delivery of the electroporating pulse, since LVs of a total duration of 400 ms remain efficient for all this long period of time). It is not possible to say towards the “electroporated” membrane (a membrane with “holes”) because high levels of permeabilisation, under the same experimental conditions, only last for 300 seconds (5 minutes) [13]. 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 [13,15,16]. Safety of the procedure was also demonstrated as only minor perturbations of muscle fibers physiology were reported [17]. For rat and mouse muscles, 1 HV of 700 V/cm followed 1 second later by 1 pulse of 80 or 100 V/cm and a duration of 400 ms (or alternatively 8 pulses of 50 ms) are recommended [16].

DNA ELECTROTHERAPY IN LIVER

DNA transfer in liver, using short pulses, was described in 1996 [18] (this was the second paper relating DNA electrophoresis in vivo, after the article by Titomirov et al in 1991 [19], 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 transfectable by simple hydrostatic pressure [20]. Recent data indicates that using long LVs (for example 4 LV of 100 ms) at field strengths of 500 V/cm and a duration of 400 ms (or alternatively 8 pulses of 50 ms) are recommended [16].

DNA ELECTROTHERAPY IN TUMORS

The first tissue to which DNA was transferred by means of long electric pulses was tumors transplanted in the flank of mice [21]. 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 [21]. 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) and with very different abundance of extracellular matrix [21]. Injection is more or less easy, reproducible and complete, depending on the consistency of the tumor (for example, experimental melanomas like the B16 melanoma are soft, inflatable tissues while fibrosarcomas are hard and breakable due to differences in the extracellular matrix of the cells). 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 square wave pulses (but the voltage was adapted to obtain a ratio of the voltage applied to the electrodes distance of 600 V/cm) [23] or using HV and LV combinations [16].

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 [24]. 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 [25]. 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. More recently, specific electrodes have been designed to treat brain tumors [26].

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. For this combination of gene electrotransfer and electrochemotherapy, most of the published work has been performed using DNA coding for either the IL-2, the GM-CSF or the IL-12. 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 [27]. 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 [28].

Painless approaches or methods to control the sensations

Animals are treated after the induction of general anaesthesia using standard laboratory protocols. However, the translation of DNA electroporation 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 short (100 µs) pulses are used to treat solid tumors in patients by 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 no 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 [29]. The same procedures have been applied before delivering genes in humans using HV + LV combinations. Pain was prevented, which means that the same procedures should be valid for Electrogenetherapy.

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 [30]. One of the trials is already closed. It reported good antitumor effects [31]. Other clinical trials involve DNA vaccination using plasmid DNA injection and electric pulses delivery.

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**NOTES**
Drug and gene delivery in the skin 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 contains 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 demonstrated that application of high voltage pulses permeablizes the stratum corneum and enhances 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., LHRRH 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 permeablization 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.
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 result in a 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 electrophoresis, GFP expression was also reported but was very low and restricted to the epidermis.

Duration of expression after electrophoresis depends on the targeted tissue. In contrast to the skeletal muscle where expression lasts for several months, gene expression is limited to only a few weeks into the skin. For example, after intradermal electrophoresis 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 electrophoresis 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 electrophoresis. 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.


Vaccination is another interesting application of electrophoresis into the skin. Intradermal electrophoresis 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.

Electrophoresis of DNA encoding either IL-2, IL-12 or an antiangiogenic compound for the treatment of melanoma is currently tested in clinical trials.

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Development of devices and electrodes

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Abstract: Since first reports on electroporation, numerous of electroporation based biotechnological and biomedical applications have emerged. 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 is inherently linked also to the electrodes choice.

INTRODUCTION

Since first reports on electroporation (both irreversible and reversible), 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 between them was used, and cells in suspension were placed in-between [1]. 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 [2]–[5]. 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 transfer 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, thousands 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. 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 [6]. 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 interesting in his/her research. Irrespective of the choice, it has to be linked also to the electrodes choice [7]–[9].

THERAPEUTIC AND TECHNOLOGICAL APPLICATIONS OF ELECTROPORATION

Nowadays electroporation is widely used in various biological, medical, and biotechnological applications [10]. Tissue ablation relying on irreversible electroporation is less than a decade old, but its efficacy is promising especially in treating non-malignant tissue, in the field of water treatment where efficacy of chemical treatment is enhanced with electroporation, in food preservation where electroporation has proven, in some cases, to be as effective as pasteurization [11]. 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.

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. Number of pulses that are usually delivered is 8. These can be delivered at pulse repetition frequency of 1 Hz or 5 kHz [12]. New technological developments were made available for in treating deep seated tumours, where 3000 V, 50 A and 100 μs pulses are being delivered [13]. Recent advances in treating liver metastasis, bone metastasis and soft tissue sarcoma have been reported [14].

TISSUE ABLATION BY NON-THERMAL IREVERSIBLE ELECTROPORATION
The ablation of undesirable tissue through the use of irreversible electroporation has recently been suggested as a minimally invasive method for tumor removal but could also be used in cardiac tissue ablation instead of RF heating tissue ablation or other tissue ablation techniques [15]. Similarly as in electrochemotherapy pulses of 50 or 100 μs with amplitudes up to 3000 V are used. The number of pulses delivered to the target tissue is however considerably higher. If in electrochemotherapy 8 pulses are delivered, here 96 pulses are used. Pulse repetition frequency needs to be low 1 or 4 Hz in order to avoid excessive heating [16].

GENE ELECTROTRANSFER
Exogenous genetic material can be delivered to cells by using non-viral methods such as electroporation. 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 [17]. Although no consensus can be reached, it can however be stated that longer pulses are generally used in gene transfection than in electrochemotherapy. Furthermore, two distinct roles of electric pulses were described. In experiments where several short high voltage pulses (e.g. 8 × 100 μs at 1000 V) were followed by long low voltage pulses (e.g. 1 × 100 ms of 80 V) [18]. It was demonstrated 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.

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 connection of neighboring cells, which is followed by electroporation 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 [19]. 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.

ELECTROEXTRACTION
Electroporation can be used to extract substances (e.g. juice, sugar, pigments and proteins) from biological tissue or cells (e.g. fruits, sugar beets, wine and yeast). Electroextraction can be more energy and extraction efficient, and faster than classical extraction.
methods (pressure, thermal denaturation and fermentation) [20]–[24].

**ELECTRO- PASTEURIZATION AND STERILIZATION**

Irreversible electroporation can be used in applications where permanent destruction of microorganisms is required, i.e. food processing and water treatment [25]. Still, using irreversible electroporation 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). This is one of the reasons why short (in comparison to medical applications) in the range of 1-3 µs are used. Especially industrial scale batch or flowthrough exposure systems may require huge power generators with amplitudes up to 40 kV and peak currents up to 500 A. Although batch and flow-through processes are both found on industrial scale, flow-through is preferred. Such mode of operation requires constant operation requiring higher output power of pulse generators [26].

**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 electroporation [27]. 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 [28]–[32]. 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, advanced numerical models were build, which take into consideration also tissue conductivity increase due to tissue or cell electroporation. These advanced models describe E distribution as a function of conductivity ω(E). In this way models represent electroporation tissue conductivity changes according to distribution of electric field intensities [33].

**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 [27]. 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.

Nowadays there are numerous types of electrodes that can be used for electroporation in any of the existing applications [34]–[37]. 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 starting point and 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 course of 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 through 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 [38]. Besides electroporation, electric pulses also cause side effects like Joule heating and electrolytic contamination of the sample, which further leads to increased sample conductivity [39].

Figure 2: Examples of commercially available electrode for electroporationabilization. Electrodes belong to the following group: A1 and A2 – parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes and E – coaxial electrodes. Electrodes A1 and B are produced by IGEA, Italy and are used for clinical applications of electrochemotherapy and electrotansfection. Electrodes A2, C and E are used for different in vitro applications and are produced by: E – Cyto Pulse Sciences, U.S.A.; A2, C and also D that are used for in vivo applications, are produced by BTX Hardware division, U.S.A.

ELECTRIC PULSES
For better understanding and critical reading of various reports on electroporation phenomenon and electroporation based applications, complete disclosure of pulse parameters needs to be given. Electric pulses are never “square” or “rectangular”, but they are characterized by their rise time, duration/width, fall time, pulse repetition frequency. Rise time and fall time are determined as time needed to rise from 10% to 90% of the amplitude, drop from 90% to 10% of amplitude, respectively. Pulse width is most often defined as time between 50% amplitude on the rise and 50% amplitude on the fall. Pulse repetition frequency is the inverse of the sum of pulse width and pause between two consecutive pulses. These may seem trivial when discussing pulses of 1 ms, but become an issue when discussing ns or even ps pulses [40], [41]. Shapes other than “rectangular” have been investigated with respect to electroporation efficiency [42]. It was suggested exposure of cells to pulse amplitudes above given critical amplitude and duration of exposure to this above critical value seem to be determining level of membrane electroporation irrespective of pulse shape. Exponentially decaying pulses are difficult to be considered as such but were predominantly used in 80s for gene electrotransfer. Their shape was convenient as the first peak part of the pulses acts as the permeabilizing part, and the tail of the pulse acts as electrophoretic part pushing DNA as towards and potentially through the cell membrane [18].

ELECTROPORATORS – THE NECESSARY PULSE GENERATORS
Electroporator is an electronic device that generates signals, usually square wave or exponentially decaying pulses, required for electroporation [1]. Parameters of the signal delivered to electrodes with the treated sample vary from application to application. Therefore, in investigating of electroporation phenomenon and development of electroporation based technologies and treatments it is 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 required pulse parameters in a reliable manner. Moreover, electroporator must be safe and easy to operate and should offer some possibilities of functional improvements. Clinical electroporators used in electrochemotherapy of deep-seated tumors or in non-thermal tissue ablation are also equipped with ECG synchronisation algorithms which minimizes possible influence of electric pulse delivery on heart function.

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 $U = I \cdot Z$. 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 [43] and updated in 2010 [3] in a manuscripts that describe techniques of signal generation required for cell/tissue electroporation.

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 plasmid DNA (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 or gene transfection efficiency and survival can also be obtained by changing field orientation in the sample using special commutation circuits that commute electroporation pulses between the electrodes [34], [36], [44].

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. The pulse characteristics determined as optimal or at least efficient and the tissue/sample will than determine the architecture of the pulse generator, whether it will be a Marx generator, Blumlein, or... [6].
CONCLUSIONS

Electroporation has been studied extensively until now, and a number of applications has been developed. 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 situations. 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 [1]. Cliniporator (IGEA, s.r.l. Carpi, Italy) was certified in EU (CE mark) as a medical device and is offered on the market along with standard operating procedures for electrochemotherapy of cutaneous and subcutaneous tumors. NanoKnife (AngioDynamics, Queensbury, USA) was certified in EU and approved by the FDA for surgical ablation of soft tissue, including cardiac and smooth muscle. Some electroporators are now available under the license for clinical evaluation purposes: Cellectra, Elgen, Medpulse, Cliniporator VITAE, DermaVax, EasyVax, Ellisphere, TriGrid [4].

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 his/her specific biotechnological or biomedical application, or for a general purpose pulse generator which will allow to generate “almost” all what researcher may find interesting/necessary in his/her research. Irrespective of the choice, this has to be linked also to the electrodes choice and tissue/sample conductivity.

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NOTES

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Electrofusion of cells: tools for new therapies
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INTRODUCTION
Cell membranes protect its cytoplasmic content from external agents and prevent their mixing when two cells are in close contact due to short distance repulsive forces (electrostatic and hydration forces). The membrane cohesion of cells can be destabilized when short and intense electric pulses are applied to cells. A new transient permeant state can be induced which allows the cytoplasmic delivery of hydrophilic compounds (drugs, protein, oligonucleotides, plasmids). Previous lectures along this workshop give the description of the present knowledge on electropermeabilization.

This new organization of the membrane supports a spontaneous fusion process when two cells are in contact [3]. This was first described in the early 80’s. But this was obtained under the contact first protocol (cells are previously brought in contact and then the electric pulse train is delivered) [10, 11, 17, 21]. But in fact the fusion is obtained even if the contact is obtained between cells already electropermeabilized [18, 21]

MOLECULAR MECHANISMS
The conclusion of the “pulse first” procedure is that the repulsive interfacial forces between the two surfaces vanished in the permeabilized state. A clear cut reorganization of the membrane surface is present affecting the water associated forces (so called repulsive hydration forces).

Electrofusion was proposed to be the results of coaxial pore coalescence [1, 19]. This was supported by the putative theory of toroidal pores supporting permeabilization. The concept was valid as long as it was postulated that pores were created just in face of each other on the two partner cells. But the toroidal pores are short lived and are not present under the pulse first approach. It was therefore proposed that the fusogenic state was linked to a more global alteration of the interfacial region a collective effect as shown by the P31 NMR studies [8]. It can be predicted that the alteration of the lipid domains on the cell surface is one of the main driving forces in electrically mediated membrane coalescence. It was reported that pure lipid vesicles can be electrofused [5, 13].

A modulation of the yield in electrofusion is brought by interfacial proteins as shown by the effect of proteases [14]. This further supported by in vivo electrofusion [9] Hydration repulsive forces are under the control of the osmotic forces. This affects of course electrofusion. A higher yield is obtained under hypoosmolar conditions [15 24]

PROTOCOLS
Electrofusion is always obtained under electric pulsing conditions inducing reversible electropermeabilization. The viability is fully preserved.

The differences between the approaches are in the protocols used to bring the partners in contact. This can be a natural biological contact the addition of chemicals bringing aggregation, a biochemical manipulation of the surfaces of the two partners (to improve their specific recognition) [7] or physical methods such as dielectrophoresis [18], sucking on filters [12], attachment on dishes [24] or mild centrifugation [21] Pulse first fusion can only proceed by dielectrophoresis or centrifugation

Pulsing parameters are those inducing electropermeabilization. The field intensity is therefore dependent on the size of the partners. A big advantage is associated with the pulse first approach, where the two partners are pulsed separately under their own specific conditions [16]. Pulse duration is always short (0.1 ms) and the number of pulses and the delay between them are classically 8 and 1 s.

Buffers should be chosen of a low conductance (to limit the Joule heating and improve the electrostatic interactions) and slightly hypoosmotic [24,25].

A strong control is brought by the cell physiology, such as the different thickness of extracellular matrices [9]. This may explain some unsuccessful trials [26].

APPLICATIONS AND DEVELOPMENTS
Hybrid cells are formed which allow a content mixing between the two partners and a sharing of the membrane surface [3]. The technology is rather straightforward [2]: Electropulse the cells under controlled conditions, bring them in a «soft» contact, let them do the membrane coalescence. Recent experiments showed that this was possible ex vivo as well as in vivo [4].

The lecture will describe the state of the art on the present knowledge on the process affecting the
membrane. A survey of the present clinical applications will be given [22]. Besides the hybridome formation, its use for cancer immunotherapy is under development by back injection to the patient of the product of electrofusing its own dendritic and tumor cells (6).

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INVITED LECTURERS
Microbial Inactivation by Pulsed Electric Fields: Fundamentals and Applications

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Abstract: Pulsed electric field technology (PEF) is viewed as one of the most promising nonthermal methods for inactivating microorganisms in liquid foods. The treatment consists of the application of pulses of high voltage and short duration to a food placed between two electrodes. This voltage results in an electric field that induces the permeabilization of microbial cytoplasmic membrane and, as consequence, the loss its semipermeable barrier properties. Evidence of electroporation of microbial cytoplasmic membrane caused by PEF has been demonstrated using different techniques such as detection of leakage of intracellular material, measurement of osmotic response or fluorescent dye exclusion assays. The capability of PEF to inactivate microorganisms at temperatures that do not affect the sensorial and nutritional food properties is very attractive to the industry for food preservation. As bacterial spores are resistant to PEF treatments, the main applications of this technology for food preservation must be focussed on pasteurization. In order to use the PEF technology as a pasteurization process, it is necessary to estimate its efficacy against pathogenic and spoilage food-borne microorganisms. To achieve this objective, there is a need to accumulate knowledge about the critical factors affecting microbial inactivation and to understand the mechanisms involved in the microbial PEF inactivation. This paper reviews the current state of the art in microbial inactivation by PEF. Particular attention is devoted to the proposed microbial inactivation mechanisms and the different parameters that influence microbial inactivation by PEF.

