Proceedings of the

Electroporation based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Ljubljana, Slovenia
November 16-22, 2014

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Electroporation based Technologies and Treatments 2014

REPUBLIC OF SLOVENIA
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Edited by:

Peter Kramar
Damijan Miklavčič
Lluis M. Mir

Organised by:

University of Ljubljana
Faculty of Electrical Engineering

Institute of Oncology, Ljubljana

Supported by:

Bioelectrochemical Society
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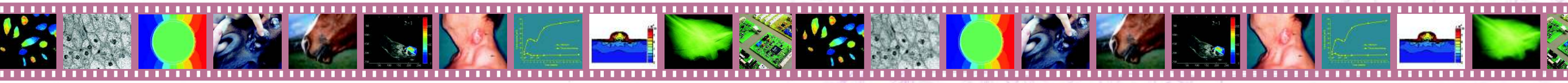

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Proceedings of the

COST TD1104

Electroporation-Based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

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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 eleven years the Course has been attended by 422 participants coming from 31 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 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) and to the Bioelectrochemical Society. We also would like to thank Igea (Italy), Iskra Medical (Slovenia), BIA Separations (Slovenia), Mediline (Slovenia) and βtech (France), 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 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 Lluis M. Mir

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LECTURERS' ABSTRACTS

Resting and Induced Transmembrane Voltage

Tadej Kotnik
University of Ljubljana, Faculty of Electrical Engineering, Ljubljana, Slovenia

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.[1] Fortunately, this complication does not affect the steady-state voltage induced on the plasma membrane of such cells, which can still be determined analytically.

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

RESTING TRANSMEMBRANE VOLTAGE

Under physiological conditions, a voltage in the range of –90 mV up to –40 mV is always present on the cell membrane [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],

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

where E is the electric field in the region where the cell is situated, R is the cell radius, and θ 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 $\theta = 0°$ and $\theta = 180°$ (the "poles" of the cell), and in-between these poles it varies proportionally to the cosine of θ (see Fig. 1, dashed).

The value of $\Delta\Phi_m$ given by Eq. (1) is typically established several μs 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],

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

where τ_m is the time constant of membrane charging,

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

with σ_i, σ_m and σ_e the conductivities of the cytoplasm, cell membrane, and extracellular medium, respectively, ε_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 μs.

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.[2] 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.

[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.

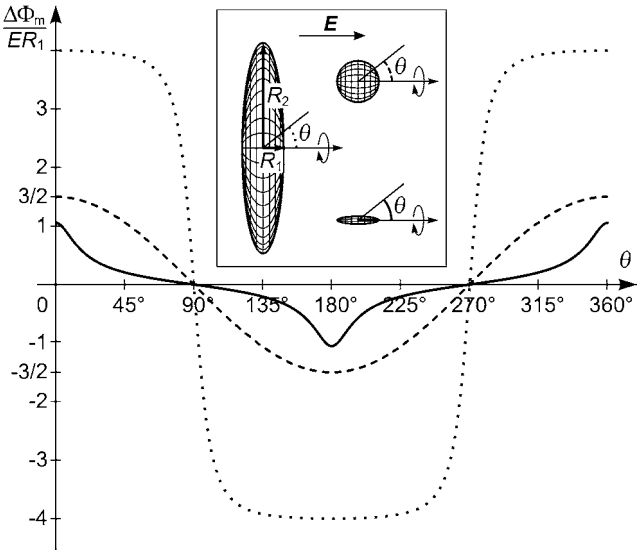

Figure 1: Normalized steady-state $\Delta\Phi_m$ as a function of the polar angle θ for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate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

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

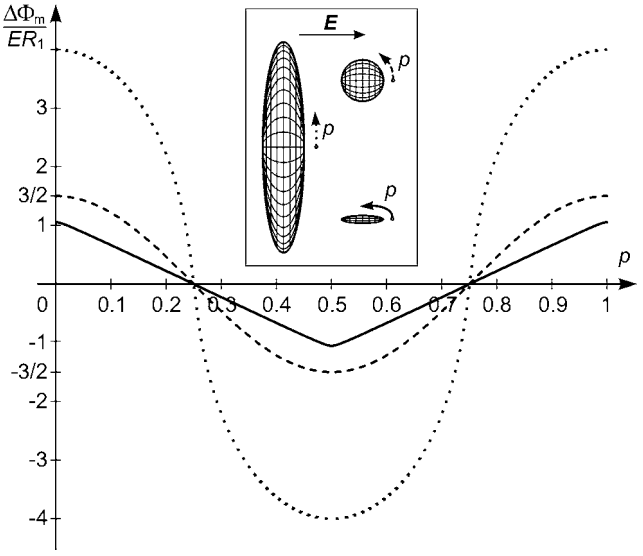

Figure 2: Normalized steady-state $\Delta\Phi_m$ as a function of the normalized arc length p for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: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

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.

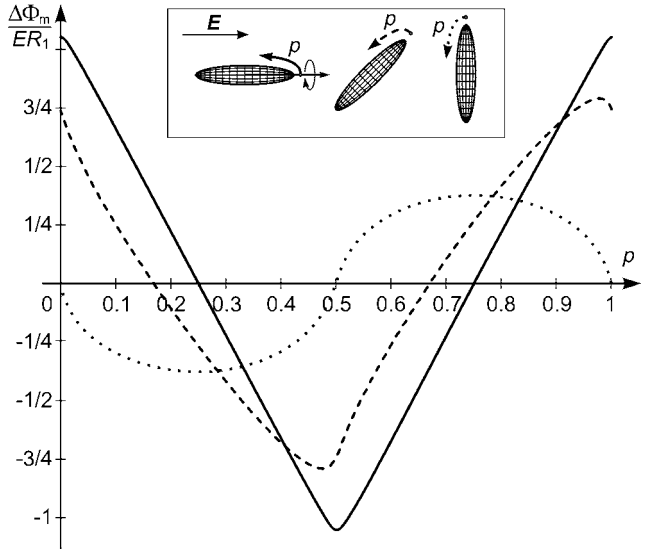

Figure 3: Normalized steady-state $\Delta\Phi_m(p)$ for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

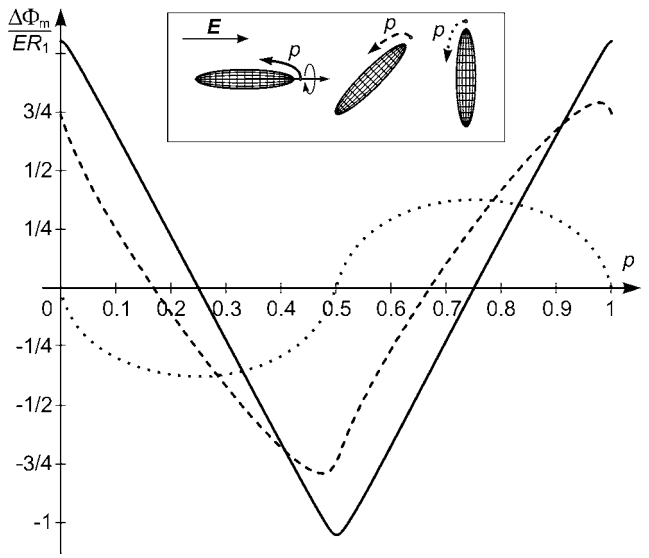

Figure 4: Normalized steady-state $\Delta\Phi_m(p)$ for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

Irregularly shaped cells

For a cell having an irregular shape, the induced transmembrane voltage cannot be determined exactly, as for such a geometry Laplace's equation is not solvable analytically. Using modern computers and finite-elements tools such as 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.

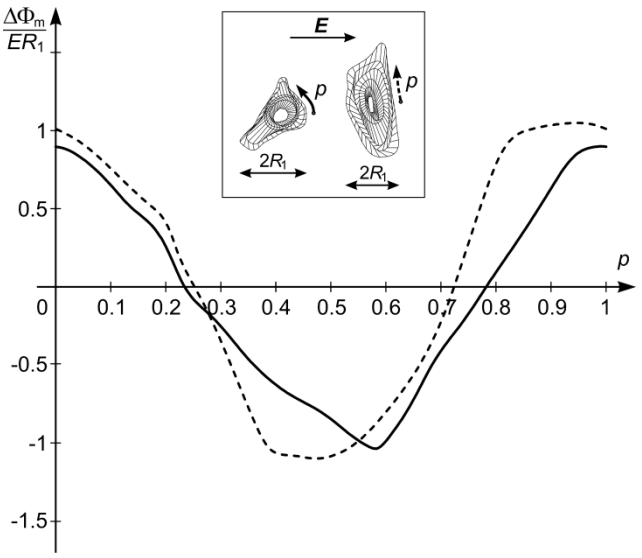

Figure 5: Normalized steady-state $\Delta\Phi_m(p)$ for two irregularly shaped cells growing on the flat surface of a Petri dish.

Cells in dense suspensions

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

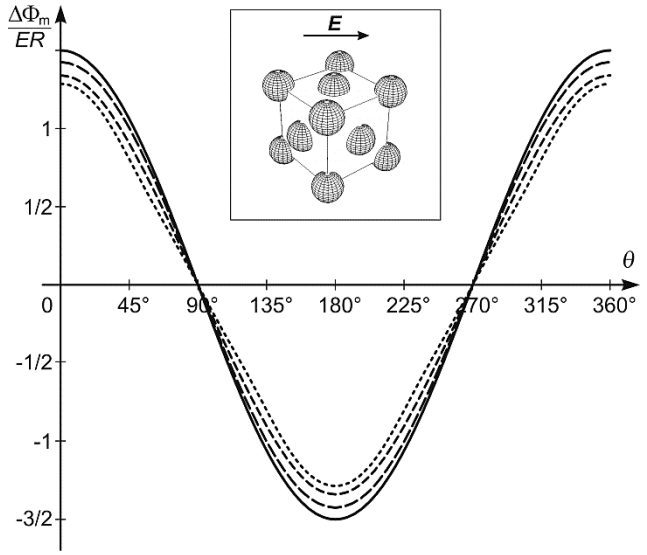

Figure 6: Normalized steady-state $\Delta\Phi_m(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by Eq. (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (with 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 (τ_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, τ_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 τ_m, as well as for rectangular pulses much longer than τ_m, the amplitude of the induced voltage remains unaffected. However, for AC fields with the period comparable or shorter than τ_m, as well as for pulses shorter than τ_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].

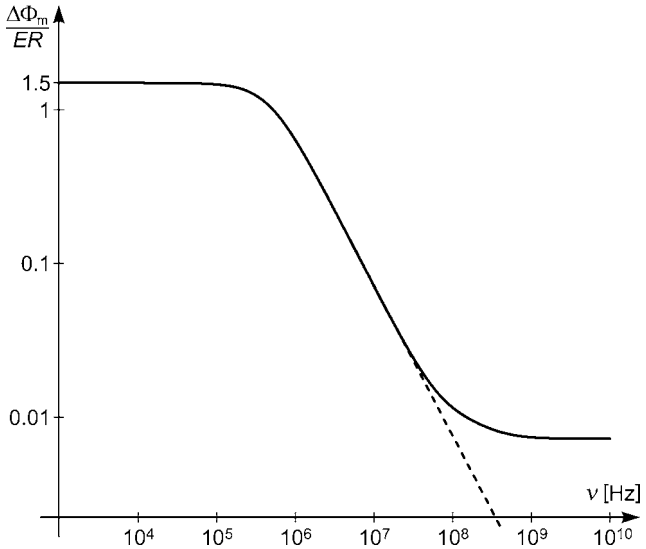

Figure 7: The amplitude of normalized steady-state $\Delta\Phi_m$ as a function of the frequency of the AC field. The dashed curve shows the first-order, and the solid curve the second-order Schwan's equation. Note that both axes are logarithmic.

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NOTES

Electric Properties of Tissues and their Changes During Electroporation

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Abstract: Passive electric properties of biological tissues such as permittivity and conductivity are important in applied problems of electroporation. The current densities and pathways resulting from an applied electrical pulse are dictated to a large extent by the relative permittivity and conductivity of biological tissues. We briefly present some theoretical basis for the current conduction in biologic materials and factors affecting the measurement of tissue dielectric properties that need to be taken into account when designing the measurement procedure. Large discrepancies between the data reported by different researchers are found in the literature. These are due to factors such as different measuring techniques used, the fact that macroscopic tissue properties show 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].

BIOLOGICAL MATERIALS IN THE ELECTRIC FIELD

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

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 ε_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, ε, and a conductivity, σ. 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 ε_0 =8.85 x 10-12 F/m. For other media:

$$\varepsilon = \varepsilon_r \varepsilon_0$$

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

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

Consider a sample of material which has a thickness, d, and cross-sectional area, A (Figure 1).

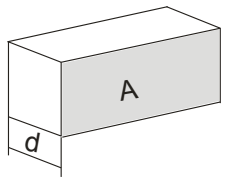

Figure 1: A considered theoretical small part of a material.

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):

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

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

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

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

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

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

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

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

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

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

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

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

We can also denote total current as:

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

We can define a complex-valued, relative permittivity:

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

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

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

We can consider the conductivity of a material as a measure of the ability of its charge to be transported throughout its volume 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, σ* and ε* 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.

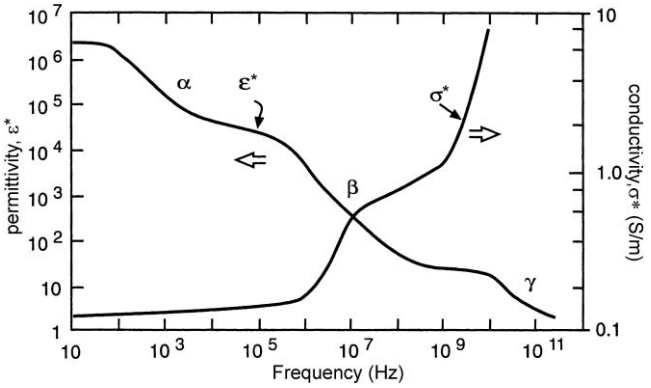

Figure 2: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.

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).

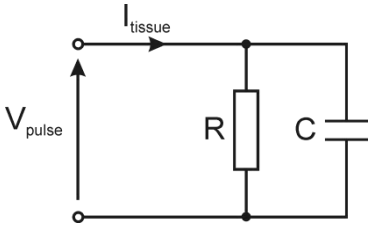

Figure 3: Parallel RC circuit: a theoretical representation of tissue response to electic pulses.

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 dispersions 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].

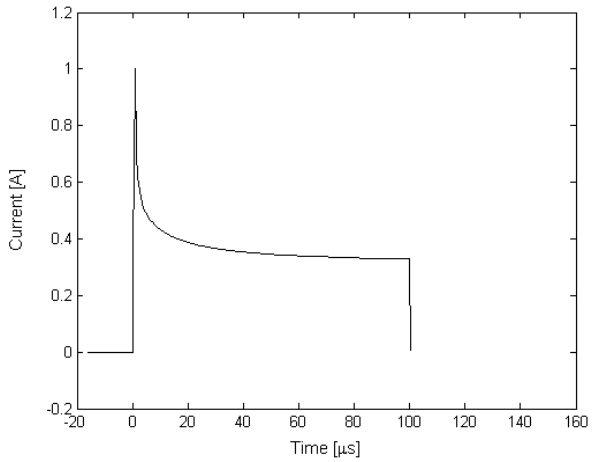

Figure 4: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs having the rise time of 1 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

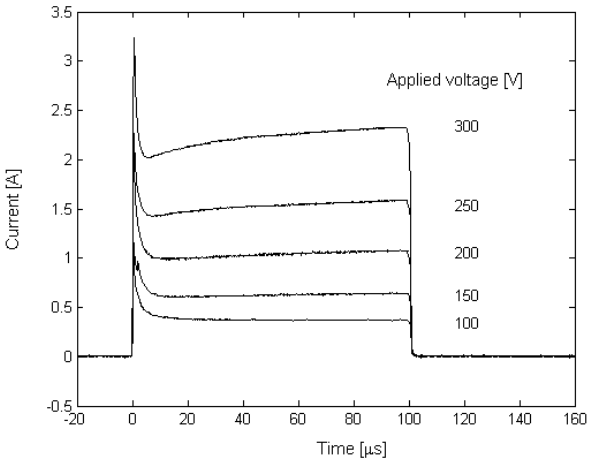

Figure 5: Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [8]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

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]. In measuring ex vivo tissue and phantom tissue made of gel like material [15] using MREIT we were able to demonstrate that electric conductivity changes due to membrane electroporation are amplitude dependent and occur in tissue only but not in phantom tissue. 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 [16].

Further, in applications where electric pulses to skin or tissues underneath (such as subcutaneous tumor) are applied externally, through skin, one might expect high (too high) voltage amplitudes needed in order to breach the highly resistive skin tissue and permeabilize tissues underneath. Namely, tissues between the electrodes can be seen as serially connected resistors. Applying voltage on such a circuit (voltage divider) causes the voltage to be distributed between the resistors proportionally to their resistivities [17]. 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 membrane permeabilization [18]. To further support this hypothesis, we described this process with a numerical model, taking into account the changes of tissue bulk electrical properties during the 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 entirely permeabilized, in some areas the electric field is also above the irreversible threshold.

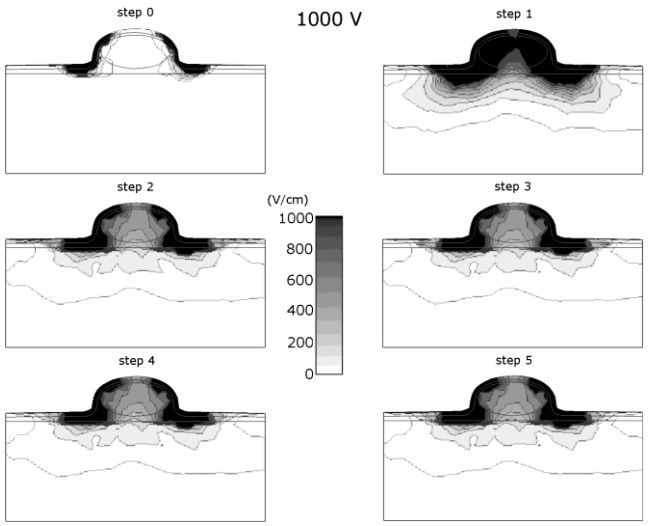

Figure 6: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [18]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact 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 [19]. 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 [20].

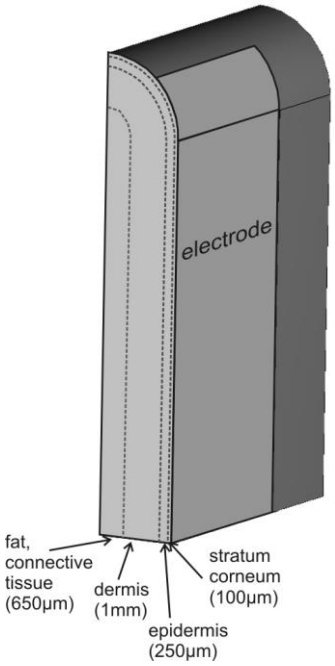

Figure 7: Schematics of a skinfold as described in a numerical model. Only one quarter of the skinfold is presented here.

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 [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 [24], 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 [25].

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NOTES

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-capacitive 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, $t + \Delta t$, 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.