INTRODUCTION

Microorganisms are commonly present in foods. Depending on the food composition and environmental factors some of these microorganisms are able to grow causing food spoilage. Microbial deterioration of food is evidenced by slime formation, and changes in the appearance, texture, odour and flavour [1]. Additionally, foods may also contain food poisoning causing microorganisms without presenting any sign of food spoilage. While microbial food spoilage is a huge economical problem (about 25% of the world’s food supply is lost from microbial spoilage), food-borne illnesses are an enormous public health concern worldwide with severe direct and indirect economic consequences.

The available means of combating the deleterious effects of microorganisms are to prevent contamination, to slow their growth or to kill them. The most effective way to preserve food spoilage and to assure microbial safety is to prevent the contamination of raw material and foods at the primary production step and during processing. However, the large number of possible sources of microbial contamination makes microbial control by preventing the access of microorganisms enormously difficult.

Food preservation technologies use in the food industry are based on the prevention of microbial growth or on the inactivation of microorganisms. In many cases, foods are preserved by inhibiting or slow down microbial activity through those factors that most effectively influence the growth and survival of microorganisms such as temperature, water activity, addition of preservatives, pH or atmosphere modification. When these procedures are used, the microorganisms present in foods are not destroyed being metabolically active and viable. As estimates of the infection rate of some pathogenic microorganisms are very low, growth of these harmful microorganisms in foods is not necessary to cause infection [2]. Therefore, the application of a treatment aiming at microbial destruction is of primary importance for producing safe foods. Heating is the method of microbial destruction more frequently used in the food industry. Thermal processing is a very effective technology for microbial inactivation; however excessive heat treatment may cause undesirable effects on foods such as protein denaturation, non-enzymatic browning and loss of vitamins and volatile flavor compounds [3]. In order to reduce the negative effects of heat treatments in foods, alternative technologies capable of inactivating microorganisms at temperatures below those used during thermal processing are being demanded by the food industry [4]. Pulsed electric field technology (PEF) is viewed as one of the most promising nonthermal methods for inactivating microorganisms in foods. The treatment consists on the application of pulses of high voltage and short duration to a food placed between two electrodes. This voltage results in an electric field which intensity depends on the voltage delivered and the gap between the electrodes. PEF cause some type of structural rearrangement of the cell membranes that
consists on the formation of local defects or pores (electroporation) that lead to an increment of the cell membrane permeability to ions and macromolecules. Depending on the intensity of the treatment applied (external electric field, treatment time) and the cell characteristics (size, shape, orientation in the electric field) the viability of the electroporated cell can be preserved by recovering the membrane integrity, or the electroporation can be permanent. Permanent electroporation causes inactivation of vegetative forms of microorganisms at temperatures below those used in thermal processing [5].

MECHANISMS OF INACTIVATION BY PULSED ELECTRIC FIELDS

The mechanism underlying microbial inactivation by PEF has not been fully elucidated although it is believed that damage in the cytoplasmatic membrane of microorganism is the main cause of microbial inactivation. The maintenance of the microbial homeostasis requires that the cytoplasmatic membrane acts as an intact semipermeable barrier that separates the cell from its environment. The local defects or pores created by the application of an external electric field lead to the loss of the membrane integrity and uncontrolled molecular transport across the membrane may occur. These events may abolish the homeostatic capacity and they will eventually lead to microbial death [6]. However, in microorganisms including bacteria and yeast, the cytoplasmatic membrane is not the only barrier that separates the cytoplasm from the environment. Cytoplasmatic membrane of Gram-positive bacteria is surrounded by a thick cell wall made of peptidoglycans and tectonic acids. On the other hand, the cell wall in Gram negative bacteria is thinner but it is surrounded by an outer membrane that differs from typical biological membrane because the main molecular constituents are lipopolysacharides. This outer membrane prevents the entrance of some molecules such as antibiotics, lytic enzymes or bacteriocins but allows low molecular weight nutrients to diffuse into the periplasmic space. Similarly to Gram-positive bacteria, yeast cells are surrounded by a cell wall. Although it has been observed differences in the effect caused by PEF in Gram positive and Gram negative bacteria, how influence the envelopes surrounding the cytoplasmatic membrane on electroporation is an aspect that requires further research.

Evidence of electroporation of microbial cytoplasmatic membrane caused by PEF has been observed using different techniques such as detection of leakage of intracellular material, measurement of osmotic response or fluorescent dye exclusion assays. The presence in the medium surrounding the microorganisms of ultraviolet absorbing material as a semi-quantitative measure of nucleic acid and proteins and of adenosine triphosphate (ATP) are the most commonly used indicator of the leakage of intracellular material. Several authors have observed leakage of intracellular compounds from different microorganisms treated by PEF treatments which were non-lethal indicating that the temporary loss of permeability control is not necessarily lethal [7]. When treatments with lethal efficacy are applied, it is observed that increasing the severity of the treatment lead to a greater leakage of ATP, nucleic acids and proteins [7-9]. This increment could be due to an increment in the number of permeabлизed cells or in the number or size of the pores induced in the electroporated population.

The ability of the cells to plasmolize is other technique that can be used to evaluate modifications in the membrane integrity of microorganisms. When intact microbial cells are suspended in an hypertonic medium, water diffuses from the cell causing a strong condensation of the cytoplasmatic content that can be determined by the increment in the optical density of the cell suspensions. Early studies showed that cells of Escherichia coli lost their ability to plasmolize after application of a PEF treatment [10].

Figure 1: Protocol for propidium iodine staining of microorganisms to detect reversible and irreversible electroporation.
One of the most used methods to measure the permeabilization of the microbial membrane is the exclusion of dyes. For this purpose, the most commonly used probe is the fluorescent molecule propidium iodide (PI). PI is a hydrophilic small molecule (660 Da) that is only to able to entry in permeabilized microbial cells. This technique has been used to analyze individual cells with epifluorescent microscopy or flow cytometry and to analyze the whole population using spectrofluorometer procedures. Using flow cytometry techniques it has been observed that when the PI is added after the PEF treatment there is a linear correlation between the number of permeabilized and inactivated cells for E. coli, Listeria innocua and Lactobacillus plantarum [7,9]. However, in the case of the yeast Saccharomyces cerevisiae it was observed that treatments that increased the permeability to PI did not necessarily caused loss of viability. In order to detect reversible and irreversible permeabilization caused by PEF, the fluorescence intensity of the microbial suspensions was compared when the PI was added before or after the PEF treatment [11]. Permanent permeabilization was detected adding the PI after the treatment and non-permanent permeabilization when the fluorescent intensity of the suspension was higher when the PI was added before than after the treatment (Figure 1). Using this protocol it has been demonstrated that the membrane permeabilization was involved in microbial inactivation by PEF but the membrane electroporation and its relationship with the microbial inactivation depended on the treatment medium pH and the characteristics of microbial envelopes. At pH 7 and 4, the loss of viability for Gram-positive bacteria investigated such as Listeria monocytogenes and L. plantarum was correlated with an irreversible loss of membrane integrity. However, for the Gram-negative bacteria (E. coli and Salmonella senftenberg), inactivation was correlated with the proportion of reversible and irreversible electroporated cells indicating that reversible changes in the cytoplasmatic membrane of Gram-negative bacteria also may cause microbial death. At pH 4, no correlation was observed between loss of viability and membrane permeabilization for Gram-negative bacteria being the proportion of permeabilized cells lower than the inactivated ones. This behavior could be a consequence of that at low pH, the size of the pores caused by PEF treatment was smaller than those requires to PI uptake or that the outer membrane prevented the entrance of PI.

The capability of the microorganism of recovering the damage caused by PEF in the cytoplasmatic membrane has been correlated with the occurrence of sublethal injury after the PEF treatment. Sublethally injury population fail to survive and multiply in harsh environments tolerant by untreated cells so comparison of cell counts of PEF treated samples on selective (harsh environment) and nonselective media is the most conventional technique to detect the occurrence of sublethal injury (Figure 2). Early studies indicated that microbial inactivation by PEF was an all-or-nothing effect because after the treatment alive or dead cells were detected but not sublethally injured ones [8,12]. However, currently it is well established that PEF causes sublethal injury depending on the microorganisms and pH of the treatment medium [13]. Generally, greater number of sublethally injured cells were detected in a population of Gram-negative bacteria when treated by PEF at pH 4 that at pH 7 but in Gram-positive bacteria occurrence of sublethal injury was greater at pH 7 than at pH 4. The fact that the presence of sodium chloride in the recovery medium prevented the growth of sublethally injured cells of E. coli after PEF treatment and the demonstration that these damaged cells required the synthesis of lipids for injury repair supports the involvement of the citoplasmatic membrane on the microbial inactivation by PEF [14].

![Figure 2: Protocol for detecting sublethally damage microorganisms after application of a pulsed electric field treatment.](image-url)
Recovery media added with bile salts is generally used to evaluate the permeabilization of the outer membrane of Gram-negative microorganisms. Using this technique, several authors have reported that PEF did not affect the permeability barrier of the outer membrane of the bacteria surviving the treatments [7,8,12]. However, recently the occurrence of sublethal injury in both cytoplasmatic membrane and outer membrane in cells of *Enterobacter sakazakii* treated by PEF especially in media of low pH has been observed [30]. This damage in the outer membrane facilitated the antimicrobial activity of citral in cells of this microorganisms treated by PEF.

### FACTORS AFFECTING MICROBIAL INACTIVATION BY PEF

The microbial inactivation by PEF has been found to depend on many factors. In order to define the processing conditions required to inactivate spoiling and pathogenic microorganisms, the influence of these factors must be understood. Critical factors affecting microbial inactivation can essentially be classified into three groups: processing parameters, microbial characteristics and treatment medium characteristics (Table 1).

**Table 1**: Main process parameters affecting microbial inactivation by pulsed electric fields

<table>
<thead>
<tr>
<th>Process parameters</th>
<th>Microbial characteristics</th>
<th>Treatment Medium Characteristics</th>
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<tbody>
<tr>
<td>Electric field strength</td>
<td>Strain</td>
<td>Composition</td>
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<td>Treatment time</td>
<td>Specie</td>
<td>Conductivity</td>
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<td>Pulsed width</td>
<td><em>Growth conditions:</em></td>
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<td>Specific energy</td>
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### Processing parameters

The most typical processing parameters that characterize PEF technology are electric field strength, pulse shape, pulse width, number of pulses, pulse specific energy and frequency.

The distance between the electrodes of the treatment chamber and the voltage delivered define the electric field strength that is generally reported as kV/cm. The different theories proposed to explain the mechanisms of initiation of cell electroporation agree that a specific transmembrane voltage threshold (from 0.5 V to 1 V) exists for the manifestation of the electroporation phenomenon [15-17]. Transmembrane potential generated in a cell by application of an external electric field depends among other factor on the intensity of the electric field strength and on the cell size. The external electric field strength required to reach the transmembrane voltage threshold is called critical electric field strength. Due to the size of microbial cells (1-10 μm) is smaller than the eukariote plant cells (40-200 μm) the critical electric field intensity to induce electroporation of microbial cells is much higher than for induce electroporation in eukaryote cells of plant or animal tissues (> 5 kV/cm). This fact involves that microbial inactivation requires more powerful equipment and higher energetic cost than electroporation of animal or plant cells.

Generally, studies on microbial inactivation by PEF have been conducted in the range of 10 to 30 kV/cm because application of higher electric field strengths have technical limitations, especially at industrial scale, and may cause the dielectric breakdown of the food material.

Treatment time is defined as a function of the duration of pulse width and the number of pulses applied. It is generally reported in μs. In square waveform pulses, pulse width corresponds to the duration of the pulse, but in exponential decay pulses the time required for the input voltage to decay to 37% of its maximum value has been adopted as the effective pulse width. The survival curves (log10 of survivors along the time) at constant electric field strength are characterized by a fast inactivation in the first moments of the treatment and then the number of survivors slowly decreases as the number of pulses applied becomes longer (Figure 3).

![Figure 3](image-url): Typical survival curves corresponding to microbial inactivation by pulsed electric field treatments of different intensity (E₁, E₂, E₃).

Specific energy of the treatment (energy applied per mass unit) depends on the voltage applied, pulse width, number of pulses and resistance of the treatment chamber that varies according to their
geometry and conductivity of the treated material. Commonly, it is reported in KJ/Kg. This parameter permits to evaluate energy costs of the PEF process and, consequently, to compare the energy efficiency of PEF with other inactivation technologies. The specific energy has been proposed as a control parameter of PEF process, especially when exponential decay pulses are used due to the lack of precision in the measurement of the pulse width [18]. Microbial inactivation by PEF increases with specific energy but when different treatments of the same specific energy are compared in terms of microbial inactivation those applied at higher electric fields are more effective [19-20]. Therefore to a precise characterization of PEF treatments both specific energy and electric field strength should be reported.

There is some controversy concerning the influence of the pulse shape, width, and frequency on PEF microbial inactivation. It is generally accepted that square wave pulses are more efficient than exponential decay ones because the characteristic slow decaying rate causes long tail section that is ineffective to kill the microorganisms in the food material. Some authors have reported that when treatment of the same duration are applied with pulses of different width or at different frequencies, longer pulses and higher frequencies are more effective [21,22]. However these two parameters apparently exert no influence on microbial inactivation when the temperature rise of the medium caused by the application of longer pulses of higher frequencies is avoided [23] Microbial inactivation by PEF is usually enhanced when the temperature of the treatment medium is increased, even in ranges of temperatures that are not lethal for microorganisms (Figure 4) [24]. This effect has been attributed to changes in the phospholipid bilayer structure of the cell membranes, from a gel-like consistency to a liquid crystalline state that is caused by the temperature increase. However, further studies are required to demonstrate this fact.

**Microbial characteristics**

Microbial inactivation by PEF depends on microbial properties such as the type of microorganism, characteristics of the cell envelopes (Gram-positive or Gram-negative), cell size and shape. Generally, it has been reported that bacteria are more PEF-resistant than yeast, Gram-negative microorganisms are more sensitive than Gram-positive microorganisms, and coccus more resistant than rods. However, it seems that the intrinsic microbial resistance is more important than the effect of the microbial characteristics in determining the microbial sensitivity to PEF. When the PEF-resistance of different microorganisms is compared under the same experimental conditions it is observed that some yeast cells are more PEF-resistant than some bacteria, some Gram-positive microorganisms more sensitive than Gram-negative microorganisms, and some yeast species and some rod bacteria more resistant than some coccus bacteria [5].

![Figure 4: Influence of treatment temperature on inactivation of Escherichia coli by pulsed electric fields (30 kV/cm, 50 μs).](image)

The PEF resistance of different strains of bacterial species may vary greatly. It has been observed that depending of the strain and pH of the treatment medium the inactivation of different strains of the same microorganism may range from 0.1 to 4.5 Log10 CFU/ml [25]. As the PEF resistance of the different strains depended on the pH of the treatment medium, the target microorganisms to define treatment conditions for PEF pasteurization could be expected to be different for foods, depending on their pH.