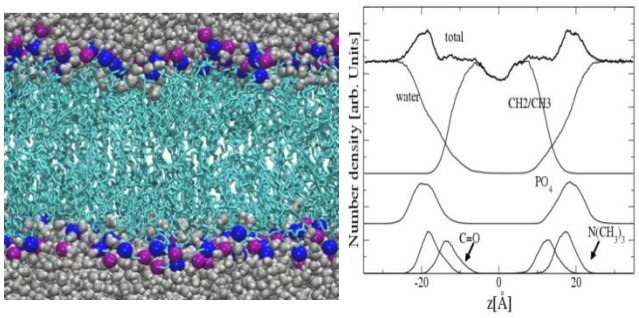

Figure 1: *Left:* configuration of a Palmitoyl-Oleyl-Phosphatidyl-Choline (POPC) hydrated bilayer system from a well equilibrated constant pressure MD simulation performed at 300K. Only the molecules in the simulation cell are shown. Water molecules (O gray; H white) and the Phosphate (blue) and Nitrogen (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 DIRRECT 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 $\vec{F} = q_i \vec{E}$ 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:

$$\Delta\phi(z) = \phi(z) - \phi(0) = -\frac{1}{\epsilon_0} \iint_0^z \rho(z'')dz''dz' .$$

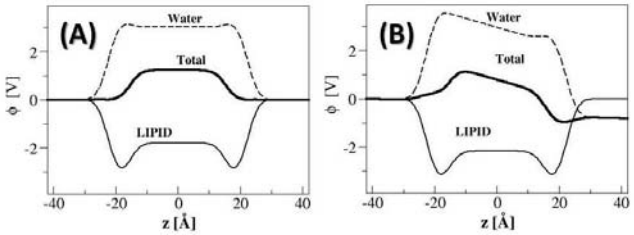

Figure 2: Electrostatic potential profiles $\phi(z)$along the membrane normal **z** of a POPC lipid bilayer (A) at rest and (B) subject to a transverse electric field $\vec{E}$. **z**=0 represents the centre of the lipid bilayer and the arrow the bilayer-water interfaces. Are shown the contribution from water, lipid and the total electrostatic profile. Note that the TM voltage ΔV (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 $\vec{E}$ induces an overall TM potential $\Delta \boldsymbol{V}$ (*cf.* Fig 2). It is very important to note here that, because of the MD simulation setup (and the use of PBCs), $\vec{E}$ induces a voltage difference $\Delta \boldsymbol{V} \approx |\vec{E}|.L_Z$ 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 ~ 10 nm. The electric field (0.1 V.nm^{-1}) applied to the POPC bilayer induces $\Delta \boldsymbol{V}$ ~ 1V.

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 hunder 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 pre-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 (~1 to 3 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 interstingly, 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 last 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]

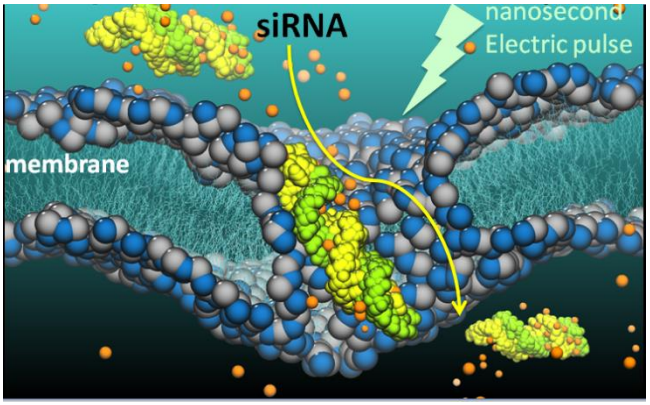

Figure 3: A single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance for the design of protocols aimed at using nanosecond-pulse siRNA electro-delivery in medical and biotechnological applications

The eletroporation process takes place much more rapidly under higher fields, without a major change in the pore formation characteristics. The lowest voltages reported to electroporate a PC lipid bilayer are ~ 2 V [29, 79]. Ziegler and Vernier [30] reported minimum poration external field strengths for 4 different PC lipids with different chain lengths and composition (number of unsaturations). The authors find a direct correlation between the minimum porating fields (ranging from 0.26 V.nm^{-1} to 0.38 V.nm^{-1}) and the membrane thickness (ranging from 2.92 nm to 3.92 nm). Note that estimates of electroporation thresholds from simulations should, in general be considered only as indicative since it is

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 $\boldsymbol{\Delta V}$ 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 x 210 x 210 Å^3) was chosen large enough to avoid interaction between the central liposome and its replica, resulting

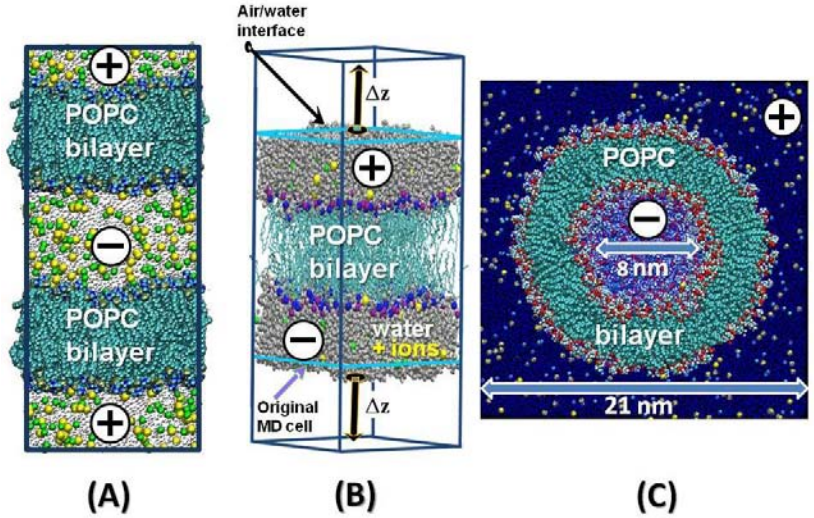

Figure 4: Molecular dynamics simulations set-ups of three systems using the charge imbalance method. (A) The double bilayer setup: two lipid bilayers are separated by electrolyte baths at 1M NaCl salt concentration. Note that due to the use of PBCs (drawn box) the upper and lower electrolytes are in contact. Q_s is imposed between the central water bath and the two others. (B) The single bilayer set-up: here one single bilayer is surrounded by water baths (maintained at 1M NaCl). The original MD cell represented the classical set-up and the large cell that allowing for the creation of water air interfaces. Q_s is imposed between the lower and upper bath. (C) The Liposome setup: A small spherical liposome is embedded in a 1M NaCl electrolyte. Q_s is imposed 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 $\Delta \boldsymbol{V}$. 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 $\Delta \boldsymbol{V}$.

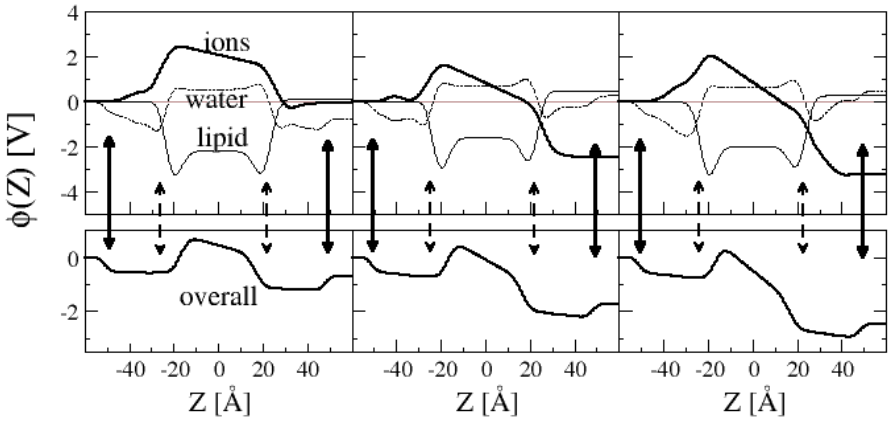

Figure 5: Components of the electrostatic potential profiles along the lipid bilayer normal Z of a POPC membrane estimated from at the initial stage of the MD simulations of the system at 1 M NaCl salt concentration using the single bilayer method. Z=0 represents here the center of the lipid bilayer, 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.

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 $\Delta \boldsymbol{V}$ from which the capacitance can be estimated as $C = Q_s . \Delta \boldsymbol{V}^{-1}$. 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 μF.cm^{-2} which is in reasonable agreement with the value usually assumed in the literature *e.g.* 1.0 μF.cm^{-2} [85, 88] and with recent measurements for planar POPC lipid bilayers in a 100 mM KCl solution (0.5 μF.cm^{-2}).

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 $\Delta \boldsymbol{V}$ 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).

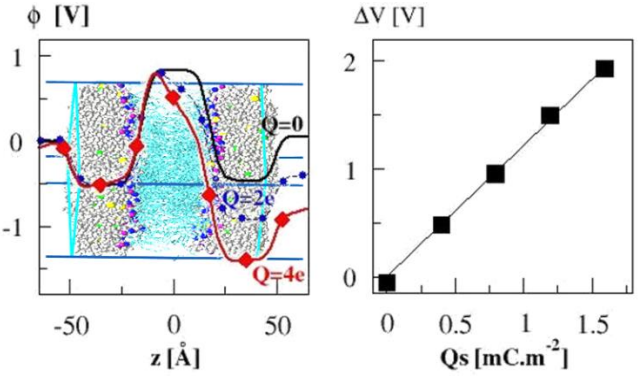

Figure 6: *Left:* Electrostatic potential across a POPC lipid bilayer for different net charge imbalances Qs between the upper and lower electrolytes from MD simulations considering the setup of Fig. 5. ϕ(z), is estimated as an in-plane average of the EP distributions (Eq. 1). As a reference it was set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

electrolyte. *Right:* TM potential ΔV as a function of the charge imbalance Qs par unit area. The capacitance of the bilayer can be derived from the slope of the curve.

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 $\Delta \boldsymbol{V}$. 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 $\Delta \boldsymbol{V}$ 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].

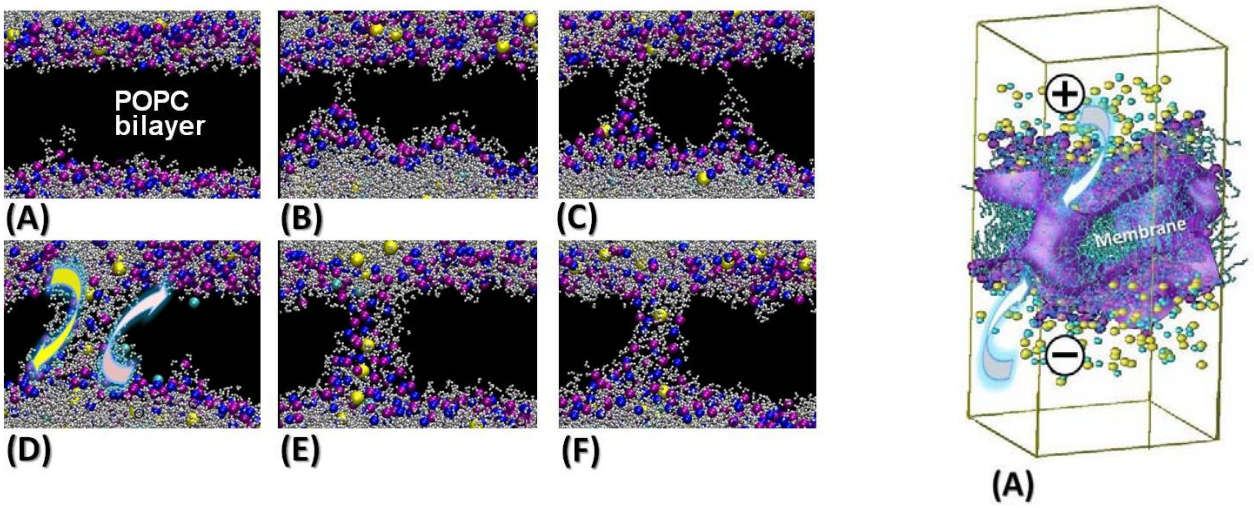

Figure 7: *Left* Sequence of events following the application of a TM voltage to a POPC lipid bilayer using the charge imbalance method (panels A to F). Note the migration of Na+ (yellow) and Cl- (cyan) ions through the formed hydrophilic pores that are lined with lipid phosphate (magenta) and nitrogen (blue) head group atoms. Panel F represents the state of a non conducting pore reached when the exchange of ions between the two baths lowered $\boldsymbol{Q_s}$ and therefore $\Delta \boldsymbol{V}$ to values ≈ 200 mV. *Right* Topology of the nanometer wide hydrophilic pores formed under high transmembrane $\Delta \boldsymbol{V}$ imposed by the charge imbalance method in the planar bilayer (A) and in the liposome (B). The arrows highlight the subsequent ionic flow through the pores.

DISCUSSION

In order to determine the detailed mechanism of the pore creation, it is helpful to probe the electric filed 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 ΔV 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.

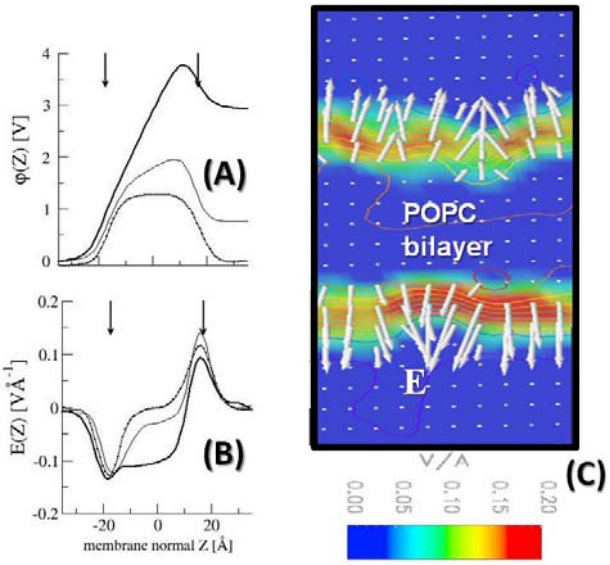

Figure 8: (A) Electrostatic potential profiles across a lipid bilayer subject to electric fields of 0.0 V.nm^{-1} (dotted line) 0.06 V.nm^{-1} (thin line) and 0.30 V.nm^{-1} (bold line). (B) Corresponding electric field profiles. (C) 2d (out of plane) map of the electric field distribution. The local 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 ~ 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.

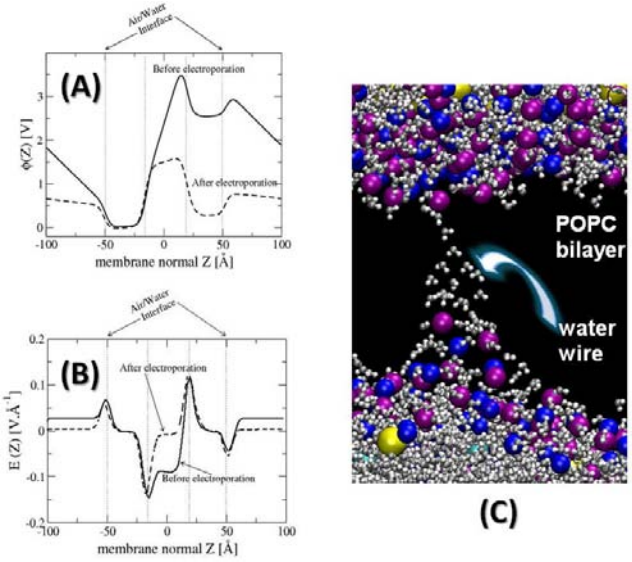

Figure 9: (A) Electrostatic potential profiles across a POPC lipid membrane subject to a charge imbalance (single bilayer set-up) before (solid line) and after (broken line) electroporation. (B) Corresponding electric field profiles. (C) Snapshot taken from the MD simulation of the lipid bilayer subject 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 lipid bilayers are rather well optimized. Despite their 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

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 (λ_m), the pulsing buffer (λo) and the cytoplasm (λ_i), the membrane thickness, the cell size (r) and packing. Thus,

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

in which θ designates the angle between the direction of the normal to the membrane at the considered point on the cell surface and the field direction, E the field intensity, r the radius of the cell and f, a shape factor (a cell being a spheroid). Therefore, $\Delta\Psi_i$ is not uniform on the cell surface. It is maximum at the positions of the cell facing the electrodes. These physical predictions were checked experimentally by videomicroscopy by using potential difference sensitive fluorescent probes [4-6]. This is valid with dilute cell suspensions. In dense systems, self shielding in the cell population affects the local field distribution and reduces the local (effective) field distribution [7]. Stronger field intensities are needed to get the same induced potential. Another factor affecting the induced potential differences is the shape or the cell and their relative orientation to the field lines. When the resulting transmembrane potential difference $\Delta\Psi$ (i.e. the sum between the resting value of cell membrane $\Delta\Psi o$ and the electroinduced value $\Delta\Psi i$) reaches 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 θ close to 0 or π. $E_{p,r}$ is such that:

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

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

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

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

These theoretical predictions are experimentally directly supported on cell suspension by measuring the leakage of metabolites (ATP) [9] or at the single cell level by digitised fluorescence microscopy [10, 11]. The permeabilized part of the cell surface is a linear function of the reciprocal of the field intensity. Permeabilization, due to structural alterations of the membrane, remained restricted to a cap on the cell surface. In other words, the cell obeys the physical predictions! The area affected by the electric field depends also on the shape (spheroid) and on the orientation of the cell with the electric field lines [12]. Changing the field orientation between the different pulses increases the fraction of the cell surface which is permeabilized.

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

The field strength controls the geometry of the part of the cell which is permeabilized. This is straightforward for spherical cells (and validitated 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 Ep 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 Ep) are applied. Another characteristic is that the 'loading' time is under the control of the cell size [14].

A-3 Forces acting on the membrane

The external electrical field pulse generates a net transient mechanical force which tends to stretch the spherical membrane [15]. This force appears due to Maxwell stresses existing in the spherical dielectric shell which cause deformation. The total radial force acts on the membrane during the transient process and tends to stretch the microorganism. It can even lead to rupture of the membrane resulting in the death of the microorganism [16]. But as the cellular elasticity is based upon the actin cytoskeleton, this stretching would affect the internal cell organization

B- Structural Investigations

B-1 P31 NMR investigations of the polar head region of phospholids

NMR of the phosphorus atom in the phosphatidylcholine headgroup was strongly affected when lipid multilayers were submitted to electric field

pulses. It is concluded that the conformation of the headgroup was greatly affected while no influence on the structure and dynamics of the hydrocarbon chains could be detected [17]. On electropermeabilized CHO cells, a new anisotropic peak with respect to control cells was observed on 31 P NMR spectroscopic analysis of the phospholipid components [18]. A reorganization of the polar head group region leading to a weakening of the hydration layer may account for these observations. This was also thought to explain the electric field induced long lived fusogenicity of these cells..

B-2 Structural approaches with advanced technologies

Atomic Force Microscopy (AFM) has been extensively used to image live biological samples at the nanoscale cells in absence of any staining or cell preparation. [19]. AFM, in the imaging modes, can probe cells morphological modifications induced by EP. In the force spectroscopy mode, it is possible to follow the nanomechanical properties of a cell and to probe the mechanical modifications induced by EP. transient rippling of membrane surface were observed as consequences of electropermeabilization and a decrease in membrane elasticity by 40% was measured on living CHO cells [20]. An inner effect affected the entire cell surface that may be related to cytoskeleton destabilization.

Due to the nonlinear and coherent nature of second harmonic generation (SHG) microscopy, 3D radiation patterns from stained neuronal membranes were sensitive to the spatial distribution of scatterers in the illuminated patch, and in particular to membrane defect formation formation. higher scatterers (membrane alterations) densities, lasting < 5 milliseconds, were observed at membrane patches perpendicular to the field whereas lower density was observed at partly tangent locations [21, 22]. Higher pore densities were detected at the anodic pole compared to cathodic pole.

C-Practical aspects of electropermeabilization

C-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. But as for other macroscopic damage to a plasma membrane, electropermeabilization has been shown to cause internal vesicles (lysosomes) to undergo exocytosis to repair membrane damage, a calcium mediated process called lysosomal exocytosis. Membrane resealing is thus a cellular process.

C-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 electropermeabilized part [9]. This gives the following expression for a given molecule S and a cell with a radius r:

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

where Φ(S, t) is the flow at time t after the N pulses of duration T (the delay between the pulses being short compared to t), P_s is the permeability coefficient of S across the permeabilized membrane and ΔS is the concentration difference of S across the membrane. Ep depends on r (size). For a given cell, the resealing time (reciprocal of k) is a function of the pulse duration but not of the field intensity as checked by digitised videomicroscopy [9]. A strong control by the temperature is observed. The cytoskeletal integrity should be preserved [24]. 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.

C-3 Cellular responses

Reactive oxygen species (ROS) are generated at the permeabilized loci, depending on the electric field parameters [25]. These ROS can affect the viability. This is a major drawback for the transfer of sensitive species (nucleic acids). Adding antioxydants is a safe approach [26].