Culture conditions of microorganisms also influence microbial inactivation by PEF. Generally, it has been reported that microorganisms at the exponential phase of growth are more PEF sensitive than those at the stationary phase [26,27]. The higher size of cells in the exponential phase could explain this difference in resistance. On the other hand, microbial resistance to PEF depends on the cultivation temperature. It has been reported that cells growth at temperatures lower that the optimal one are more PEF sensitive than those grown at optimal temperature. Variation in lipid composition of cultures growth at different temperatures could be the reason of this behavior.
Treatment medium characteristics

Generally studies on microbial inactivation by PEF have been conducted with microorganisms suspended in liquid media. Microbial inactivation in solid media has received less attention. The influence of the electrical conductivity and the pH of the substrate on microbial inactivation have been widely investigated. Several studies have reported that the conductivity of the treatment medium affects microbial inactivation. However, it is unclear if the conductivity influences electroporation or if the effect observed is a consequence of the influence of conductivity on the intensity of the PEF treatment applied. A change in conductivity modifies the resistance of the treatment chamber and it may cause changes in the electric field strength and the pulse width and total specific energy of the pulses. In a range of conductivity from 0.5 to 4.0 mS/cm, which corresponds to the conductivity of most of liquid foods, it has been observed that the conductivity did not affect microbial inactivation when the input voltage and input pulse width was modified in order to obtain the same treatment (electric field strength and treatment time) in media of different conductivities [19,20].

Published research indicates that microbial PEF resistance varies considerably depending of the pH of the treatment medium. Researchers have reported that a variation of the pH of the treatment medium can increase, reduce, or have no effect in modifying the microbial sensitivity to PEF [5]. Generally, Gram positive microorganisms are more PEF resistant in media of neutral pH than in acidic conditions, and Gram negative ones are more resistant in media of acidic pH that in neutral conditions [28]. This effect of the pH on microbial resistance has been confirmed in both buffers and liquid foods. The mechanism that explains these differences seems to be related to the occurrence of sublethal membrane damage by PEF. It has been observed that when Gram-positive bacteria are treated in neutral media their ability for repairing sublethal injury caused by PEF is higher than that when treated in low pH media. On the contrary, the higher PEF ability for repairing sublethal injury in Gram-negative bacteria occurs when they are treated in acidic pH media.

The possible protection or sensitization to electric fields conferred by different foods components such as carbohydrates, lipids or proteins, etc. has been investigated for different authors [29]. However, the different treatment conditions and media used make it difficult to obtain definitive conclusions to this respect. For example, while some authors determined that microbial resistance increased with the fat content of milk others found that microbial inactivation were independent of the fat or protein content when buffers were used as treatment media. On the other hand, a protective effect that made Escherichia coli more PEF resistant has been reported as consequence of the presence of organic acids in both buffers of pH 4 and fruit juices [31].

FOOD PASTEURIZATION BY PEF

Inactivation of vegetative cells of bacteria and yeast by PEF has widely been demonstrated. However, the few studies conducted on the inactivation of bacterial spores by PEF describe these structures as resistant to PEF treatments [32,33]. Therefore, practical applications of PEF processing aim at replacing thermal pasteurization as a means of killing vegetative microorganisms rather than sterilization. Pasteurization refers to a treatment used for food preservation which aims to inactivate pathogenic forms of vegetative microorganisms. Although the main objective of PEF pasteurization is to guarantee food safety, a large proportion of the population of vegetative spoilage microorganisms is also inactivated by the treatment. Therefore, PEF treatments may extend the shelf-life of foods [34,35]. However, the treatment is not capable of achieving commercial sterility because spores or other non-public health significant microorganisms can be present; thus, other preservation techniques, such as refrigeration, atmosphere modification, the addition of preservatives, or a combination of these techniques, will be required to preserve the quality and stability of the food during its distribution and storage [36].

PEF is gaining interest as a gentle method of food preservation of heat sensitive foods such as fruit juices. Demands by consumers for fresh-like and natural taste foods have promote the introduction in the market of nonpasteurised fruit juices produced from fresh fruit and distributed under refrigeration. As psychrotrophic microorganisms are able to grow at refrigeration temperatures, the shelf-life of these fruit juices is very short (around 7 days). On the other hand, several outbreaks associated with the consumption of unpasteurized juices have demonstrated that these products can be a vehicle of foodborne illness caused by pathogens such as Salmonella spp or E. coli O157:H7 [37].

Commercial exploitation of PEF for food pasteurization requires proof that PEF promotes a level of microbial safety that is equal to that made possible via traditional processing. A 5 Log10 reduction of the most resistant microorganism of public health significance has been established by the U.S. Food and Drug Administration (FDA) concerning fruit juice pasteurization [38]. Studies to
evaluate the application of PEF for microbial decontamination at room temperature have shown that to obtain these levels of reduction it is necessary to apply long treatments (i.e., >100 μs) at high electric field strengths (i.e., ≥30 kV/cm) [19,20,3 9]. At commercial scale, technical and economical limitations exist in applying these intense treatments in continuous flow. However, several studies confirmed that the application of PEF at moderate temperatures provides the possibility of obtaining substantial microbial inactivation of pathogenic microorganisms that are particularly PEF resistant with a short residence time (less than 1 s) at moderate electric field strengths (25 kV/cm). Under this treatment condition the fruit juice shelf life is confirmed that the application of PEF at moderate temperatures provides the possibility of obtaining substantial microbial inactivation of pathogenic microorganisms that are particularly PEF resistant with a short residence time (less than 1 s) at moderate electric field strengths (25 kV/cm). Under this treatment condition the fruit juice shelf life is

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Javier Raso received his PhD in 1995 at the University of Zaragoza (Spain) where he is currently professor of Food Technology and former Director of the Pilot Plant of Food Science and Technology. He has been visiting researcher of the Microbiology Department at Unilever Research in Bedford (UK), of the Department of Food Biotechnology and Food Process Engineering at Technical University of Berlin (Germany) and of the Biological Systems Engineering Department at Washington State University (USA). His areas of research are in the field of food preservation and processing by thermal and non-thermal technologies such as ultrasound, high hydrostatic pressure, pulsed electric fields and combined processing. Research interest is focused in critical factors affecting efficacy of technologies, kinetics and mathematical modeling, process optimization and mechanisms of action. He has been involved in a number of EU and national funded projects in these topics and he is the author of more than 80 peer-review papers. He is co-author of the book “Pulsed Electric Fields Technology for the Food Industry” and he is serving in the editorial board of the “Innovative Food Science and Emerging Technologies” journal. He is currently Vice-chair of the COST Action TD1104 “European network for development of electroporation-based technologies and treatments (EP4Bio2Med)”.

NOTES
Gaining access to intracellular compartments by electroporation

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Abstract: Cell membranes provide functional barriers that define cell compartments. This includes the outer or plasma membrane (PM) which separates inside from outside, and also a variety of single and double membranes of organelles. For several decades large electric field pulses have been used to perturb the PM in order to deliver ions and molecules into a cell’s cytoplasm. The mechanistic hypothesis of electroporation (EP) is generally accepted, but many important quantitative features are not fully established. A temperature rise is inescapable, but is usually not a significant contributor to creating pores. In this sense, electroporation is a non-thermal effect. Here we show that the transient aqueous pore hypothesis already has significant quantitative successes. The temporary pores are capable of providing access, not only to the cytoplasm, but also to a variety of organelles. We argue that intracellular transport of ions and molecules between organelles is likely the basis of non-thermal cell death, including accidental necrosis and involving aspects of both caspase-dependent and caspase-independent intrinsic apoptosis. These cell death processes are relevant to ongoing investigation and application of irreversible electroporation (IRE) and nsPEF (nanosecond pulsed electric fields). Both are approaches to drug-free, non-thermal tumor ablation that can spare critical structures such as major blood vessels. Overall, our goal is the creation and use of cell-level models that provide useful predictions of complex EP behavior, for which we show recent examples. Our motivation is the prospect of achieving approximate descriptions of electrodiffusive molecular transport, both during and after a pulse. These can aid interpretation and design of experimental protocols, a productivity enhancing capability.

INTRODUCTION

Although widely used in research laboratories, biotechnology development and in clinical medicine, [1,2] electroporation (EP) is not yet well understood quantitatively. This is important. The strong interplay between experiment and theory/modeling that characterizes most scientific research is minimal. The parametric space of EP is very large. Even a relatively simple strength-duration map (Fig.~1) for experimental conditions reveals that about three orders of magnitude of field strength and nine orders of magnitude of time are involved [3]

A more complete EP parameter space includes characteristics of pulses (strength, duration, waveform shape, pulse number), geometry and material of electrodes (the usual way of creating electric fields), properties of colorimetric and fluorescent dyes, and the detection limits and accuracy of the corresponding optical devices. These affect which pulses are used. Figure 1 is simplified, based only on strength and duration.

Basic properties of cells also contribute to this space. Molecular dynamics (MD) studies indicate that the composition of lipids in various cell membranes is important. Continuum models show the importance of cell shapes and size distributions in vitro, the shape and proximity of cells in tissue, as well as the wide variety of different cell types (mammalian, plant and microbial).

Figure 1: EP Strength-Duration map [3].

Taken together this means that comprehensive quantitative understanding still eludes us. There are, however, successes. This is partially illustrated below by the following experimental findings and corresponding modeling.

Continuum modeling successes

There are several examples of successful descriptions of cell EP behavior, mostly electrical and poration behavior, but recently also some of molecular
transport. Below these are presented chronologically, based on when theoretical modeling results were first presented.

**Artificial planar bilayer membranes**

One early bilayer membrane (BLM) experiment shows very different electrical behavior as the pulse changes, with four distinguishable outcomes [4]. These are reversible electrical breakdown, incomplete irreversible electrical breakdown, rupture (irreversible breakdown) and simple passive charging. A dynamic pore model provides approximate quantitative electrical behavior descriptions that display these four outcomes [5].

A related “cubic cell” model based on two planar membranes addresses three basic behaviors: electrical, poration and molecular transport. In addition to transmembrane voltages and pore density distributions, it estimates propidium iodide (PI) transport by electrical drift [6], with comparison to quantitative experimental results for PI uptake in individual members of a cell population [7].

**Cell models**

An early cell model [8] shows that electroporation (EP) holds down the transmembrane voltage. The model shows that the angular distribution of the PM (EP) holds down the transmembrane voltage. The cell models show that even reversible electrical breakdown, incomplete irreversible electrical breakdown, rupture (irreversible breakdown) and simple passive charging. A dynamic pore model provides approximate quantitative electrical behavior descriptions that display these four outcomes [5].

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Different experiments reveal a common qualitative feature: The PM resistance is significantly decreased and only slowly (seconds to minutes) recovers after a pulse. This corresponds to prolonged, reversible post-pulse PM depolarization. Cell models show that even though poration occurs mainly near the poles for conventional EP (pulse duration longer than about 1 μs for typical mammalian cells), $U_m$ approaches zero over the entire PM after the pulse. This can be explained by pores shunting the resting potential source [8,11-14].

Experiments with megavolt per meter, sub-microsecond pulses (nsPEF or nanosecond pulse electric fields) were often initially reported to cause intracellular effects without poration of the PM [15,16], but some did not [17]. This apparent contradiction is consistent with modeling that suggests supra-EP, the appearance of very many but small pores for very large, sub-microsecond fields [11,12]. A large number of small PM pores can provide a large membrane conductance while still restricting transport of larger solutes.

Many nsPEF experiments together suggest that cells can be killed by intrinsic apoptosis, and that the mitochondria are likely involved [18-22]. An initial model with explicit irregular shaped and positioned organelles is consistent with the supra-EP concept, and shows that as field strength is increased smaller organelle membranes are porated along with the PM [12], a candidate mechanism for initiating apoptosis. A subsequent model for an important experiment for a 95 kV/cm, 60 ns pulse [10] shows that EP-based conduction currents through the PM dominate after ~4 ns.

Experimental determinations of Lucifer Yellow (445 Da, charge number $z_{LY} = -2$) transport into a cell were analyzed by a model with diffusion [24]. Using a cylindrical molecule approximation $r_{LY} = 0.61$ nm, $l_{LY} = 1.46$ nm for solute radius and length, respectively) the measurements are also consistent with a cell model with dynamic pores, and solute electrodiffusion within a cell’s cytoplasm and through pore populations both during and after pulsing [25].

Conventional EP (1 ms rectangular pulse) experiments with sea urchin eggs provide $U_m$ measurements based on a voltage-sensitive dye. A dynamic pore cell model [13] reports results which explain all the observed experimental results [26] but the electroporation asymmetry, a major accomplishment.

In addition to the many nsPEF experiments showing apoptosis, experiments using unusually large conventional EP (single “exponential” pulses; 8.1 kV/cm maximum; 40 μs time constant) also report apoptosis [27]. A cell model with explicit organelle representation shows that this strength-duration pulse should electroporate both the outer and inner mitochondrial membranes [28] This is a plausible basis for both caspase-dependent and caspase-independent intrinsic apoptosis [29] as reported for nsPEF exposures [30].

Quantitative modeling of Ca$^{++}$ uptake has also been accomplished, based on electrophoretic transport of extracellular Ca$^{++}$ into a cell during a pulse [31]. This is consistent with experimental observations [32].

A quantitative description of propidium$^{++}$ cell uptake has also been achieved [33], but for nsPEF conditions with reversible EP [17].

Overall, these summaries reveal a progression of successful use of cell level EP models to explain...
Experimental results. With the relatively recent introduction of molecular dynamics (MD) modeling capability is becoming even better.

**EP behavior during and after a pulse**

There is a significant difference between electrical conditions during and after a pulse. As shown in the “life cycle of a pore population” (Fig. 6), during the pulse $U_m$ initially rises rapidly, reversible electrical breakdown (REB) occurs due to a burst of pore creation, and $U_m$ rapidly drops to a quasi-plateau (~0.5 V; Fig. -5). After that, depending on pulse duration, pore populations evolve, leading to both simple (nsPEF) and complex (conventional EP) population behaviour [14].

The EP literature is also distributed extremely unevenly between experimental studies during and after a pulse. This poses a significant problem for understanding of what electroporation is, and what electroporation does. Our EP community goal should be to address this basic problem.

The basic problem is easy to state: If we do not understand what happens during a pulse, when pores are made, and then expand and contract, how do we interpret measurements carried out after a pulse? During a pulse $U_m$ is supra-physiological (~ 0.1 V), with a typical value 5-fold larger (Fig. 5A), and this can drive electrodiffusion of solutes through sufficiently large pores. Post-pulse determinations miss this, except for determining how many solutes have entered into, or departed from, a cell.

Post pulse experiments are of two types: (I) Those that respond to what happened during the pulse (e.g. drug delivery, initiation of accidental necrosis or intrinsic apoptosis), and (II) those that measure and characterize the post-pulse membrane properties. Somehow, it is important to connect these two approaches to the interactions that take place during the pulse.

A simple observation is relevant: If nothing happened during the pulse, the post-pulse studies would not have much to consider. Yet the connection between “during” and “after” is often not really addressed in post-pulse studies, except by a few modeling efforts that treat both during and after explicitly.

The phenomenon of electroporation is a single, continuous process, with complex behavior and dramatic changes at both the beginning and end of the life cycle of pore populations for typical pulses (Fig. 6 life cycle) [14]. Here, to provide examples, we focus on “during”.

**Multiple pulses**

Often experiments use more than one pulse, sometimes tens or even hundreds. Clearly these “pulse trains” can be important, needed to get a desired outcome. If the interval between pulses is less than the mean pore lifetime, memory effects are expected. Unsurprisingly, the main change occurs between the first and second pulse, with progressively lesser changes for additional pulses (Son et al., unpublished). If established this would justify the approximation of simple additive contributions after the first few pulses.

A refinement would apply this approximation by using the second pulse contribution. In any case, the modeling results to date provide a likely justification for multiple pulses: a larger number of transported solute(s) are needed between cell compartments. If so, modeling provides support for the wide use of many (10 to 1,000, occasionally even more) pulses.

**METHODS -- GENERAL**

We use a biophysical modeling approach that is based on non-equilibrium processes. More specifically, we build models that impose potential differences (voltages) which drive non-equilibrium current densities and their associated spatially distributed electric fields [25,34,35]. This approach sets the stage for incorporating other non-equilibrium processes, most particularly chemical reactions.

**Family of prior cell EP models**

Our present models are members of an evolving family of continuum models that describe (1) electrical behavior, (2) poration and (3) molecular transport. The spatial scales range from ~5 nm (approximate cell membrane average thickness) to many micrometers (single to multiple cells) [8,11-14].
Multi-membrane models

The three-cell model in Fig. 2 is an example of a general feature of the meshed transport network (MTN) method [35]. Progressively more complicated models can be created. This shows that multiple membrane models can be created, here different size plasma membranes. Alternatively, multiple membrane models can provide representations of single and double membrane organelles [23].

Not surprisingly, the main limitation is computer power. Extension from 2D to 3D is possible. Our experience, however, is that more insight can presently be gained by constructing and solving complicated 2D models than by going to 3D. At some point 3D will become increasingly compelling. There is no fundamental barrier to doing that, just resources. In the meantime, many aspects of the EP hypothesis can be partially tested by 2D MTN models.

Cylindrical cell model construction

Our models are built from membrane geometries, passive models for bulk aqueous media within cell compartments and complex dynamic pore model interactions. Thus, while both bulk regions and thin membranes are involved, almost all of the computational effort resides in membranes. This is fitting, because the membrane barrier to transport within a cell also resides predominantly within the membranes.

Most studies and applications of EP involve applied fields, not currents. In this general case a full understanding can be found in 2D voltage division within the system. In a very real sense, $U_m(t)$ at different membrane sites depends on both the conductive and dielectric properties of elemental volumes that comprise the system. This is most readily appreciated for entirely passive cell models [34]. But significant EP means that very large, localized conductance changes occur. It is in this context that a rather general view of “voltage division” is recognized as a way of understanding the electrical behavior.

But pore populations and molecular transport through these populations add still more complexity. This involves: (1) electrical behavior, (2) poration behavior and (3) molecular transport, which can together be usefully described by the present MTN model [25,35].

Although many model geometries are possible we presently emphasize the simplest closed, curved membrane – a cylindrical membrane [14].

Fig. 3 shows the geometry of an isolated 2D cylindrical cell membrane. The membrane is located at the interface between light and dark blue regions (left panel), with partially zoomed meshing (center panel) and and the full system meshing 100 μm x 100 μm system scale meshing (right panel) [14,36]. As shown here, this deceptively simple curved membrane system is capable of complex behavior. At each transmembrane node pair we assign a dynamic EP model that involves both creation and destruction of pores, and also pore radius, $r(t)$, evolution governed by a pore energy landscape (Fig. 4).

The pore energy landscape is also shown (Fig. 4). This landscape presents the pore energy as a function of pore radius (interior, minimum radius of a pore near the membrane mid-plane), and also the local transmembrane voltage, $U_m$. This landscape is populated with pores by injecting pores according to an absolute rate equation that describes the average rate of pore creation near the cusp ($r_p = 0.65$ nm). The same landscape has an exit for pore removal - stochastic pore death that occurs according to a second absolute rate equation. Expansion and contraction of dynamic pores is governed by the landscape, itself dependent on the local $U_m$. 

Figure 3: Cell system geometry.
For widely used pulses, pores are created during an initial burst associated with reversible electrical breakdown (REB; Fig. 5A, ~1 μs). If the pulse duration is sufficiently long pores can evolve to form size-distributed populations. These can contain distinguishable small-pore and large-pore subpopulations (Fig. 6).

These subpopulations have significantly different solute transport properties, which is directly relevant to accessing various compartments of cells.

**Figure 4:** Pore energy landscape for selected $U_p$ [14].

**Figure 5:** A: $U_m(t)$; B: $U_m(θ)$; C: Pore Density at $t = 99$ μs.

**RECENT RESULTS AND DISCUSSION**

**Electrical behavior**

For compactness we show a spatially averaged transmembrane voltage, $U_m$ ($t$) (Fig. 5A), and also equipotentials within both the intra- and extra-cellular spaces (Fig. 5C). This provides some sense of what occurs at cell membranes (essential to EP), and also within aqueous media of cell compartments (less important).

**Poration behavior**

The number of pores per membrane area is the “pore density”. This quantity provides a rough estimate of the degree of poration. But it is rough because it does not explicitly include pore sizes. Nevertheless, it is a useful intermediate guide to understanding the complex behavior of a porating curved membrane that approximates a cell plasma membrane (PM).

An example of spatially distributed poration is shown in Fig. 5C, with the white regions within the membrane indicating the pore density (pores per area). The three values depicted in white are 10, 10^3, and 10^5 pores per square micrometer but width varies continuously.

For nsPEF pores are created extremely rapidly. The resulting pore size distributions at local membrane sites are dominated by small pores. Even for the larger fields typically employed (10 – 100 kV/cm) the short pulse duration precludes significant pore expansion.

Fig. 6 gives an example of a life cycle of a pore population for a widely used 1 kV/cm, 100 μs electrochemotherapy (ECT) pulse. It involves many (of order 10,000) pores. Moreover, it is obtained as part of a semi-empirical model's response. By “semi-empirical” we mean that while many of the model inputs are basic, others are obtained from comparisons to experiments, mainly mammalian cell experiments [14].
The resulting pore populations typically have a peak at ~1.5 nm radius during the pulse. This is consistent with the early conclusion that megavolt per meter, sub-microsecond pulses create mostly small pores [11,12,37]. However, for the longer pulses of conventional EP more complex behavior is predicted, with the emergence and subsequent contraction of a large pore subpopulation a major event [14]. The transient existence of a large pore subpopulation is a candidate mechanism for both the introduction and release of macromolecules through membranes.

**Molecular transport behavior**

Electrodiffusion of charged solutes is expected to occur simultaneously with poration and elevated \( U_m \) [38]. Poration creates temporary aqueous pathways of various sizes (see pore population examples; Figs. 6, 7), and \( U_m \) provides a local driving force near (spreading resistance fields) and within (internal pore fields) that support electrodiffusion through a porated membrane [25].

In the bulk aqueous regions defined by curved membranes there is also electrodiffusion, but this contribution is not rate limiting. Why? Because even for nsPEF that heavily porates membranes, the barrier to transport is still the membrane. This is true even for the ubiquitous small ions \( \text{Na}^+, \text{K}^+ \) and \( \text{Cl}^- \) that dominate electrical conductance. It is even more so for larger solutes.

Below we show recent, illustrative results for both calcein (cal) and bovine serum albumin (BSA) efflux. Both molecules are widely used in research, and neither readily passes through an unperturbed cell membrane. We use a cylindrical representation for each solute [25].

Calcein (623 Da, \( r_{cal} = 0.58 \) nm, \( l_{cal} = 1.89 \) nm, \( z_{cal} = -4 \) (simplest representation)), is relatively non-reactive, and widely used as a polar tracer. BSA (~68 kDa, \( r_{BSA} = 4.32 \) nm, \( l_{BSA} = 9.26 \) nm, \( z_{BSA} = -11 \) ) is a well characterized marker macromolecule.

We first show the “before” and “during” pore populations for a 0.8 kV/cm, 10 ms pulse with 1 ns rise/fall times (Fig. 7).
Before the pulse ($t=0$ s) there is on average $\sim 1$ pore on both the anodic and cathodic sides. These are the spontaneous pores, created by thermal fluctuations at the resting transmembrane voltage, $U_{m,\text{rest}} = 50$ mV. These pores contribute insignificantly to BSA transport. For BSA there is also insignificant release after the 10 ms pulse, even though there is a thermalized, slowly decaying population of small pores (not shown, similar to Fig. 6G-H).

In Fig. 8 the solute, $s$, transport rate, $dn_s/dt$, over the entire membranes is very different for calcein (initial concentration, 1 mM) and for BSA (initially, 1 $\mu$M). During the pulse the initial concentration difference accounts for much of those value differences. However, after the pulse, BSA ceases to efflux, while the smaller calcein (green) rate first increases, then plateaus. Our models use the approximation of determining electrical behavior solely through a single small ion that represents the bulk conductivity within an aqueous compartment. Another approximation introduces the transference number so that electrodiffusion of a charged solute is computed from the field only due to the conduction of ubiquitous small ions. Finally, electrodiffusion uses established meshing with non-linear discretization [25,38].

Together these approximations allow our models to simultaneously determine (1) time-dependent $U_m(t)$ values and (2) the corresponding dynamic pore populations at different cell membrane sites, and use these to compute electrodiffusion of charged solutes within bulk media of cellular compartments and through rapidly changing porated membranes. Neutral solutes (e.g. sucrose, inulin) can also be treated, using only the diffusion contribution.

The above example shows calcein efflux during a conventional EP pulse for a 10 $\mu$m radius cylindrical cell. A 1 ns rise time is possible because common commercial cuvettes can have this or even shorter rise times in ordinary arrangements [39].

We use a simulation box that is much larger than the cell [40], but display only a small region near the cell (Fig. 2). We expect a significant efflux of calcein during the pulse, because this solute is highly charged compared to its moderate size, and large pores evolve. This example (Fig. 9) shows that $\sim 66\%$ of the calcein is lost during the pulse. After the pulse pores are small, and the efflux rate drops significantly (Fig. 8).

A second example is BSA for the same pulse. In this case we are interested mainly in the fractional depletion. The model is independent of the absolute concentration value, so we somewhat arbitrarily choose 1 $\mu$M. We assume that BSA is initially uniformly distributed within the cytoplasm. We then solve the model to answer the question: What fraction of the BSA is released? This example shows that $\sim 28\%$ is released.

These two examples (Figs. 9 and 10) demonstrate part of what the present model can do. By choosing a long (10 ms) conventional EP pulse with reversible electroporation, we find that significant fractions of charged molecules, even macromolecules, may be released from a cell. More studies are underway.

**General implications**

There is growing recognition of the use of EP to access intracellular compartments, for both scientific research and technical applications. In addition to drug and gene delivery into cells, there is increasing interest in EP for transport of solutes between different cellular compartments, as this may lead to apoptosis.

One hypothesis is that in a multiple pulse protocol calcium can be released from the endoplasmic reticulum while calcium also enters through a porated PM. With subsequent entry into porated mitochondria resulting in swelling and eventual rupture of the mitochondrial outer membrane (MOM). This releases “death molecules” that trigger apoptosis [41,42]. This maybe be possible for both nsPEF and for some relatively large conventional EP pulses [3].

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Alternatively, significant amounts of cytoplasmic molecules may be released by the multiple pulses used in IRE (irreversible electroporation) for non-thermal tumor ablation. The end result may be a chemical imbalance that is lethal, but membrane poration itself may be reversible on short time-scales.

Another motivation is controlled release of molecules in microfluidics (but we show lysis may not be required) [41]. Still another is investigation of the molecules in microfluidics (but we show lysis may not be required) [41].

These topics give increasing motivation for a quantitative understanding of molecular transport into and from intracellular compartments due to EP.

ACKNOWLEDGEMENT

Supported by NIH grant GM063857 to JCW, and graduate research fellowships from the National Science Foundation and the Harvard-MIT Division of Health Sciences and Technology to KCS.

Due to space limitations, this partial review of our and some other groups modeling cites only some of the many relevant publications, particularly experimental studies. We acknowledge their importance and regret not being able to incorporate them.

REFERENCES


Electroporation based research and resource efficient process development for healthy foods

Dietrich Knorr

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INTRODUCTION

Historically it is of interest to mention that electric fields have been applied for pasteurization of milk on an industrial scale as early as 1925. Pulsed electric fields (PEF) have been used for permeabilization of biological membranes in Germany by Doevenspeck and have also been applied on an industrial scale by the Krupp Company.

After the “re-discovery” of PEF most food related research was aimed towards a replacement for conventional thermal processing for the inactivation of vegetative microorganisms. In addition, work on the permeabilization of plant membranes has been initiated and meanwhile a vast amount of potential applications have been explored. Recently, industrial scale equipment for pasteurization of juices as well as for permeabilization of potatoes (50t/h capacity) has been developed and introduced to the food industry. The wide range of applications explored is demonstrated in Table 1.

Table 1.

<table>
<thead>
<tr>
<th>Food &amp; Biotechnology Applications</th>
<th>Irreversible permeabilization</th>
<th>Reversible permeabilization</th>
<th>Stress induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass transfer</td>
<td>aggregation (fat, starch)</td>
<td>High pressure</td>
<td></td>
</tr>
<tr>
<td>Microorganisms (selective)</td>
<td>nutrient retention</td>
<td>light</td>
<td></td>
</tr>
<tr>
<td>Biopolymers</td>
<td>enzyme retention</td>
<td>temperature</td>
<td></td>
</tr>
<tr>
<td>Texture/structure</td>
<td>freshness retention</td>
<td>ultrasonic</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>permeabilization change</td>
<td></td>
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<tr>
<td></td>
<td>high pressure</td>
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<td>temperature</td>
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<td>ultrasonic</td>
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</table>

The wide range of applications has been demonstrated in Table 1.

Developments and modeling of treatment chamber designs will be presented and sustainability issues as well as food chain integration of PEF discussed.

Finally, consumer acceptance and legal issues will be shown and possible changes in PEF nomenclature described. To demonstrate the need for name clarification the number of terms used for PEF is shown in Table 2. Suggestions for a new terminology and consumer responses are provided in Table 3.
CONCLUSIONS

Food applications for PEF technologies are numerous and the experience gained can and should be transferred to the medical and pharmaceutical fields. Due to the low energy input needed it is a sustainable technology which can contribute to new areas such as bio-economy and re-vitalization of alternative food and feed processing concepts such as single cell proteins or leaf protein concentrate production. This could be one approach to tackle the future challenge of feeding the world.

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[3] Soliva-Fortuny, R., Balasa, A., Knorr, D., Martin-Belloso, O., Effects of pulsed electric fields on bioactive compounds in foods: a review. Trends in Food Science & Technology 20 (2009) 544-556.

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He was Research Associate at the Department of Food Technology in Vienna, Austria, Visiting Scientist at the Western Regional Research Centre of the US Department of Agriculture, Berkeley, USA; at the Department of Food Science Cornell University, Ithaca, USA and of Reading University, Reading, UK.

Prof. Knorr is Editor of the Journal “Innovative Food Science and Emerging Technologies”.

He is President of the European Federation of Food Science and Technology, member of the Governing Council International Union of Food Science and Technology, Member of the International Academy of Food Science and Technology.

He has published approximately 500 scientific papers and holds 7 patents and is one of the ISI “highly cited researchers”.

Table 3.

<table>
<thead>
<tr>
<th>Micro electric pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro pulse is a gentle, low energy consuming and short process for pasteurization of foods as well as for enhancing mass transfer (e.g. expression, extraction, drying) and increasing secondary metabolite production (e.g. antimicrobial or antioxidative substances in plant foods).</td>
</tr>
<tr>
<td>It is based on applying electrical treatments in the microsecond range.</td>
</tr>
</tbody>
</table>

- During the survey the technology descriptions given in the attachment were used.
- The ‘micropulse’ name has better associations (indulgence, short, modern) than pulsed electric field (associated with radiation, unhealthy, unnatural, rough).
- The ‘micropulse’ is in no way associated with electricity among the respondents. After getting information on the two technologies the name ‘micropulse’ was also preferred in scoring to PEF.
- Consumers expressed their definite need of getting informed about the technologies even if this leads to uncertainty and untrust because of eventual lack of comprehensive knowledge.
- Consumers do not regard the name ‘micropulse’ misleading in comparison to PEF, though the electrical feature of the treatment should be mentioned.
- The survey was conducted in two preliminary focus groups: the preferred technologies were HPP and micropulse.
DNA Vaccination Protocols Assisted by Electrotransfer

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Abstract: Due to recent advances in understanding the biology of the immune system, a more rational design of vaccines and vaccination strategies such as those based on gene transfer have been proposed. Plasmid DNA vaccines offer several advantages in comparison to traditional vaccines such as safety, tolerability and feasibility in manufacture. Nevertheless, plasmid DNA vaccines need further implementation. Recent data suggest electrotransfer as a useful tool to improve DNA-based vaccination protocols. Its ability to induce a higher DNA uptake and a stimulation of both humoral and cellular immunity, leads to consider electrotransfer not only as a delivery strategy but also as a good adjuvant in naked DNA vaccination protocols. DNA vaccination mediated by electrotransfer is successfully used in pre-clinical trials protocols, and its efficacy and tolerability has been demonstrated in Phase I clinical trials. This therapeutic approach seems to be promising for application to veterinary medicine and into a wide range of infectious and cancer human diseases.