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 [27].

There is a loss of the bilayer membrane asymmetry of the phospholipids on erythrocytes[28].

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) [23].

CONCLUSION

All experimental observations on cell electropermeabilization are in conflict with a naive model where it is proposed to result from holes punched in a lipid bilayer (see [29] 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) [30, 9]. SiRNA are only transferred by the electrophoretic drag present during the pulse [31]. DNA plasmids are accumulated in spots on the electropermeabilized cell surface during the pulse and slowly translocated in the cytoplasm along the microtubules by a metabolic process [32, 33]

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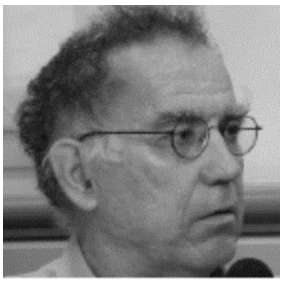

Teissié Justin was born 24 March 1947 in Poitiers, France. Got a degree in Physics at the Ecole superieure de Physique et de Chimie Industrielles de Paris (ESPCI) in 1970. Got a PhD in Macromolecular Chemistry on a project on fluorescence detection of action potential under the supervision of Prof. Monnerie (ESPCI) and Changeux (Institut Pasteur) in 1973. Got a DSC in Biophysics on a project on fluorescence characterisation of Langmuir Blodgett films in Toulouse in 1979. Was a Post Doc at the Medical School of the John Hopkins University in Baltimore in 1979-81. Present position: Directeur de recherches au CNRS emeritus. Author of more than 200 papers.

NOTES

NOTES

Nanoelectropulses: Theory and 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.

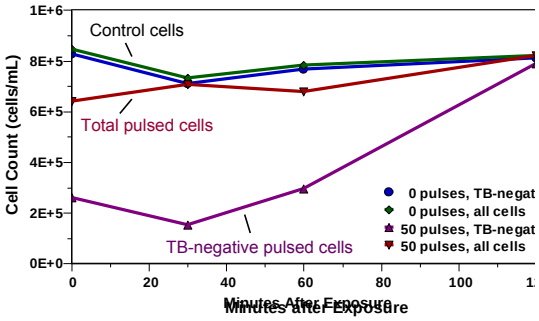

Figure 1. Nanoelectropulsed Jurkat T lymphoblasts recover over 2 hrs from initial Trypan blue permeabilization after exposure to 50, 20 ns, 4 MV/m pulses at 20 Hz.

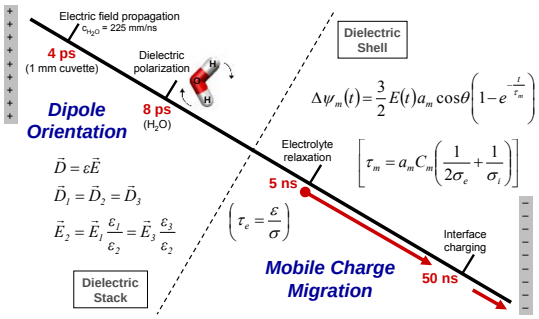

Figure 2. Timeline representing the sequence of events following electrical polarization of a biological tissue or aqueous suspension of cells. The sub-nanosecond regime can be modeled by the dielectric properties of the system. For longer times the distribution of fields and potentials is dominated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

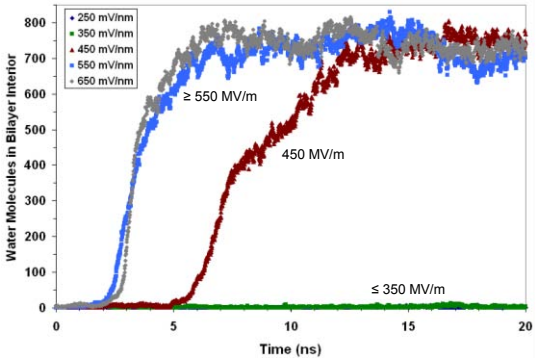

Figure 3. Electric field-driven intrusion of water into a simulated lipid bilayer.

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].

Nanosecond bioelectrics and the dielectric stack model. Figure 2 depicts a time line of events in an aqueous suspension of living cells and electrolytes between two electrodes after an electric pulse is applied. Water dipoles re-orient within about 8 ps The field also alters the electro-diffusive equilibrium among charged species and their hydrating water, with a time constant that ranges from 0.5 to 7 ns, 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.

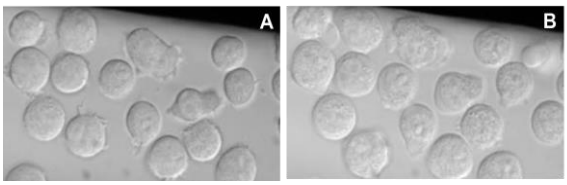

Figure 4. Differential interference contrast (DIC) images of Jurkat T lymphoblasts before (A) and 30 s after (B) exposure to 5 ns, 10 MV/m electric pulses (30 pulses delivered at 1 kHz). Note pulse-induced swelling, blebbing, and intracellular granulation and vesicle expansion, results of the osmotic imbalance caused by electropermeabilization of the cell membrane.

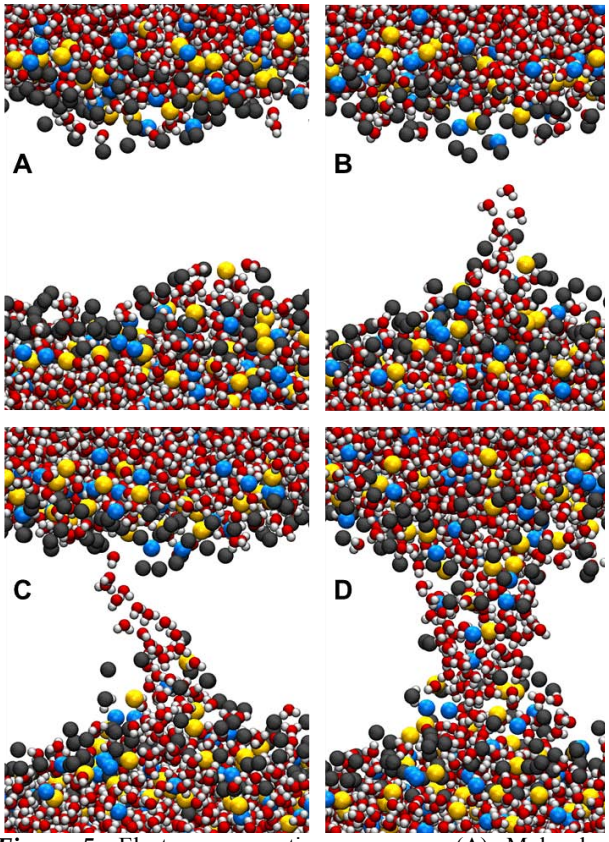

Figure 5. Electropore creation sequence. (A) Molecular dynamics representation of a POPC lipid bilayer system. Small red and white spheres at the top and bottom of the panel are water oxygen and hydrogen atoms. Gold and blue spheres are head group phosphorus and nitrogen, respectively, and large gray 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

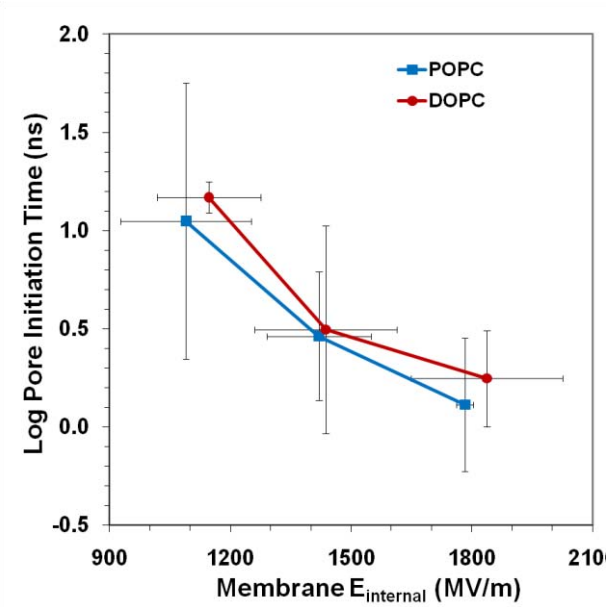

Figure 6. Electropore initiation time is a nonlinear function of the magnitude of the porating electric field. Pore initiation time (time required to form the water bridge shown in Fig. 1C) is exponentially dependent on the applied electric field, expressed here as the electric field observed in the lipid bilayer interior in molecular dynamics simulations. Error bars are standard error of the mean from at least three independent simulations. Data are from Tables 4 and 5 of [32].

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,

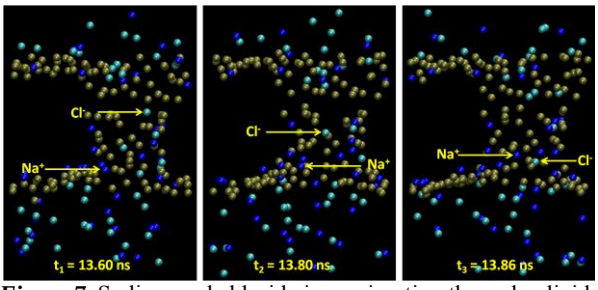

Figure 7. Sodium and chloride ions migrating through a lipid nanopore in the presence of an external electric field.

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

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,

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

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

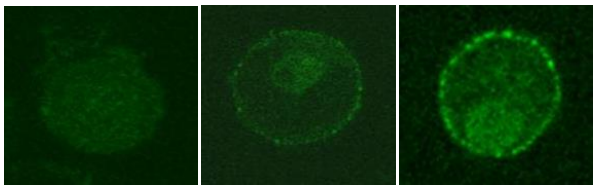

Figure 8. Immunocytochemical labeling of dopamine-β-hydroxylase (DβH) using an anti-DβH antibody coupled with a fluorescently-tagged 2° antibody. DβH is externalized by exocytotic fusion of vesicles with plasma membrane. Left panel, control. Center panel, 2 min after treatment with the pharmacological stimulant DMPP. Right panel, 2 min after a single, 5 ns, 5 MV/m pulse.