TRADITIONAL VACCINES AND DNA VACCINES

Vaccination is historically one of the most important methods for the prevention of infectious diseases in humans and animals.

Traditionally, a vaccine is known as a preparation of attenuated or killed microorganisms or of subunit vaccines (purified components of a pathogen including the protein-conjugated capsular polysaccharides, toxoids, cell-free extracts, recombinant proteins and stand-alone capsular polysaccharides) administered for inducing active immunity to a specific disease.

Two types of immunization exist with intrinsic differences between them: prophylactic vaccination initiates a response against an antigen to which the immune response is naïve, leading to a long-term memory cell maintenance and protective efficacy; therapeutic vaccination stimulates the immune system to a chronically displayed antigen, leading to a clearance of an established infection.

Several infectious diseases can be prevented by vaccines produced with conventional approaches. These methods are based on the cultivation in laboratory conditions of the microorganism from which single components are isolated individually by using biochemical, microbiological and serological techniques. Each antigen is produced in pure form either directly from the bacterium or using the DNA recombinant technology, and finally tested for its ability to induce an immune response [1].

Conventional approaches provided the basis of vaccinology and led to great achievements such as the eradication of smallpox and the virtual disappearance of diseases like diphtheria, tetanus, poliomyelitis, pertussis, measles, mumps, rubella and invasive Haemophilus influenzae B, increasing the life quality and expectancy [2]. Nevertheless, they present major disadvantages such as to be time-consuming and, more important, to be impractical in some circumstances due to the difficulty in cultivating some microorganisms in vitro and to the fact that even attenuation may result in detrimental or unwanted immune responses [3]. Moreover, in many cases the antigens expressed during infection are not produced in laboratory conditions, as well as the proteins that are most abundant and easily purified are not necessarily protective antigens and, in any case, only few molecules can be isolated and tested simultaneously [1].

The last decade has witnessed a revolution in the approach to vaccine design and development. These advances include new knowledge in the biology of the immune system that allows a more rational design of vaccines. Due to these studies, new vaccination strategies are emerging.

DNA vaccines have been proposed as a promising approach for introducing foreign antigens into the host for inducing protective immunity and has revealed a number of advantages over conventional vaccinations. They are for definition vectors based on bacterial plasmids engineered to express the disease-specific antigen using promoter elements active in mammalian cells, without the addition of surrounding chemicals or a viral coat. The main advantages of these vaccines respect to conventional vaccines are safety and production in large amounts, as well as stability at different temperatures, their flexibility in design. More importantly, vaccination with naked plasmid DNA encoding antigens was proved to be able to stimulate both cellular and humoral immune responses [4].
Due to their characteristics, plasmid DNA vaccines can be employed as prophylactic strategy to treat viral, bacterial or parasitic infections, or used for infectious diseases, cancers, Alzheimer disease, allergy, and autoimmune disorders [5].

Respect to cancer diseases, is known that conventional therapeutic approaches are invasive and they often cause unpleasant side effect on patients, sometime prolonging patient survival only for short time. Immunisation with tumour-associated antigens or tumour-specific antigens can represent a potential alternative to other therapies when the tumor antigen is known.

Nevertheless, because of their poor immunogenicity, plasmid DNA vaccination strategies need further implementations. Current experimental data obtained in preclinical protocols suggest DNA vaccine efficacy may be increased significantly by several strategies such us optimization of plasmid vectors, antigen selection and optimization of epitopes able to bind to MHC class I and II molecules, use of co-stimulatory molecules and genetic adjuvants, delivery methods among which, electrotransfer is considered as a very efficient tool for intramuscular DNA transfer [6].

In pre-clinical trials, gene electrotransfer is successfully used in prime-boost combination protocols and its tolerability and safety has been demonstrated also in Phase I clinical trials.

**MECHANISM OF ACTION OF DNA VACCINES**

The crucial event responsible for the initiation of an immune response against a foreign antigen is recognition by specialized cells namely the antigen presenting cells (APCs), uptake and presentation of the antigen to naive lymphocytes and induction of effector T helper (Th), cytotoxic (CTL) and B lymphocytes.

The gene sequence of interest is cloned in a plasmid vector that can be delivered intradermally, subcutaneously or intramuscularly.

The delivery of DNA vaccine into skeletal muscle is highly preferable as it represents approximately the 30% of the body mass, is provided by good vasculature and it is easy to access. More importantly, muscle cells are characterized by stable, large syncytial cells containing several nuclei that can actively take part in immune reactions.

Once the plasmid enters the nucleus of transfected local cells, such as myocytes and resident Antigen Presenting Cells (APC), expression of plasmid-encoded antigen takes place. This expression is followed by generation of foreigner antigens as proteins that have been converted to peptide strings. These antigens, synthesized by the host, can become the subject of immune surveillance in the context of both Major Histocompatibility Complex (MHC) class I and clas II molecules of APCs. Antigen-loaded APCs travel to the draining lymphnodes where they present antigenic peptide-MHC complexes combined to signals from costimulatory molecules to naïve cells. This interaction provides the necessary secondary signals to initiate an immune response and to activate and expand T cells or, alternatively, to activate B cells and antibody production cascades [5].

By this mechanism of action, DNA vaccines, differently from traditional vaccines, elicit both humoral and cellular immune responses.

**ROLE OF ELECTROTREATMENT IN DNA VACCINATION**

An important limitation of intramuscular DNA vaccines is their weak performance in large animals as regards the low DNA transfection efficiency due to the tissue barriers encountered [7]. For this reason, novel and safe delivery systems have been developed to further improve the vaccine delivery, in terms of efficiency and immunogenicity.

In several reports, DNA delivery mediated by electrotransfer (ET) was described as an effective tool in eliciting immune response in small and large animal models [8,9], with numerous studies proving that this technique is effective in the stimulation of humoral and cellular immunity [10] and in the increase of DNA delivery [11].

One of the first demonstration of the applicability of ET for gene transfer into a target organ was carried out in 1999 by Mir et al. [12] who proved the high-efficiency of gene transfer into skeletal muscle. Application of electric fields for a short duration on muscle cell surfaces, is supposed to induce transient and localized destabilization of the cell membrane. Then, a higher DNA uptake in vivo is possible thanks to the perturbation provoked in the cell membrane leading to a permeabilization phenomenon and electrophoretic movement of DNA molecules into the target cells. Moreover, if ET is applied in muscle cells, these work as a platform for antigen production within the skeletal muscle [13]. A combination of both these events facilitates target cell transfection, so achieving a higher synthesis of the transfected gene and an intensification of the immune response to the encoded protein.

Despite these evidences, details on possible mechanisms responsible for the positive effect of ET on the immune response to DNA vaccines have not completely characterised [14]. Nevertheless, numerous findings are clarifying ET mechanism [15]. Lot of studies are also devoted to explore the most
appropriate and tolerable parameters suitable for application in human patients [16].

Recently, our studies have reported ET as crucial event through which, inducing transient morphological changes and a local moderate damage in the treated muscle, is possible to generate an early production of endogenous cytokines responsible for signalling danger at the local level. The activation of a danger pro-inflammatory pathway and the recruitment of inflammatory cells result in T lymphocyte migration, indicating electrotransfer per se able to recruit and trigger cells involved in antigen presentation. These ability to induce a higher DNA uptake and a stimulation of both humoral and cellular immunity, leads to consider ET not only as a delivery tool but also as a good adjuvant in naked DNA vaccination protocols [17].

Moreover, in our experience, the combination of hyaluronidase (HYA) with electrotransfer (ET) of DNA vaccine enhances transfection of muscular fibres so increasing expression of the encoded antigen [18]. We wanted to analyze the events occurring in the first two weeks following electrotransfer of the muscle in the presence of hyaluronidase, to verify whether HYA could have a role in the local inflammatory response induced by ET. Our results demonstrated that HYA amplifies ET effect in terms of inflammatory cells recruitment enhancing the early release of IL-1β, TNF-α and IL-6 cytokines. In contrast HYA does not induce Th-1/Th-2 cytokine production, confirming the DNA vaccine is indispensable to induce mediators of antigen-specific immune responses. In the muscle treated with HYA plus ET, we observed inflammatory cell migration in a time window between day 4 and 7 following cytokine induction.

These conclusions can be useful in the choice of prime-boost intervals for optimizing ET-based DNA vaccination protocols [19].

ELECTROTRANSFER IN DNA VACCINATION PROTOCOLS

Plasmids encoding therapeutic genes or DNA vaccines administered by in vivo electrotransfer are tested in some Phase I and Phase II clinical trials and in lot of in preclinical studies. Clinical trials in human patients are focused on cancers (Melanoma, Leukemia, Prostate) and on infectious diseases (HIV, HCV, HPV, Influenza virus and Avian influenza).

For an extensive description of these protocols, see [20,21,22].

DNA vaccination protocols in association with ET have been carried out in the mouse for the therapy of various cancer types such as breast, melanoma and lymphoma. In Balb/C mice progressive clearance of breast malignant lesions induced by Her2/neu antigen and complete protection of all 1-year-old mice have been achieved [23].

Intratumoral injection of plasmid encoding IL-12 with ET in mice with B16.F10 melanoma resulted in the cure of 47% of tumour-bearing mice, and 70% of cured mice were resistant to challenge with B16.F10 cells [24]. Furthermore, it has been shown that gene transfection by intradermal plasmid injection of IL-12 assisted by EP in the peritumoral area of soft tissue sarcoma is able to induce an antitumor response [25]. In a mouse model of lymphoma, ET was demonstrated to improve the animal immune response against the tumour. In particular a DNA plasmid encoding for the variable region of the heavy and light immunoglobulin chain antigen of the BCL1 lymphoma cell line was fused to a peptide domain of the tetanus toxin Fragment C and was used for immunizing animals prior to tumour challenge [26]. A prime/boost approach with naked DNA followed by DNA plus EP increased dramatically the antibody levels against the lymphoma. A similar prime-boost approach was successfully employed in therapeutic vaccination of mice inoculated with the 38C13 lymphoma cell line. The vaccinated mice showed a considerable longer survival rate when the vaccine was administered in the presence of ET [27].

Proof of the importance of animal studies for developing more efficacious and safe ET protocols for human disease therapy, emerges from the numerous works investigating the potential of ET in the administration of HIV vaccines [28,29]. Electrotransfer has also been used to deliver Ad6 vectors and a nucleic acid containing an inactive NS5B RNA-dependent RNA polymerase region of hepatitis C virus (HCV) [30].

Another promising area of study is that of inflammatory diseases, in particular rheumatoid arthritis, where ET of plasmids encoding for human tumor necrosis factor alpha–soluble receptor I variants (hTNFαR-Is) were shown to exert protective effects, with decreased joint destruction in the ankles [31]. Other studies are investigating the use of EP for vaccination against influenza virus. An optimized H5N1 hemagglutinin (HA)-based DNA vaccine administered by intramuscular ET was shown to elicit antibodies that neutralized a panel of virions from various H5N1 viruses and protected the immunized mice from H5N1 virus challenges [32]. The development of naked DNA, coupled or not to electrotransfer technology in animals, not only provides the basis for human studies but also has practical application in veterinary medicine.
In the last decade, some DNA vaccines have been licensed for non-human applications: West Nile virus for horses [33], infectious haematopoietic necrosis virus for salmon [34], growth hormone releasing hormone for swine [35], and treatment of canine melanoma [36].

The initial results in veterinary medicine seem effective. Most clinical trials based on DNA vaccination assisted by electrotransfer are showing promising results. Therefore, it is important to support further researches about the efficacy of DNA vaccines administered by electropermeabilization, in order to successfully translate this new approach in the practical use for human patients.

REFERENCES


NOTES

Emanuela Signori, MSci, PhD, is the group leader of the Laboratory of Molecular Pathology and Experimental Oncology at CNR-IFT, and acting professor of general pathology at University Campus Bio-Medico of Rome, School of Medicine. She has been visiting researcher of the Gene Targeting Unit at the Imperial College in London (UK). She has also been visiting professor at: VIB, Center for Biology of Disease, Catholic University of Leuven (BE); School of Medicine, University of Plovdiv BG); University of Buenos Aires and CONICET (AG); CNRS-IPBS, laboratory of Biophysique Cellulaire and University Paul Sabatier, Toulouse (FR).

She is member of international societies and serves as editorial board member and peer-reviewer in scientific journals. She is author of more than 40 international scientific publications and 4 book chapters.

Since 2000 her research activity is focused on the development of preclinical protocols of DNA vaccination administered by electrotranfer in models of human diseases.

She is a member of the COST Action TD1104 “European network for development of electroporation-based technologies and treatments (EP4Bio2Med)".
STUDENTS’ ABSTRACTS
Comparison of sinusoidal versus square wave pulses for electroporation

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INTRODUCTION
Irreversible Electroporation (IRE) is a non-thermal ablation technique that involves delivering a series of unipolar electric pulses to permanently permeabilize the cell membrane of cancer cells through an increase in transmembrane potential, leading to cell death [1]. Clinically, IRE requires the administration of paralytic agents during treatment to prevent muscle contractions that are associated with the delivery of electric pulses [1]. High-frequency irreversible electroporation (H-FIRE) utilizes bipolar bursts of electrical energy with individual pulse durations that are two orders of magnitude shorter (duration ≈ 1 μs) than in IRE. It has been shown that H-FIRE non-thermally ablates tissue without causing muscle contractions [1]. In the past, models have been used to predict the effects of arbitrarily shaped electroporation pulses on cell membrane conductivity to predict the outcome of experiments and optimize the efficiency of electroporation [2]. In the present study, we investigated the efficiency of sinusoidal versus square wave pulses for H-FIRE.

METHODS
In-vitro experiments with human prostate cancer (DU-145) cells were conducted to identify percent electroporation for the two wave shapes. Bench top instruments such as function generator, oscilloscope and a 100 um electrode slide were used to achieve electric fields of 2000V/cm to 2500 V/cm and pulse widths between 1 to 10us. Propidium Iodide and Calcein AM dyes were used to stain the dead and live cells respectively. A microscope camera and the ImageJ program were used to capture and count the number of stained cells. % electroporation was defined as a ratio of the number of live cells to the number of dead cells multiplied by 100.

RESULTS
It was observed that the % electroporation due to square wave pulses was higher than % electroporation due to sinusoidal pulses at all the test electric fields and pulse widths. However for a given electric field, the difference in % electroporation between the two pulse shapes decreased with increasing frequency (as can be observed in the figures 2 & 3).

REFERENCES
Contribution of Electrochemical Phenomena to the Electroporation Current of Cell Suspension

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Electroporation of cells is being successfully used in biology, biotechnology and medicine. Practical problems still arise in electroporation of cells in suspension. For example, determination of cell electroporation is still demanding and time consuming task. Also electric pulses cause contamination of the solution by the metal released from the electrodes and create local enhancements of electric field, leading to the occurrence of electrochemical reactions at the electrode/electrolyte interface [1, 2, 3]. The goal of this work was the determination of the contribution of the current due to electrochemical phenomena (interface electrode/electrolyte) to the total electroporation current of cell suspension.

In our study we used electrical impedance spectroscopy to obtain an equivalent circuit model to predict the leakage current in the presence of cells before electroporation. The impedance measurement setup consisted of a two electrodes system, where one of the electrodes was used as a working electrode and the second one as a counter electrode. Cuvettes with different distance between electrodes (1, 2 and 4 mm) made of Aluminium parallel plate electrodes were used. Electroporation buffers with different conductivities were used without cells and in presence of CHO cells with a concentration of 10⁷ cells/ml. Impedance spectroscopy measurements were performed by means of Agilent 4284 Precision LCR Meter in the 20 Hz to 1 MHz frequency range.

During the pulses the electric current and voltage were measured and stored on the oscilloscope (LeCroy, 400 MHz) using a current probe (LeCroy 50 A, New York) and a voltage probe (LeCroy 6 kV, New York). A train of eight square pulses of 100 μs duration with 1 Hz repetition frequency were used. Pulse amplitude was varied between (0 to 500 V) resulting in up to 2.5 kV/cm electric field strength as estimated by voltage-to-distance ratio.

Based on Nyquist plots, an equivalent circuit was found to nicely fit the electrical impedance spectroscopy data. The circuit include the double layer capacitance formed at the electrode/electrolyte interface due to the electrode polarisation and the charge transfer resistance due to the occurrence of electrochemical reactions. The parameters of the equivalent circuit for the medium in presence and without cells were determined. The leakage current in time domain was determined by using the Inverse Laplace Transform according to the equivalent circuit model. The simulated current of the medium (without cells) was compared to the measured current of the medium, the simulated current reproduce the current measured during the application of electric pulses. The leakage current was determined by simulation using the same method and taking in account the presence of cells in the medium before electroporation.

The current bypassing the cells was predicted and subtracted from the total measured current to determine the real current due the pore formation and ionic flux during the electric pulse application. The result was correlated to the permeabilization determination by the Propidium Iodide (PI) uptake.

ACKNOWLEDGEMENTS:

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REFERENCES

Electroporation numerical models – research and application

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INTRODUCTION

C3M is a company specialized in development of custom numerical solutions in wide range of application areas, including engineering, converging sciences, biomedicine etc.

The company’s activities in the field of electroporation can be divided into two branches. First is development of specialized Finite Element Method (FEM) model of electroporation process in collaboration with Laboratory of Biocybernetics, Faculty of Electrical Engineering, University of Ljubljana. The second one is development of treatment planning tool based on analytical solution for electric field between pairs of electrodes [1] for IGEA S.p.A.

RESEARCH

FEM numerical model including correlation between electric field strength and tissue electrical conductivity has been developed in AceFEM [2] (Figure 1). Detailed studies of numerical method behaviour, electrode spacing and applied voltage on several simple and complex geometries were made. Benchmark results of these simulations are in line with results from other researchers [3].

APPLICATION

Detailed simulations require long calculation times, that slow down electroporation treatment planning. By using analytical solution instead of complex FEM model we were able to develop an efficient treatment planning tool Pulsar (Figure 2), that couples analytical solution [1] and specialized electrode placement optimization method.

Pulsar allows physician to quickly and efficiently plan electroporation treatment. DICOM image can be used in background to simplify lesion shape drawing and determining forbidden zones (areas where electrodes cannot be placed). Solution can be calculated either for free-hand electrode placement or placement using electrode placement masks.

Goal of electrode placement optimization is to reach sufficient lesion poration with minimum number of electrodes in order to minimize treatment time, invasiveness and expenses.

REFERENCES

Atomic Force Microscopy: a tool to study bacterial cell-wall disruption induced by Pulsed Electric Field

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INTRODUCTION

Atomic Force Microscopy (AFM) is a polyvalent tool that allows biological and mechanical studies of full living microorganisms, and therefore the comprehension of molecular mechanisms at the nanoscale [1]. This technology is particularly relevant to study the variations of morphological and mechanical cell-wall properties in bacteria induced by an environmental stress or drugs [2].

Pulsed Electric Field (PEF) is efficient for bacteria eradication [3]. However, the studies on cell-wall disruption, induced by PEF, are limited. To get a better understanding of the phenomena, we choose to combine AFM and biological studies, in order to explore the biophysical mechanisms involved in bacterial eradication by PEF.

METHODS

*Bacillus pumilus* bacteria were cultivated at 37°C in classical medium. Sporulation of *B. pumilus* was obtained at the same temperature in sporulation medium. PEF experiments were carried out for a bacterial concentration of 107 bacteria per mL. The PEF parameters used were: 1000 or 10000 pulses, 5 μs, 7500 V/cm at a frequency of 1 kHz and a conductivity of 500 μS.

AFM images of bacteria were recorded in liquid with Nanowizard III form JPK (JPK Instruments, Berlin, Germany), in Quantitative Imaging (QI) mode, using MLCT cantilevers.

RESULTS

The first experiment, described below, was a feasibility study to observe morphological characteristics of bacteria by AFM.

*Figure 1*: AFM (QI) height images of *B. pumilus* in vegetative form (A) and in spore form (B).

The second step was to find the best conditions of PEF for eradication, according to bacterial phase growth and bacterial forms. In this aim we performed, after PEF experiments, colony counting tests to measure the diminution of viability.

Table 1: Determination of viability rate (in log10) of *B. pumilus* after PEF in exponential growth, in stationary phase and in spore form.

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<th>Exponential growth</th>
<th>Stationary phase</th>
<th>Spores</th>
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<td><em>n</em> = 1000</td>
<td>2.5 log 10</td>
<td>3.5 log 10</td>
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These results shown a more sensitivity to PEF of bacteria in exponential growth phase, with a loss of viability of 2.5 log10 with only 1000 pulses. For an efficient eradication in stationary growth phase, 10000 pulses are needed. The spores were the most resistant to PEF with only 1 log 10 of decrease with 10000 pulses.

CONCLUSION

We demonstrate here, the potentiality of AFM to observe *B. pumilus* in vegetative and spore forms. Furthermore, we found the best PEF parameters to eradicate different form of *B. pumilus*. The next step will be to study morphological and mechanical properties by AFM after pulses eradication.

REFERENCES


Utilization of non thermal technologies to induce food allergens modifications

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INTRODUCTION

Food allergy is an anomalous response of the immune system against some substances called antigens contained in many foods. The reaction between the antigen and the antagonist antibody is influenced by different parameters among which the structure of the allergen plays the major role. As an alternative to allergen-free diets, processing technologies were investigated and are currently in use able to induce the structural modification of the allergenic proteins.

However, the traditional technologies used to modify the protein structure have also detrimental effects on the organoleptic properties of foods, thus reducing the quality of the products perceived, and in some case show only a weak effect on the allergenic power of the proteins.

For this reason the aim of the project proposed is to investigate the use of innovative technologies such as High Hydrostatic Pressure (HHP), Pulsed light (HILP) and Pulsed Electric Fields (PEF), also in combination with hydrolysis, to reduce the allergenic power of some allergenic proteins, namely albumin, casein, etc.

METHODS

Common allergens (casein, BSA) solubilized in buffer are processed with different technologies at several processing conditions and the level of modifications induced determined through chemical-physical and rheological characterization. In particular structural changes of samples were measured using rheological tests in both stationary and dynamic regime (strain sweep test, frequency sweep test and temperature ramp test), texture analysis, DSC, FTIR, and free SH-groups measurements.

The hydrolysis assisted by non-thermal technologies is characterized in terms of extent of hydrolysis, SDS-page investigation, peptide profile analysis, and the modelling of enzymatic kinetics is also set up.

RESULTS AND FIGURES

HHP treatment, the first non thermal method investigated, is able to induce protein denaturation and aggregation. In fact, loss and storage moduli (Fig.1) of BSA treated by HPP increase with increasing the pressure and treatment time [1]. Protein unfolding influences enzymatic hydrolysis of allergens (Fig. 2) exposing some peptide bonds in the external part of the globular protein. Results show that the degree of hydrolysis is higher in HHP treated samples than in those processed at atmospheric pressure. However, at very high pressure levels the enzymes inactivation takes place and the hydrolysis degree is reduced [2], also due to protein aggregation.

Figure 1: Storage and loss moduli of BSA after HPP (700-900 MPa, 25 min).

Figure 2: Hydrolysis degree of BSA treated at different pressures with chymotrypsin and trypsin enzymes.

A similar characterization will be carried out on proteins treated by PEF/ hydrolysis assisted by PEF to verify the effects of this technology on allergens denaturation/aggregation. Since polypeptide chains in protein possess a strong dipole moment, they can be potentially affected by an external electric field which induces an increase in the dielectric constant of protein, which in turn can cause polypeptide unfolding [3].

REFERENCES

In Situ Detection of Changes in Lipids Environment using Raman Effect

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This abstract presents the main aims of my PhD.

INTRODUCTION
Electroporation is a consequence of an interaction between pulsed electric field and membranes leading to the uptake of molecules (including water) through the membrane. Many applications based on this biophysical have been developed in medicine and in the food industry during the last 20 years.

PhD OBJECTIVES
Although electroporation has been known for decades, the underlying mechanisms leading to the membrane electroporation are still not fully understood [1]. One of the most credible hypotheses of the membrane destabilization is the oxidation of the membrane phospholipids due to electrical excitation [2]. The aim of my PhD is to verify this hypothesis and quantify the oxidative phenomenon using Raman spectroscopy.

MATERIALS AND METHODS
The Raman effect is an inelastic light scattering depending on vibrational modes of the chemical bonds of the sample [3]. In the light of quantum mechanics, each type of chemical bond in a sample can be decomposed in vibrational modes associated with a vibrational frequency $\Omega_{\text{vib}}$. A sample is defined intrinsically by a spectral signature depending on this decomposition and reflecting its chemical bond types. When a sample is excited by a laser beam, there are two kinds of light scattering characterized by different frequencies: Rayleigh and Raman scattering (Figure 1).

![Figure 1: Rayleigh and Raman scattering.](image)

The spontaneous Raman spectroscopy consists in exciting a sample with laser beam to probe its spectral signature by detecting the Anti-stokes or Stokes transitions.

Coherent Anti-Stokes Raman Spectroscopy (CARS) is a non-linear third-order optical process. It involves the mixing of four waves in order to increase the Raman signal by exciting coherently the vibrational modes and stimulating the anti-stokes transition (Figure 2).

![Figure 2: CARS excitation and relaxation.](image)

A CARS system synchronized with an external pulse generator allows acquiring short band frequency of the sample during and after the pulse with a high time resolution [4].

This study will be performed on two study models: Giant Unilamellar Vesicles (GUV) of phospholipids, which are a simple mimic of cell membranes and DC-3F (chinese hamster lung fibroblast cell) a classical study model in electroporation.

PERSPECTIVES
Comparing the spectral signature of the samples (GUV and DC-3F) before and after electroporation, the spontaneous Raman spectroscopy will allow the determination of the most critical band frequencies to characterize electroporation. The CARS spectroscopy permits to quantify the evolution of those critical bands during the pulse and immediately after in order to acquire specific temporal information.

REFERENCES
Investigation of refractive index of electroporated cells using digital holographic microscopy

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INTRODUCTION

Part of a PhD project starting in October 2013, the present study is dedicated to the investigation of optical and mechanical properties of electroporated cells and vesicle lipid models (giant unilamellar vesicles, GUVs).

Several methods are intended to be used: optical tweezers, digital holographic microscopy (DHM) [1] and a combined dielectro-optical trap [2].

By using DHM we propose evaluating important biophysical cell parameters like shape, volume and refractive indices of cells and GUVs consecutive to various electroporation conditions.

DHM METHOD DESCRIPTION

DHM is an interferometric technique visualizing morphological and structural parameters dynamic of transparent specimens without labels or staining, with a nanoscale axial sensitivity and under milliseconds time resolution [3]. It is already used in biology, microstructure characterization, fast processes monitoring etc. The validity of the refractive index measurement DHM-based technique is demonstrated at single cell level: erythrocytes, streptococcus [4], using its ability to find minute phase retardation induced on the transmitted wavefront by the transparent specimen.

The two steps involved in DHM are: hologram recording (experimentally) and image reconstruction (digitally). The experimental setup is based on the Mach-Zehnder interferometer, with the investigated object positioned in one arm. Identical microscope objectives are used in both reference and object arms, to have the same wavefront curvatures in the recording plane (CCD sensor).

Based on the Fresnel-Kirchhoff integral, the reconstruction algorithm delivers the 3D image of the object starting from one single hologram (an intensity image).

SETUPS

For electroporation Electro Cell B10 rectangular bipolar pulses generator (Toulouse) (1 kV max pulse amplitude, max 10 A electric current, min 5 μs pulse duration) will be used. Two consecutive holograms will be acquired for the same area of the sample, situated in two isomolar extracellular solutions of different refractive indices (appropriate values). For fluid changes, a microfluidic system (Cell MicroControls, Norfolk) is adapted to the object stage of DHM system. The DHM device located at Politehnica University consists in:

- experimental off-axis setup for digitally recording of the microscopic objects hologram on high resolution CCD (2048x2048 pixels with 7.4μm pitch) or high speed CMOS video camera (maximum 675 000f/s),
- dedicated commercial software based on Fresnel transform to simulate the laser beam propagation through the hologram to obtain the 3D image of the reconstructed object and quantitative information about the cell intracellular content, via its refractive index values in each pixel.

PERSPECTIVES

- Fundamental research for investigation of optical properties and physical parameters of cells during and after a dynamic process like electroporation.
- Extension and improvement of DHM method to determinate if the refractive indices present significant variations between normal and pathological cells. The optical methods need to be combined with genetic and immunological cell exploration for developing clinically oriented research.

REFERENCES

Electric field-induced yeast cells permeabilization using micro- and nanosecond duration pulses

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INTRODUCTION
Yeasts, Saccharomyces cerevisiae, have been considered prototypical eukaryotic cells, ideally suited for use in studies of many of the basic phenomena of eukaryotic life and some of their fundamental properties [1, 2]. The yeast cells are surrounded by cell walls, which provide them not only with protection from osmotic stress, but are also important for their defence against toxic compounds, for self-recognition and for flocculation [3, 4]. It has to be mention that yeast cell wall playing main role in the natural aggregation of yeast cells. The purpose of this study was to investigate the permeability of viable yeast cells after exposure to PEF using the indirect TPP⁺-selective probe-based method.

MATERIALS AND METHODS
The yeast S. cerevisiae strain SEY6210 suspended in the electroporation buffer containing 1 M sorbitol 20 mM Tris-HCl buffer, pH 7.4 was used.

Two different experimental pulsed electric field generator was used. A programmable high-voltage square-wave electric pulse generator was used for the electroporation of the yeast. The device was designed in our center and it is able to generate a single and sequence of single electrical pulses with widths from 3 μs to 10 ms and amplitudes up to 4 kV. It has variable energy storage capacitor thus accumulation of high energy is avoided. LCD screen for the display of the pulse waveform during experiment makes the unit easy to operate. In order to generate a single electric pulses having discrete durations from 10 ns to 92 ns and amplitude up to 18 kV.

A combined tetraphenylphosphonium selective electrode was used for TPP⁺ accumulation analysis. This electrode has been described previously [5].

RESULTS
The experimental investigation of TPP⁺ absorption by yeast proved that the rate of TPP⁺ accumulation was accelerated by PEF, because the TPP⁺ concentration in the suspension decreased up to several times compared with that in the initial suspension.

To describe and modelling the TPP⁺ accumulation process the second order kinetic equation was used. The characteristic time constants of the kinetics processes were obtained by the fitting procedure. The time constants were 48.6 min for yeasts untreated by PEF and 19.8 min for those affected by PEF. Moreover, evaluation of the TPP⁺ kinetics demonstrates that the PEF affects the rate of TPP⁺ uptake, but not the TPP⁺ accumulation level. Applying a 60 ns duration PEF pulses, the TPP⁺ absorption rate increase up to 65 times comparing with the untreated yeast cells (Fig. 1). It has to be mention, that increasing the strength of pulsed electric field up to 200 kV/cm, the survivor rate was decreasing only by 0.6 log.