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

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. 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, Usual methods for detecting cell electropermeabilisation in vivo are reported here. The electrodes type and their positioning, which influence electric field distribution in the tissue as well as electropermeabilization level and extent in the tissue, are important issues that may require treatment planning procedures in the case of the treatment of internal tumor masses. The body, like the tissues, is also complex since nerves, muscles and large vessels can be located in the area affected by the electric pulses. These various anatomical constraints must be considered to avoid (or to limit) the sensations (pain), the contractions of the muscle and potential haemorrhage caused by penetrating electrodes insertion. These secondary effects can be circumvented and the applications of in vivo electropermeabilization (antitumor electrochemotherapy, electrogenethrapy, DNA vaccination, …) are rapidly developing.

INTRODUCTION

Tissues, as well as the body, are complex structures. A given tissue mainly contains, of course, 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. However, 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. Clearly, the 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 electropermeabilized 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 metabolisation.

Therefore, marker must be an injectable product that will be non-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 electropermeabilization 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 electropermeabilized cells, as described here below for each of them.

At least the following molecules have been used:

BLEOMYCIN

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

Using ^{57}Cobalt-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 electropermeabilization *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.

^{51}Cr-EDTA

^{51}Cr is also a gamma emitter but its half-life is very short and the ^{51}Cr-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 ^{51}Cr-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* electropermeabilisation level allowed to determine reversible and irreversible thresholds [7,9], to show differences between internal and external electrodes [10], to show differences between pulses of different durations thresholds [7,9],to show similarities between the same tissue in different species thresholds [7,9,10] and to demonstrate differences between different tissues [10].

PROPIDIUM IODIDE

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

$^{(99m)}$Tc-DTPA

Radiolabelled diethylenetriaminepentaacetic acid (DTPA) was used to trace the distribution and internalisation of a hydrophilic drug after *in vivo* electropermeabilization [12]. The drug accumulation in the treated volume was 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 electropermeabilization.

During the pulses, there are important changes in the conductivity of the tissues exposed to electropermeabilizing 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 electropermeabilization is achieved, the current will progressively increase. In a multidisciplinary study in which conductivy changes were paralleled to results of muscle and liver permeabilization achieved with the quantitative ^{51}Cr-EDTA test, Cujkati 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 preventing the generation of damages in the tissue that would have been provoked by the inadequate voltage, keeping the electropermeabilisation 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 electropermeabilization [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 bioimpedance 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 1200V/cm (determined by the absence of cell killing by the electric pulses alone) [5]. Moreover the comparison was done with the electropermeabilisation 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 *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 *in vitro* on isolated cells. Indeed, *in vitro*, resealing time depends on the temperature and, at about 37°, cells become impermeable in less than one minute. *In vivo*, muscle 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 electropermeabilized 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.
- Since muscle contractions cannot be avoided (using high pulse repetition frequencies, they can be reduced to a single contraction even if trains of several pulses are delivered, but the amplitude of the contractions cannot be reduced using the present technology), the synchronisation of the electric pulses delivery to the cardiac rhythm has been proposed and implemented when in vivo electropermeabilisation affects the heart or tissues located close to it [17].

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 ulterior experiments). A good fit was found between the percentages of tissue exposed to fields of strength above a given value and the ^{51}Cr-EDTA uptake values at different field strengths.

A numerical three-dimensional model was proposed in 2000, and it was topologically validated using the bleomycin qualitative test described here above [1]. This model has been quite important to define electrodes geometry since it showed that in the case of needle electrodes, the diameter of the needles is of the utmost importance to have a more or less heterogeneous distribution of the electric field between the electrodes (and these differences could then be experimentally demonstrated). The model has been refined: it has been possible to made a numerical model of the dynamics of tissue permeabilisation *in vivo* [18]. 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) [19,20]. 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 [21]. 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 since the threshold values are significantly different in the two main orientations (parallel or perpendicular) [22].The use of appropriate conductive gels is also recommended in some situations for the convenient treatment of surface tissues like exophytic tumor nodules [23].

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NOTES

Electroporation in Electrochemotherapy of Tumors

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Abstract: Electrochemotherapy consists of chemotherapy followed by local application of electric pulses to the tumor to increase drug delivery into 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 new approaches, 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].

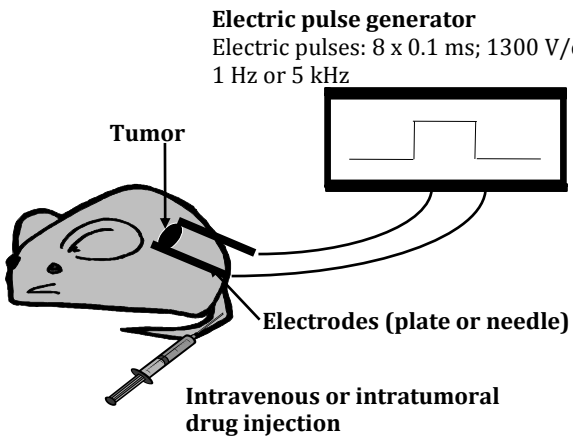

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumorally at doses which do not usually exert an antitumor effect. After an interval which allows sufficient drug accumulation in the tumors, electric pulses are 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 tumours can be effectively treated by plate electrodes, whereas appropriate electric field distribution in the deeper parts of the tumour is assured by using needle electrodes [7-9].

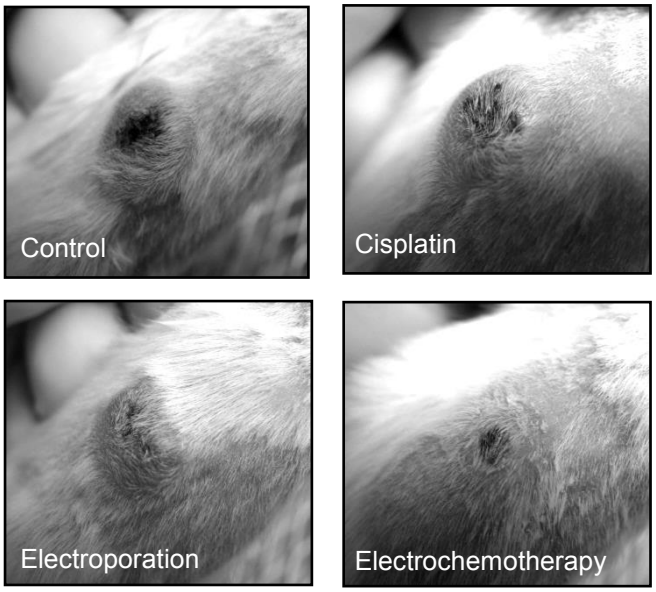

Figure 2: Example of good antitumor effectiveness in SA-1 tumors after electrochemotherapy with cisplatin. Cisplatin was given intravenously (4 mg/kg), 3 min thereafter 8 electric pulses were applied to the tumor with plate electrodes. Electric pulses were applied in two directions; 4 pulses in one and 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 tumours were up to 2-4 fold higher than in those without application of electric pulses [1-4].

Besides membrane electroporation, which facilitates drug transport and its accumulation in the cell, other mechanisms that are involved in the 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. Recently, some new drugs and chemicals were used in combination with electric pulses in preclinical studies, such as Mitomycin C, Ruthenium compounds and Calcium. The results of the studies in mice shown positive effects [25-27]. 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 [28,29]. 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 [30,31].

The application of electric pulses was shown to modulate tumor blood flow. Both reduced blood flow and lowered partial oxygen pressure (pO_2) in the tumors are consequences of the applied electric pulses [32]. The reduced pO_2 can activate bioreductive drugs to exhibit a cytotoxic effect on hypoxic cells [33]. 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 [34].

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 electrotransfer 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 [35-38].

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, hemangioma, hemangiosarcoma, perianal tumors, neurofibroma and sarcoids in dogs, cats, hamsters, rabbits and horses. Recent reports demonstrated successful treatment of different neoplasms in companion animals and sarcoids in horses either of electrochemotherapy alone or in combination with other treatment, mainly surgery [39-47]. 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.

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She is the author of more than 100 articles in peer-reviewed journals.

NOTES

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, like basal and squamous cell tumors of head and neck. 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 and primary tumors, of pancreas, 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. Recent reviews elaborate on its technology and biomedical applications in medical practice [1,2].

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 [3], which was thereafter followed by several others [4-42]. 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 [41-64]. 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 electro-chemotherapy according to tumor type, drug used, route of administration and type of electrodes, was tested [42]. 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 [43].

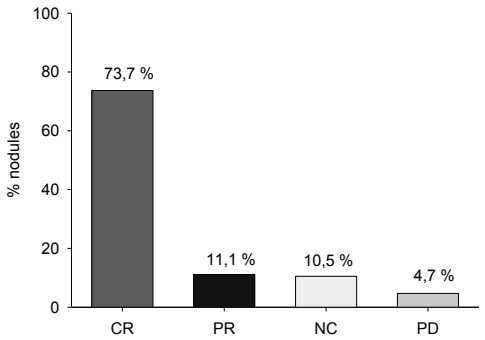

Figure 1: Treatment response of tumor nodules treated by electrochemotherapy in the ESOPE project. CR: complete response; PR: partial response; NC: no change; PD: progressive disease.

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 [59,60]. In general, electrochemotherapy provides a benefit to patients especially in quality of life [60], because electrochemotherapy is nowadays used predominantly in palliative intent [59,60].

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 [65].

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 [42,43,59,60].

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 [43].

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 140 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 in systematic review and meta-analysis [66]. 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 electro-chemotherapy, 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 [67].

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 [68].

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 [69,70]. 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 [72]. In this prospective pilot study 29 metastases in 16 patients were treated in 16 electrochemotherapy sessions. Radiological evaluation of all the treated metastases showed 85% complete responses and 15% partial responses. In a group of 7 patients that underwent a second operation at 6-12 weeks after the first one, during which electrochemotherapy was performed, the histology of resected metastases treated by electrochemotherapy showed less viable tissue ($P=0.001$) compared to non-treated ones [70].

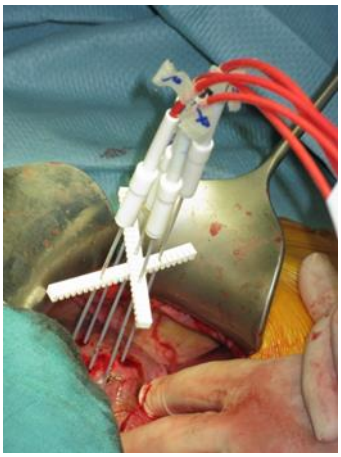

Figure 2: Electrochemotherapy of liver metastasis. Electrodes were inserted into the tumor and around the tumor in healthy liver tissue and connected to electric pulse generator. Electric pulses were delivered between the pairs of electrodes according to the treatment plan.

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 Electro-chemotherapy 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 [72].

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 [73]. The feasibility and effectiveness was elaborated also in recent clinical study by Campana et al. [74].

Recently also a study from Herlev Cancer Center has demonstrated that even big chest wall breast cancer recurrences can be treated successfully by electrochemotherapy [75].

The last but not least also electrodes for treatment of brain tumors are developed [76,77]. They will enable treatment of brain tumors, minimally invasively. The clinical trial is was launched.

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

Electrotransfer of DNA vaccine

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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.

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NOTES

Gene electrotransfer *in vitro*: a 30 years old story

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Abstract: Cell membranes can be transiently permeabilized under application of electric pulses. This process, called electropermeabilization or electroporation, allows hydrophilic molecules, such as anticancer drugs and DNA, to enter into cells and tissues. Open questions still exist about the behavior of the membranes both while the field is on (µs to ms time range) and after its application (from seconds to several minutes and hours). Also, there is a lack of understanding on how molecules are transported in complex environments, such as those found in tissues. As our objectives are to give a complete molecular description of the mechanisms, our strategy is to use different complementary systems with increasing complexities (model membranes, cells in culture, spheroids and tissues in living mice) and different microscopy tools to analyze the processes. Single cell imaging experiments revealed that the uptake of molecules (nucleic acids, antitumor drugs) takes place in well-defined membrane regions and depends on their chemical and physical properties (size, charge). 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 (1-5). This strategy is nowadays used in clinics to treat cancers. Vaccination and oncology gene therapy are also major fields of application of DNA electrotransfer (6, 7). Translation of preclinical studies into clinical trials in human and veterinary oncology has started (8-10). The first phase I dose escalation trial of plasmid interleukin electroporation has been carried out in patients with metastatic melanoma and has shown encouraging results (11).The method has also been successfully used for the treatment of dogs and horses (8, 12). 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 (13), many of the mechanisms underlying DNA electrotransfer remain to be elucidated (14, 15). 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 at a molecular resolution consists on using different imaging tools, to directly visualize the processes, and different experimental models with increasing complexities (from vesicles to tissues).

MEMBRANE ELECTROPERMEABIZATION

The use of video microscopy allows visualization of the permeabilization phenomenon at the single cell level. Propidium iodide uptake in the cytoplasm is a fast process that can be detected seconds after the application of electric pulses. Exchange across the permeabilized membrane is not homogeneous on the whole cell membrane. It occurs at the sides of the cells facing the electrodes in an asymmetrical way where it is more pronounced at the anode-facing side of the cells than at the cathode (Figure 1), i.e. in the hyperpolarized area than in the depolarized area, which is in agreement with theoretical considerations.

Electropermeabilization can be described as a 3-step process in respect with electric field: (i) before electropulsation, the plasma membrane acts as a physical barrier that prevents the free exchange of hydrophilic molecules between the cell cytoplasm and external medium; (ii) during electropulsation, the transmembrane potential increases which induces the formation of transient permeable structures facing the electrodes and allow the exchange of molecules; (iii) after electropulsation, membrane resealing occurs.

A direct transfer into the cell cytoplasm of the negatively charged small molecules such as siRNA was observed on the side facing the cathode. When added after electropulsation, siRNA did not penetrate the cells. Therefore, electric field acts on both the permeabilization of the membrane and on the electrophoretic drag of the charged molecules from the bulk into the cytoplasm. The mechanism involved

is clearly specific for the physico-chemical properties of the electrotransferred molecule (16).

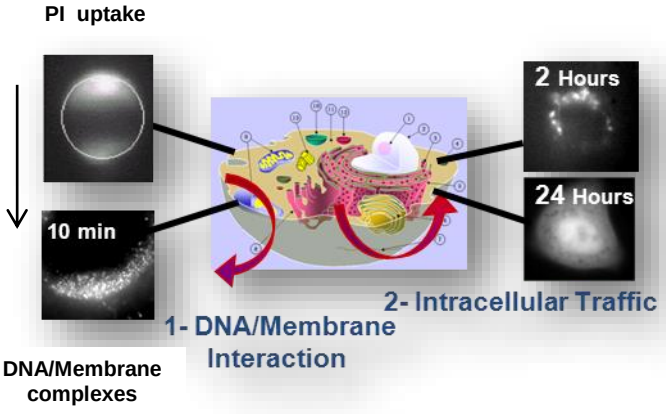

Figure 1: **Molecule electrotransfer mechanisms.** Left: During electric pulses application: Plasma membrane is electropermeabilized facing the 2 electrodes (PI uptake). DNA aggregates are formed. This interaction takes place only on the membrane facing the cathode. Right: About 2 h after electric pulses application, DNA molecules are present at nucleus level. Finally, eGFP expression is detected for hours. The arrow indicates the direction of the electric field.

Progress in the knowledge of the involved mechanisms is still a biophysical challenge. One of our recent objectives was to detect and visualize at the single-cell level the incidence of phospholipid scrambling and changes in membrane order (17, 18). The pulses induced the formation of long-lived permeant structures and resulted in a rapid phospholipid flip/flop within less than 1s and were exclusively restricted to the regions of the permeabilized membrane. Our results could support the existence of direct interactions between the movement of membrane zwitterionic phospholipids and the electric field. We also performed experiments on lateral mobilities of proteins and showed that electropermeabilization affects the lateral mobility of Rae-1, a GPi anchored protein. Our results suggest that 10-20 % of the membrane surface is occupied by defects or pores and that these structures propagate rapidly over the cell surface.

We also took advantage of atomic force microscopy to directly visualize the consequences of electropermeabilization and to locally measure the membrane elasticity. We visualized transient rippling of membrane surface and measured a decrease in membrane elasticity. 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 investigate basic process of electroporation on living cells in absence of any staining (19, 20).

MECHANISMS OF ELECTROTRANSFER OF DNA MOLECULES INTO CELLS

Single-cell microscopy and fluorescent plasmids can be used to monitor the different steps of electrotransfection (16, 21). DNA molecules, which are negatively charged, migrate electrophoretically when submitted to the electric field. Under electric fields which are too small to permeabilize the membrane, the DNA simply flows around the membrane in the direction of the anode. Beyond a critical field value, above which cell permeabilization 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.

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 (22). 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 (23). 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 (24-26).

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 (27, 28). 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 (27).

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 (29). The cytoplasm is composed of a network of microfilament and microtubule systems, along with a variety of subcellular organelles present in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration means that there is substantial molecular crowding in the cytoplasm which hinders the diffusion of plasmid DNA. These apparently contradictory results might be reconciled by the possibility of a disassembly of the cytoskeleton network that may occur during electropermeabilisation, and is compatible with the idea that the cytoplasm constitutes an important diffusional barrier to gene transfer. In the conditions induced during electropermeabilisation, the time a plasmid DNA takes to reach the nuclei is significantly longer than the time needed for a small molecule. Therefore, plasmid DNA present in the cytosol after being electrotransferred can be lost before reaching the nucleus, for example because of cell division. Finally, after the cytoskeleton, the nuclear envelope represents the last, but by no means the least important, obstacle 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 electrotransferred DNA is transported in the cytoplasm towards the nucleus (26). For this purpose, we have performed single particle tracking (SPT) experiments of individual DNA aggregates in living cells (25). We analyzed the modes of DNA aggregates motion in CHO cells. We showed fast active transport of the DNA aggregates over long distances. Tracking experiments in 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.

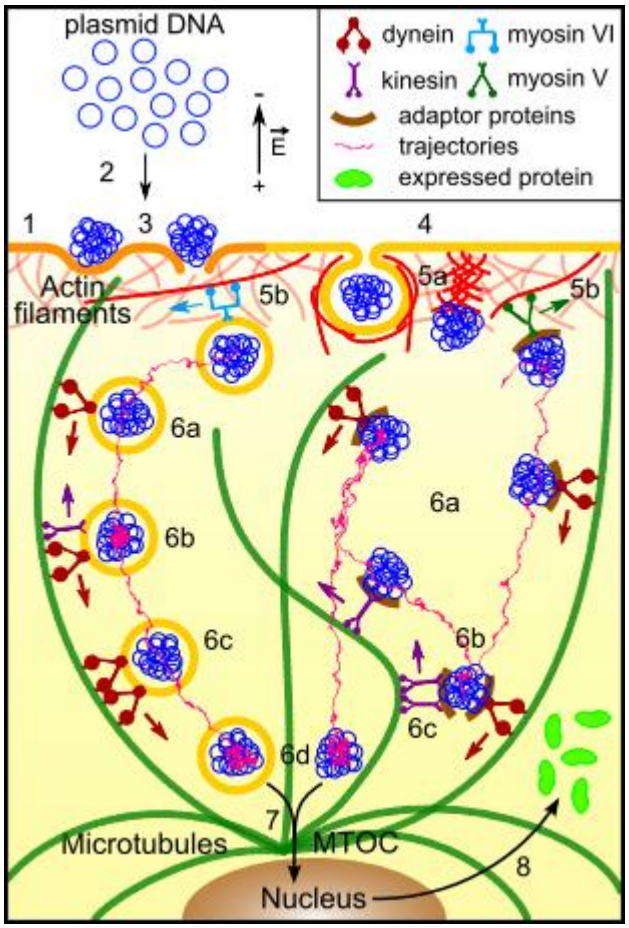

Figure 2: DNA electrotransfer as a multistep process. During the application of the electric field: (1) the plasma membrane is permeabilized (orange), (2) the DNA is electrophoretically pushed on the membrane side facing the cathode therefore (3) DNA/membrane interactions occur. DNA aggregates are inserted 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 internalized via endocytosis (DNA in vesicles). Free DNA is perhaps also internalized through electropores. For gene expression to occur (5-6), DNA has to cross the cytoplasm toward the nucleus. Our study suggests (5) actin related motion that can be in the form of (5a) actin polymerization 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 (26).