In conclusion, the experimentally observed dependence between the threshold electric field and pulse duration in the range from 5 μs to 150 μs and 60 ns can be explained by membrane electroporation, taking into consideration that the rate of TPP⁺ absorption depends on the energy of electrical pulse. Additionally, 3.5 times higher effectiveness of nanosecond pulse on the yeast cell wall permeabilization can be explain by the assumption, that nanosecond pulses can charge separated yeast cells in the aggregates of cells, unlike using microsecond duration pulses, the electric fiels is focused on yeast aggregates.

REFERENCES
Electroporation of the green Microalgae *Auxenochlorella protothecoides*

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**INTRODUCTION**

Microalgae are phototrophic microorganisms. They are able to produce many commercially interesting compounds like polysaccharides, proteins, lipids and pigments that can be used for food, feed, renewable materials and biofuel production. Compared to terrestrial plants they have higher aerial production rates, they need less water and less fertilizer and they can be cultivated on non arable land. However, many of the valuables algae produce are stored intracellular and the extraction of them involves a cell disintegration step. An efficient and sustainable method for algae cell disintegration is still not available, although a lot of research is currently going on in this field. Electroporation may be a promising alternative to conventional cell disintegration methods. In our studies we investigated the application of PEF for the cell disintegration of the fresh water microalgae *Auxenochlorella protothecoides*.

**MATERIALS AND METHODS**

The microalgae *A. protothecoides* were grown in a closed photo-bioreactor up to a cell density of 5 g/l (dry mass) and a lipid content of 15-20% (by weight). Immediately after harvest we concentrated the algae suspensions to biomass contents of 50-200 g/l and PEF treated them with square pulses (pulse duration 1 μs). Electric field strength and specific energy input varied in the following range: (23-43 kV/cm and 52 - 211 kJ/kg\(_{\text{suspension}}\)).

After PEF-treatment the microalgae were removed from the suspension by centrifugation. Dry weight, total organic carbon (TOC) and carbohydrate concentration of the supernatant were analysed. Freeze dried samples of the pellet were used for lipid extraction experiments. We performed the extraction with 70 % ethanol and determined the amount of extracted lipids gravimetrically.

**RESULTS AND FIGURES**

For all pulse parameters applied, the PEF induced cell disintegration resulted in the spontaneous release of soluble intracellular matter into the suspension. The disintegration efficiency increased with increasing specific treatment energy (Fig. 1), whereas the field strength hardly had any influence. Increasing the suspension’s biomass content did not have a negative effect on cell disintegration. According to dry mass analysis, 10 – 15% of the initial biomass remained in the supernatant. Lipids were not spontaneously released. However, in solvent extraction the lipid yield from PEF treated cells was 3 to 5 times higher compared to the control (Fig. 2).

Cell disintegration with PEF might offer the opportunity to perform selective two-step extractions in algae biorefinery concepts. In a first step, PEF treatment allows to separate water soluble intracellular substances. In a subsequent step, lipids can be extracted by solvents at high efficiency.
Microalgae – raw material for the future

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INTRODUCTION
Lipids delivered from microalgae present big potential in the production of biodiesel. In comparison to farm crops lipids (soy bean, corn, rape), microalgae fast growth and nondemanding environment conditions are suitable for their growth off the fertile farm land in waste water treatment plants, biogas plants or in the sea. The specific surface area of the algae is large which enables higher CO2 absorption rate and subsequently better photosynthesis efficiency.

When subjected to starving conditions, microalgal lipids can reach up to 50% of cell dry mass at the cost of lower yield. Starving conditions based on nitrogen limitation is the single most crucial parameter affecting lipid metabolism. In addition to lipids microalgae can be steered in higher production of omega 3 fatty acids, amino acids, proteins and other useful products which classifies them as microbioreactors, interesting also for dietary industry and pharmaceutical. Implementation of ecological and energy saving method of lipid extraction by means of electroporation makes the process of biodiesel production cheaper and potentially enables retreatment of algal biomass or selling uncontaminated algae as a dietary supplement

METHOD
The microalgae Chlorella vulgaris was first cultivated in a 9 l laboratory scale bioreactor with implemented mixing and aeration. The broth was cultivated for one week in a nitrogen low medium (modified Jaworski medium) under 16 hours of artificial light daily before it was transferred in to 60 l pilot scale photobioreactor with the same conditions. During their growth pH values and oxygen consumptions were monitored automatically. pH was regulated via computer program and enforced with CO2 blowing into the reactor. The broth was eared with 1000 l/h of air. Size of the cell was monitored daily. After 14 days of growth the average cell size measured with NIS elements BR3.10 (NIKON) was 3.8 μm.

For extraction of lipids with organic solvent after growing procedure, algal broth was left to settle for a week in refrigerator. Then the broth was centrifuged, and biomass was lyophilized. Cell membranes were disrupted with acid and lipids were extracted with petroleum ether.

For lipid extraction with electroporation the same broth was used as in the previous test. The broth was pumped from photobioreactor through the continuous flow electroporation chamber with the minimum of 600 ml/min which guaranteed no air bubble was present in the tube during the process. The conductivity of the medium was measured beforehand to avoid break thru of the electrical field. The conductivity was 1 mS/cm².

The electroporation process took place for one hour. The parameters used was voltage to distance ratio=3,5 kV/cm, I=2 A, t=100 μs, v=10 Hz. It is important to state that the shape of the pulse was square.

PRELIMINARY RESULTS
On average every cell in the broth was exposed to 21 electrical pulses, which is 2.1 seconds. After electroporation cells were left to sediment for a week in a refrigerator. The lipid content was skimmed from the top and dried in a pre-weighted vessel for three days on 30°C to avoid boiling. At the end the dry vessel was weighted and from the difference in mass the lipid content was established. From 2 l of broth with 0.2 kg/m3 of algae 0.14 g of lipids was obtained which equals to 22 % of total lipid content of dry algae mass.

REFERENCES
Design and modelling of capacitor-based nanosecond pulse generator and applicator

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INTRODUCTION
Nanosecond electroporation provides a potential way for intracellular manipulation [1]. However, the nanosecond pulse generator is quite challengeable for both research and application. A capacitor-based circuit is designed here to try to provide the pulse flexible width and amplitude.

METHODS
Different from transmission-lined based pulse generator, the capacitor one can provide more control on the pulse width by controlling the charging or discharging time [2].

The circuit is designed in the software Agilent ADS as shown in Figure 1(a). BASYS2 FPGA board acts as the source of low-voltage pulse to the gate of MOSFET STW9N150, which controls the charge and discharge of the energy storage capacitor C1. The spice models of the components are loaded into ADS to provide more closed results to the real scenario. Certain measures have been taken to protect the circuit under the high DC power supply, including the gate-side current limiting resistor, gate-source side transient voltage suppressor diode, source-side current-limiting resistor and drain-source side parallel large resistor.

The capacitance of the energy storage capacitor needs to be chosen wisely. On one hand, it should be as large as possible to provide enough energy to the load; on the other hand, it has to satisfy the requirements on the pulse repetitive rate which reduces the capacitance in order to charge or discharge more quickly. The capacitance used in this model is 0.5uF, which provides at least 5 kHz repetition rate for the nanosecond-range pulse.

RESULTS AND ANALYSIS
The results of the voltage at the load side are shown in Figure 2(a). About 90% of the DC voltage is delivered into the load. The loss is coming from the on-state MOSFET and source-side resistance. The rise and fall time of the pulse take a certain amount of time due to the delay in MOSFET. This is the inevitable effect and the main drawback compared with the transmission-line scheme. But the pulse width can be flexibly manipulated by the programme inside the microcontroller.

From Figure 2(b), a good field distribution inside the gap is observed. The quasi-uniform electric field can ensure that each cell inside can withstand almost the same electric field configuration. The work for the next step is to realize these hardware designs in the real case and conduct the further electroporation experiment.

REFERENCES
Correlation between Cancer Cell Metabolism and sensitivity to Treatment with Calcium Electroporation, Investigated In Vitro
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INTRODUCTION
Electroporation is a method used to generate a transient added permeability of the cell membrane by applying short, high voltage electric pulses through cell medium or tissue. The method can be used to facilitate transport of otherwise non-permeant ions such as calcium, through simple diffusion.

Electroporation is used in the clinic in combination with chemotherapeutic drugs for local treatment of malignant tumors[1]. Calcium used instead of chemotherapeutics may prove to be a novel anti-cancer treatment, and our study hopes to further the understanding of the mechanisms behind this treatment.

METHODS
Previous studies show that SW780, a human bladder cancer cell line, has low sensitivity for calcium electroporation with only 33% necrosis two days after treatment[2].

For determining the SW780 cell-line’s sensitivity for calcium electroporation we used pre-treatment of 0, 1 or 5mM of calcium with or without consequent electroporation with 0.8, 1.0 and 1.2kV/cm.

Electroporation was applied using an electroporator and 0.4cm wide electroporation cuvettes.

IC50 indicates the dose of calcium needed in calcium electroporation to decrease the viability with 50%. The viability is measured using MTS assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay) which is a colorimetric method for determining the number of viable cells in proliferation- or sensibility assays. MTS is a tetrizolium salt that is bioreductable to a formazan product. The absorbans of formazan at 490 nm can be directly measured from 96-well assay plates, and is directly proportional to the number of living cells in the culture.

RESULTS
The data showed dose-dependant decrease in viability in the SW780 cell-line in vitro.

The lowest calcium concentration needed for 50% cell necrosis was found at the highest voltage used with IC50 = 0.33mM at 1.2kV/cm.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>IC50 [Ca²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0kV/cm</td>
<td>Unassessable</td>
</tr>
<tr>
<td>0.8kV/cm</td>
<td>1.81 mM</td>
</tr>
<tr>
<td>1.0kV/cm</td>
<td>0.52 mM</td>
</tr>
<tr>
<td>1.2kV/cm</td>
<td>0.33 mM</td>
</tr>
</tbody>
</table>

Similar effects were obtained at 1.0 and 1.2kV/cm. Treatment with calcium alone had little effect on cell necrosis. The data shows a considerable necrotic effect of the lowest applied voltage (0.8kV/cm) without pre-treatment with calcium (Figure 1). This could be induced by low cell viability caused by extensive processing of the cell culture.

REFERENCES
In vitro cytotoxic effect of the biomaterials to the primary human cell cultures

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INTRODUCTION

In vitro cytotoxicity is crucial for evaluation of new biomaterial biocompatibility before the biomaterial can enter animal studies in order to enhance the 3R principle and to reduce the costs of animal testing. Testing the biomaterial is necessary when the biomaterial is intended for clinical use. In vitro testing using cell lines are most commonly used to assess the cytotoxicity of biomaterials. However testing on primary human cell cultures yields more accurate results since it better imitates the body conditions of the tissue that biomaterial will be in contact with upon implantation. In our study two biomaterials in development for bone regeneration on primary human cell culture were tested.

It is important to provide highly accurate and sensitive methods for measuring the number and vitality of cells in order to reliably detect cytotoxicity. Materials that are intended for medical implants should cause no toxicity in vivo. However in vitro testing systems are more sensitive thus several different methods have to be used to distinguish between no cytotoxicity or low cytotoxic responses, which will not be cytotoxic in vivo and stronger cytotoxicity, which will be noted in vivo. In our study two systems for cytotoxicity testing were used, direct contact testing system and extraction testing system [1].

EXPERIMENTAL METHODS

In direct contact testing system, the primary human mesenchymal stromal cells (hMSC) were cultivated in direct contact with biomaterial. The growth and morphology of the cells was observed for 8 days and the zone of inhibition was measured.

In extraction system the extract of the biomaterials was added to the growing hMSC. The cytotoxic effect was defined in three time points by measuring viability and enzyme activity of the testing cells relative to control.

The cells in the study were isolated in our laboratory from the donor tissues with enzymatic and mechanical degradation.

RESULTS AND DISCUSSION

In direct contact system, biomaterial 1 (Figure 1c) formed no zone of inhibition, whereas biomaterial 2 exhibited huge zone of inhibition (the entire vessel was almost deprived of cells, Figure 1 d).

In extraction testing system (Figure 2) biomaterial 1 exhibited non to low cytotoxicity, while biomaterial 2 exhibited low cytotoxicity.

In our experiments, the pH for extraction system was adjusted and pH for direct contact system was not adjusted. Therefore the reduced impact in extraction test system indicates that the cytotoxic effect in direct contact testing system was largely due to pH change, which is most obvious in biomaterial 2, since some alkaline component was dissolved into media causing greater cytotoxic effect.

CONCLUSION

Biomaterial 1 exhibits low cytotoxicity in both testing systems. On the other hand, biomaterial 2 exhibits low cytotoxicity in extraction testing system and severe cytotoxicity in direct testing system. The cytotoxicity of biomaterial 2 is largely due to pH change. Therefore the use of biomaterial 1 would be preferred over biomaterial 2.

REFERENCES

Dual-porosity model of solute extraction by diffusion from electroporated tissue

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INTRODUCTION
We present a theoretical model developed in an attempt to advance the knowledge in modelling of enhanced mass transport due to electroporation. Tissue is represented as a dual-porosity medium; one medium is the intracellular, and the other the extracellular space. Separating the two media is the cell membrane of variable permeability. The model is based on porous media theory, applied for a particular case of plant tissue electroporation, but can, with suitable modifications, be applicable in biomedicine and other fields of research concerning diffusion on tissue level. In this initial attempt at building a dual-porosity model, we focus ourselves only to diffusion of small molecules (several kDa) post-pulse, when the effects of the electric field are no longer present or is negligible. We model effects of electroporation via electric field effects on transmembrane diffusivity, and the practical example presented is that of sucrose extracted from electroporated sugar beet tissue.

THEORY
Observing inter-to-extracellular transport and diffusion of matter to the outside of a block of tissue of thickness \( h \) suspended in an aqueous solution, the mass conservation law gives the set of equations 1–2. Eq. 1 holds for extracellular concentration. In eq. 2 for the intracellular concentration.

\[
\frac{\partial c_i}{\partial t} - D_i \frac{\partial^2 c_i}{\partial x^2} - f \cdot P_m (c_i - c_e) = 0 \quad (1)
\]

\[
\frac{\partial c_e}{\partial t} + f \cdot P_m (c_i - c_e) = 0 \quad (2)
\]

In above equations, \( D_i \) is the solute diffusion coefficient in extracellular space, \( P_m \) is the membrane permeability coefficient, and \( f \) is a geometrical factor (surface-to-volume ratio). We model only a half-block of tissue due to symmetry. The boundary conditions (bc) demand that \( c_e \) be zero at \( h/2 \) (infinite dilution). On the other hand, \( c_i \) at this boundary is prescribed by eq. 2 and bc for \( c_e \). The central plane of symmetry is a no-flux boundary, setting the spatial derivative of both \( c_i \) and \( c_i \) to 0. Initial condition (ic) is assumed constant, i.e. \( c_i(x,0) = c_{i0} \), \( c_e(x,0) = c_{e0} \). The solution of eq. 1–2 at these bc and ic gives the intra- and extracellular concentration as a function of space and time, given by final model equations 3–4.

In eq. 3–4, \( P \) is short for \( f P_m \), \( K_{i2} \) are constants to satisfy initial conditions, \( \gamma_{12} \) are roots of characteristic polynomial with coefficients \( a = 1, b = 2P + \lambda_{a}^2 D_i, c = \lambda_{a}^2 D_i, \) and \( \lambda_a = (2n+1)\pi/h \). The effect of electroporation is captured in \( P_m \) which is a function of membrane geometry, pore surface fraction, and hydrodynamic hindrance imposed by the pore on the diffusing solute.

\[
c_i(x,t) = \frac{4c_{i0}}{\pi} \sum_{n=0}^{\infty} \left[ \frac{(-1)^n}{2n+1} \cos(\lambda_a x) \left( K_1 e^{\alpha_1 t} + K_2 e^{\alpha_2 t} - e^{-\alpha_1 P} \right) \right] + c_{i0} e^{-\alpha_1 P} \quad (3)
\]

\[
c_e(x,t) = \frac{4c_{e0}}{\pi} \sum_{n=0}^{\infty} \left[ \frac{(-1)^n}{2n+1} \cos(\lambda_a x) \right. \\
\left. \cdot \left( K_1 e^{\alpha_1 t} \left( \frac{2_1}{P} + 1 \right) + K_2 e^{\alpha_2 t} \left( \frac{2_2}{P} + 1 \right) \right) \right] \quad (4)
\]

RESULTS
We present results obtained using previously published experiments [1], modelled by the dual-porosity model as well as the model of diffusion out of a homogeneous plane sheet [2]. Comparison is given in Figure 1. Best fit to experimental data provides the dual-porosity model.

CONCLUSIONS
By proposing a dependence of transmembrane diffusion on the average pore radius and surface fraction of electroporated cells, we introduce the theory of electroporation to modelling of experiments conducted on biological tissue. The novelty in our approach lies in a new level of complexity introduced into the model, which provides advantage when compared to performance of models of diffusion out of a plane sheet used until now.

REFERENCES
Migration of skin dendritic cells after IL-12 electro-geno-therapy

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INTRODUCTION
Electroporation (EP) increases the efficacy of DNA vaccination by enhancing the transfection rate of the target gene in situ and subsequent activation of immune cells. In this project we propose an innovative concept based on a strong and durable expression of a gene that amplifies attraction and activation of several subsets of dendritic cells (DC) in the tumor periphery: the IL-12 cytokine. The expression is induced by electrogenotherapy (EGT) of IL-12 plasmid. We combine plasmid EGT with Partial Irreversible electroporation (PIRE) that increases the release of tumor antigens by inducing apoptosis and necrosis of tumor cells. The goal of this project is to induce an effective tumor regression in a murine melanoma model and to characterize the uptake of tumor antigens by DC, their activation, and migration from treated skin to draining lymph nodes upon treatment. Moreover, we will analyze the recruitment of new DC or other immune cells at the tumor site. Finally we will validate the combined use of EGT and PIRE to enhance the efficacy of immunity against tumor.

METHODS
In the skin, two major DC populations can be distinguished; the dermal DC characterized by the expression on their surface of the CD11c molecule and the epidermal DC that express the langerin. CD11c-eGFP transgenic mice that express the GFP allowed the specific discrimination of dermal DC. The EGT protocol we applied consists in 4 HV-MV trains of pulses (1000 V/cm, 100 μs and 250 V/cm, 20 ms). Lymph nodes (LN) and 1.5x1.5cm samples of treated and untreated skin from the back of the mouse were harvested 1, 4, 7 and 14 days after EP treatment. LN cells were resuspended in PBS 1x + 2.5% FBS. Skin samples were incubated for 24h at 4°C in dispase solution to allow dermis and epidermis dissociation. DCs were extracted from the tissues by 30 min treatment with Trypsin-EDTA at 37°C. The percentage of CD11c-eGFP+ cells was analyzed by flow cytometry.

RESULTS
Upon HV-MV treatment, we observed a continuous decrease in the percentage of DCs in the skin dermis until day 4 and an increase of this percentage to reach the basal level at day 8. In parallel, we observed an increase in the percentage of these cells in the draining lymph nodes with a maximum reached at day 4, followed by a plateau value between 4 and 7 days after treatment.

CONCLUSION
These results suggest that EGT by HV-MV pulses, by itself, induces the migration of dermal DCs from skin to the draining lymph nodes. We hypothesize that the combination of IL-12 plasmid transfection by EGT (HV-MV) with PIRE treatment of melanoma could strongly activate the skin dendritic cells and induce an effective immune response against the primary tumor and possibly against metastases.

ACKNOWLEDGMENTS
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**In vitro and in vivo effects of anti-endoglin therapy in mouse melanoma**

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**INTRODUCTION**

Endoglin is a transforming growth factor-β (TGF-β) co-receptor, which participates in the activation of a complex signaling pathway that mediates endothelial cell proliferation and migration in angiogenic tumor vasculature [1]. It is also expressed in melanoma cells, but its function is not yet well known. In our previous study, we have shown that silencing of endoglin using small interfering RNA (siRNA) molecules in *in vitro* and in *in vivo* exerts pronounced antitumor and specific antiangiogenic response in non-endoglin expressing murine mammary carcinoma [2]. To further explore antiangiogenic and antitumor effect of endoglin silencing, we evaluated its therapeutic potential in endoglin expressing melanoma cells *in vitro* and *in vivo*.

**METHODS**

Two murine melanoma cell lines, B16F1 with low and B16F10 with high metastatic potential, were used in this study. Firstly, endoglin expression in these cell lines was determined at mRNA level with quantitative real time polymerase chain reaction (qRT-PCR). Based on our previous data with siRNA, therapeutic plasmid encoding microRNA (miRNA) against endoglin, pENTR/U6 CD105 was constructed. *In vitro*, gene electrotransfer (GET) was performed by mixing 10 µg of plasmid DNA (1µg/µl), therapeutic or control pENTR/U6 SCR, with 1x10⁶ cells, which were immediately thereafter exposed to 8 high voltage electric pulses (dₚ = 2mm, 600 V/cm, 5 ms, 1 Hz). After 5 min incubation time clonogenic assay was performed, by plating cells to Petri dishes and stained 7 days thereafter. *In vivo*, tumors were induced by injecting 1.1x10⁶ B16F1 or B16F10 melanoma cells subcutaneously in the right flank of C57BL/6 mice. When tumors grew up to 3 mm in the longest diameter, GET was performed on days 0 and 2. Tumors were injected with 25 µl of plasmid (4µg/µl) and after 10 min exposed to 8 high voltage electric pulses (dₑ = 4 or 6 mm, 600 V/cm, 5 ms, 1 Hz). Tumors were measured until they reached 350 mm³ or followed up to 100 days in cases where complete regression of the tumors was achieved. Thereafter, tumor growth delay was calculated.

**RESULTS AND FIGURES**

Level of endoglin mRNA in both melanoma cell lines was relatively high and comparable to murine endothelial cell line 2H11 [2]. Both, therapeutic and control plasmid in combination with GET, significantly reduced cell survival in comparison to all other groups. This effect was more pronounced in B16F1 cells. The survival of cells treated by GET and control or therapeutic plasmid DNA was reduced to the same level, indicating that the reduced survival was predominantly due to the cytotoxicity of GET DNA alone.

*In vivo*, the growth delay of B16F1 tumors treated with either of the both plasmid DNAs in combination with GET was prolonged to the same extent. In B16F10 melanoma, the antitumor effectiveness of therapeutic plasmid combined with GET was more pronounced and resulted in significantly prolonged tumor growth delay in comparison to tumor growth delay of tumors in all other treatment groups.

**CONCLUSIONS**

Taken together, these results demonstrate that silencing of endoglin has mainly antiangiogenic effect. Therefore this therapy is a feasible approach in treatment of metastatic melanoma, although additional experiments are required.

**REFERENCES**


Characterization of patients with feline injection-site sarcoma as body condition score and as to the origin of its formation and their microenvironment.

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INTRODUCTION
The feline injection-site sarcoma (FISS) has specific characteristics that are little studied. The FISS becomes a new challenge for veterinary oncologists. [1].

FISS treatment is a hurdle to getting because of their aggressiveness, with profuse invasion of adjacent tissue, as well as the presence of anaplastic cells in its composition. Its etiology and pathogenesis remain obscure.

The high recurrence rates show necessity of better treatment. Exam analyzes of neoplasia characteristic and adherence. New imaging techniques i.e. computerised tomography (CT) plays an important role helping oncologists improve the diagnosis of several neoplasias. CT parameters are important to local staging of the tumor and prognosis. The CT provides essential information like attenuation pre and poscontrast, appearance heterogeneous, and enhancement poscontrast.

OBJECTIVE
The focus of this research first was to describe the FISS feline patient about their weight and body condition score (BCS) [2]. Second to characterize FISS by immunohistochemical examination to identify inflammatory markers and to classify it as its origin, aiming at a better prediction of treatment and prognosis. Finally, to characterize the aspect and volume of feline injection-site sarcoma, it was based in CT exam.

MATERIALS AND METHODS
Were attended 46 cases of FISS at the Service of Small Animal Surgery of the Surgery Department of the FVMAS/USP.

The cats with FISS underwent to a CT exam. They are submitted to anaesthesia and then positioned. Slices of 2 a 5 mm with 5 of increment were performed pre e poscontrast. Organs and muscles commitment, blurring of adjacent fat, presence of areas of central liquefaction, and enhancement poscontrast (heterogeneous or homogeneous) were analyzed. After the CT was decided about the possibilities of conservative or surgery treatment.

These 46 FISS were analysed histological (HE) and immunohistochemical (S-100; CD117/c-kit; Cox-2; Anti-FelV).

RESULTS
Statistical difference was found between the BCS (p=0.001) and weight (p<0.001) of FISS group and a control group. No differences were found between sex and race.

The region most commonly affected was the toracoabdominal (78%); interscapular area (13%) and pelvic limb (9%). The FISS majority presented as fibroblasts origin, while no marking for inflammatory components (CD3 and CD68). There was marking for kinases cascade (c-Kit), for COX-2, S-100 and for FelV. (Table 1).

76% patients showed heterogeneous enhancement, and after the contrast 82% showed the enhancement. Nevertheless, 58% presented muscle invasion; 36% presented blurring of adjacent fat, and 65% of the patients did not present cleavage between neoplasia and adjacent structures (muscle, bone). Only in 2(6%) patients the soft tissue mass did not invade adjacent structures, and had a cleavage with the adjacent structures. 1(3%) patient presented a punctual calcification into the mass. These findings had demonstrated the invasiveness of the FISS, and the relevance of the CT exam to aim a better surgical planning, in order to calculate the most appropriated oncology margins.

Table 1-Markers for FISS

<table>
<thead>
<tr>
<th>Immunohistochemical</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-100</td>
<td>95+</td>
</tr>
<tr>
<td>CD117/c-kit</td>
<td>23+</td>
</tr>
<tr>
<td>Cox-2</td>
<td>64+</td>
</tr>
<tr>
<td>Anti-FelV</td>
<td>41+</td>
</tr>
</tbody>
</table>

There was no influence of the parameters evaluated with clinical variables and overall survival. There was a statistically significant influence when comparing the invasion observed in CT examination and choice of the treatment (p=0.019).

CONCLUSION
The immunohistochemical analysis was essential for the FISS diagnosis. The Cox-2 and c-kit suggest the possibility of treatment with NSAIDs and drugs as inhibitor of kinases.

The cats with BCS above 5 they have a tendency to develop FISS.

REFERENCES
Study of cell markers as a prognostic factor in dogs with intranasal neoplasia treated with chemotherapy and rhinotomy

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INTRODUCTION
The nasal and sinonasal neoplasms represent 59-82% of cases of tumors in the respiratory tract of dogs, and 80% are malignant. The visualization of these neoplasms in nasal cavity, is possible by rhinoscopy, and tomographic image upside assesses the extent, however, is the histopathological analysis of biopsy which concludes the diagnosis. The treatment is still limited and discussed.

OBJECTIVE
The objective of this study is to evaluate the contribution and effectiveness of treatment of malignant neoplasms in the nasal cavity of dogs using chemotherapy alone or associated with rhinotomy, and analyze the role of cell markers as prognostic indicators through immunohistochemical study classifying these patients about the staging and survival after treatment.

METHODS
Will be used in dogs treated in Hospital College of Veterinary Medicine and Animal Science from the University of São Paulo, with clinical manifestations compatible with nasal disease, underwent rhinoscopy with collection of biopsy and CT head. Animals with histopathology confirmed by nasal malignant neoplasm of mesenchymal or epithelial origin shall be submitted to chemotherapy agent or in combination with rhinotomy. The results will be evaluated following the staging systems according to CT imaging and rhinoscopy, after the 28th day of the eighth session of chemotherapy, interspersed four applications of each drug. The prognosis of these patients will be evaluated according to the expression of cellular markers COX 2, VGF, EGFR, p53, PCNA and Ki67, correlating with disease progression and response to treatment. The quality of life will also be analyzed by specific questionnaire.

RESULTS
Until the moment, underwent CT examination of head together with rhinoscopy 17 animals with the result of malignant neoplasms. Of these animals, about 58% (n = 10) were submitted to chemotherapy, and about 23% (n = 04) by surgical procedure in conjunction with chemotherapy. Eleven patients died before they came to finalize all sessions of chemotherapy. These animals, had between 90 and 416 days of life after diagnosis of nasal proliferation by rhinoscopy. Started treatment within 60 days, and three of the patients, did not start yet any treatment. The results from the presence of cell markers, has not been evaluated. Only one case allowed perform tomographic and endoscopic control after 8 sessions, which showed a reduction in tumor mass in both diagnostic tests.

REFERENCES
PNAs as therapeutic approach against mouse ErbB2 positive breast carcinomas

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INTRODUCTION

Although extraordinary advances in the understanding of breast cancer biology have been achieved and potential molecular targets for its treatment have been identified over the last decade, therapeutic resistance and a lack of curative treatment in metastatic disease are still major hurdles to overcome. This is particularly true for the most aggressive breast cancer subtypes. As a consequence, breast cancer remains the most frequently diagnosed cancer and the leading cause of cancer death in women worldwide (http://globocan.iarc.fr). Breast cancer is not a single disease but a group of several tumor subtypes, each with a different natural history and requiring a different treatment. In breast cancer, overexpression of Human Epidermal growth factor Receptor-2 (ErbB2), mainly due to gene amplification, is found in 20% to 30% of the cases and is associated with rapid development, high metastatization and poor prognosis [1]. Innovative therapeutic options that specifically target HER-2 or other members of the HER family have recently been introduced however even if some inhibitors already exist, there is still space for the evaluation of alternative approaches, e.g. antisense regulation of gene expression [2]. In this respect PEPTIDE NUCLEIC ACIDS (PNAs) are an intriguing antisense probe as they are DNA analogues with the ability to form extremely stable complexes with complementary oligomers. This stability is related to the substitution of the normal phosphodiester with a 2-aminoethyl glycine backbone, linked by a methylenecarbonyl linkage to one of the four bases found in DNA. This non-standard backbone confers resistance to degradation by proteases and nucleases. Having once entered a cell, PNAs are retained if the target mRNA is present, otherwise they are released into the medium, untouched by the enzymatic arsenal of the cell [3]

RESULTS

We designed a PNA matching 5’-UTR of rat ErbB2 mRNA (PNA-neuT) to transiently silence the expression of transgenic rat ErbB2 oncogene overexpressed by a mouse mammary carcinoma cell line (TUBO cells). The in vitro and in vivo behaviour of TUBO cells was investigated, comparing the effect of PNA-neuT with respect to a scrambled PNA (PNA-scramble). Using the Nucleofector (Amaxa™ Lonza) electroporation system we delivered PNAs into TUBO cells with PNA-neuT transfected cells showing a 30% downmodulation of rat ErbB2 (Figure 1A e 1B). We then tested in vitro the potential of PNA as an inhibitor of proliferation (Figure 2A) and colony formation in agar (Figure 2B) upon electroporation of TUBO cells as well as testing their ability to form carcinomas in vivo after transfection (Figure 3). We are also assessing the possibility of electroporating PNA in vivo to impair the spread of TUBO induced carcinomas.

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