3) New developments.

As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA in the intracellular medium. Electrotransferred plasmid DNA, containing specific sequences could then use the microtubule network and its associated motor proteins to move through the cytoplasm to nucleus (30). 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 (31, 32). 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 (33).

LIPID VESICLES AND SPHEROIDS AS CONVENIENT (NEW) APPROACHES TO STUDY GENE ELECTROTRANSFER

New lines of research 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. GUVs (Giant Unilamellar Vesicles) represent a convenient way to study membrane properties such as lipid bilayer composition and membrane tension. They offer the possibility to study and visualize membrane processes due to their cell like size in absence of any constraint due to cell cytoskeleton. Experiments showed a decrease in vesicle radius which is interpreted as being due to lipid loss during the permeabilization process (Figure 3)

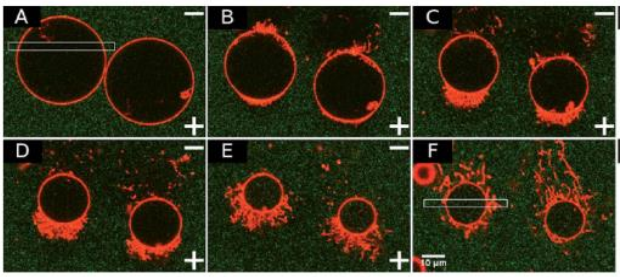

Figure 3: **Microcopic observations of GUVs submitted to electric pulses in the presence of pDNA.** From (34)

Three 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 (35). We also gave evidence that GUVs are a good model to study the mechanisms of electrofusion, with a direct interest to their use as vehicles to deliver molecules (36). However, a direct transfer of DNA into the GUVs took place during application of the electric pulses (34). 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 past few years, we decided to use an *ex vivo* model, namely tumor multicellular tumor spheroids, for the understanding of the DNA electrotransfer process in tissues.

Upon growth, spheroids display a gradient of proliferating cells. These proliferating cells are located in the outer cell-layers and the quiescent cells are located more centrally. This cell heterogeneity is similar to that found in avascular microregions of tumors. We used confocal microscopy to visualize the repartition of permeabilized cells in spheroids submitted to electric pulses. Electrotransfer of bleomycin and cisplatin confirmed the relevance of the model in the case of electrochemotherapy and doxorubicin showed its potential to screen new antitumor drug candidates for ECT. Confocal microscopy was used to visualize the topological distribution of permeabilized cells in 3D spheroids. Our results revealed that all cells were efficiently permeabilized, whatever their localization in the spheroid, even those in the core. The combination of antitumor drugs and electric pulses (ECT) led to changes in spheroid macroscopic morphology and cell cohesion, to tumor spheroid growth arrest and finally to its complete apoptosis-mediated dislocation, mimicking previously observed *in vivo* situations. Taken together, these results indicate that the spheroid model is relevant for the study and optimization of electromediated drug delivery protocols (37). 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

(38). Taken together, these results, in agreement with the ones obtained by the group of R. Heller (39), indicate that the spheroid model is more relevant to an *in vivo* situation than cells cultured as monolayers (40, 41).

CONCLUSIONS

Classical theories of electropermeabilization present some limits to give a full description of the transport of molecules through membranes. Certain effects of the electric field parameters on membrane permeabilization, 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 giving interesting new insight into the process (32). Electroinduced destabilisation of the membrane includes both lateral and transverse redistribution of lipids and proteins, leading to mechanical and electrical modifications which are not yet fully understood. One may suggest that such modifications can be involved in the subsequent transport of molecules interacting with them such as the DNA molecules. Experimental verification of the basic mechanisms leading to the electropermeabilization 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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NOTES

Gene transfer *in vivo*

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Abstract: Nucleic acids electrotransfer is the most advanced and efficient among the physical means to vectorize DNA or RNA in vivo. 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" HV pulses followed by trains of low voltage and very long duration "electrophoretic" LV 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

It is worth mentioning that the development of DNA electrotransfer [1] and its progression towards its application in the clinics [2] was the result of the general developments concerning the *in vivo* use of electric pulses to electropermeabilize mainly solid tumors after the delivery of non permeant or low permeant cytotoxic drugs (this combination was termed electrochemotherapy) [3,4] as well as normal tissues. The possibility to transfer plasmid DNA to cells *in vivo* by appropriate electric pulses (DNA electrotransfer) was therefore explored by several groups in the 90's. In this chapter we will adopt the following terminology: in terms of technology, we will use "nucleic acids (gene) electrotransfer" as the descriptions made here will be valid (with minor modifications) for all types of nucleic acids, DNA or RNA, long or short. In terms of therapeutic goals, we will use the term electrogenetherapy (electric pulses-mediated gene therapy) since a) most of the time coding DNA sequences are used at clinical level, and b) this denomination parallels the nowadays well accepted use of electrochemotherapy for the "electric pulses-mediated chemotherapy"

Methods to achieve very efficient *in vivo* DNA transfer have been developed in the last fifteen years, particularly for gene transfer to skeletal muscle or skin 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 ELECTROTRANSFER IN SKELETAL 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 ELECTROTRANSFER MECHANISMS 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 electropermeabilisation [13] but it is mandatory to inject the DNA before the electrophoretic LV pulse [5, 13]. Actually, the LV component is the real responsible for the plasmid transfer to the muscle fibers since it efficiently pushes the DNA towards the membrane, which remains still "altered" for a long period of time (up to 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 ELECTROTRANSFER IN LIVER

DNA transfer in liver, using short pulses, was described in 1996 [18] (this was the second paper relating DNA electrotransfer 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 a lot of care is necessary in experiments dealing with gene transfer in liver. Indeed, hepatocytes in vivo are easily transfected by simple hydrostatic pressure [20]. Recent data indicates that using long LVs (for example 4 LV of 100 ms) at field strengths even rather low (for example as low as 20 V/cm), there is no need for an HV. The exact reasons for such behaviour are not yet understood [16].

DNA ELECTROTRANSFER IN TUMORS

The first tissue to which DNA was transferred by means of long electric pulses were tumors transplanted in the flank of mice [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 consistence of the tumor (for example, experimental melanomas like the B16 melanoma are soft, inflatable tissues while fibrosarcomas are hard and breakable due to differnecs 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 squave 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].

DNA ELECTROTRANSFER IN SKIN

DNA electrotranfer has been analysed in several studies and using different models of skin |24, 25]. It is very important to consider also the type of electrodes and keep in mind that tissue-penetrating electrodes will require the application of lower voltages than non-penetrating ones (because the electric field generated by the former does not need to cross the stratum corneum). In the case of the use of combinations of pulses, it appears that the field amplitudes of both the HV and the LV must be higher than those optimal for DNA electrotransfer in muscle [24]. For the HV, this is easily explained considering that the diameter of the target cells is smaller than the diameter of the muscle fibers.

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 [26]. 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 [27]. 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 [28].

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 [29]. 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 [30].

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 electrotransfer to humans requires an extensive analysis of the analgesia or sedation needs. Indeed, it is convenient to avoid, as much as possible, unnecessary patient's anaesthesia. The sensations caused by HV pulses alone are well known since 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 [31]. Similar 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 [32]. The first published phase I/II trial reported good antitumor effects [33]. The corresponding phase II trial is ongoing. Other clinical trials involve DNA vaccination using plasmid DNA injection and electric pulses delivery.

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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 permeabilizes 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., LHRH or calcitonine) was shown to be enhanced. Few in vivo studies confirm the increased transport and rapid onset of action.

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

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

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

TOPICAL DRUG DELIVERY BY ELECTROPORATION

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

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

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 electrotransfer, GFP expression was also reported but was very low and restricted to the epidermis.

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

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

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

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

Electrotransfer of DNA 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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NOTES

Development of devices and electrodes

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Abstract: Since first reports on electroporation, numerous 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.

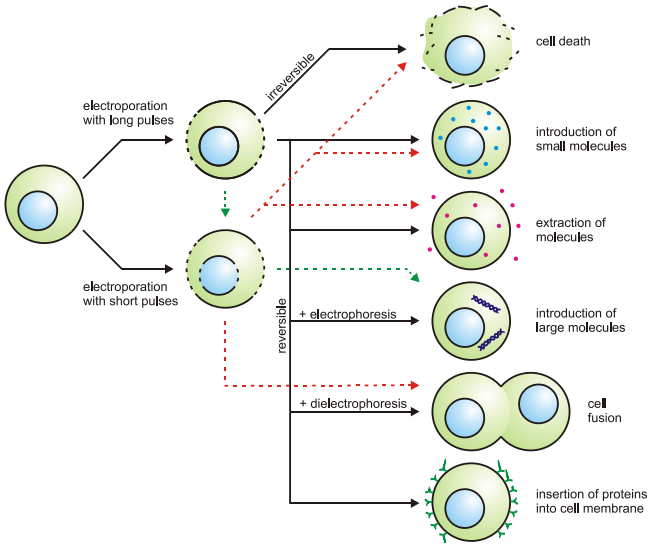

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications 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 usualy 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]. Smilarly 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 electropermeabilization. Electrotransfection can be achieved using: exponentially decaying pulses; square wave pulses with superimposed RF signals; or only long square wave pulses up 20 ms and with amplitudes ranging from 200 to 400 V [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 of 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. Skin can be an excellent target for gene transfer protocols due to its accessibility [19].

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 [20]. 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. Electrofusion of cells of different sizes can be achieved by nanosecond pulsed electric fields [21].

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) [22]–[26].

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 [27], [28]. 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 [29].

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 [30]. Therefore electric field distribution in tissue has to be estimated before the treatment, which can be achieved by combining results of rapid tests or *in situ* monitoring [31] with models of electric field distribution [32]–[36]. 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 $\sigma(E)$. In this way models represent electroporation tissue conductivity changes according to distribution of electric field intensities [37].

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 [30]. 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 [38]–[41]. 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 [42]. Besides electropermeabilization of cell membranes which increases electrical conductivity of the sample, electric pulses also cause side effects like Joule heating and electrolytic contamination of the sample, which further leads to increased sample conductivity [43].

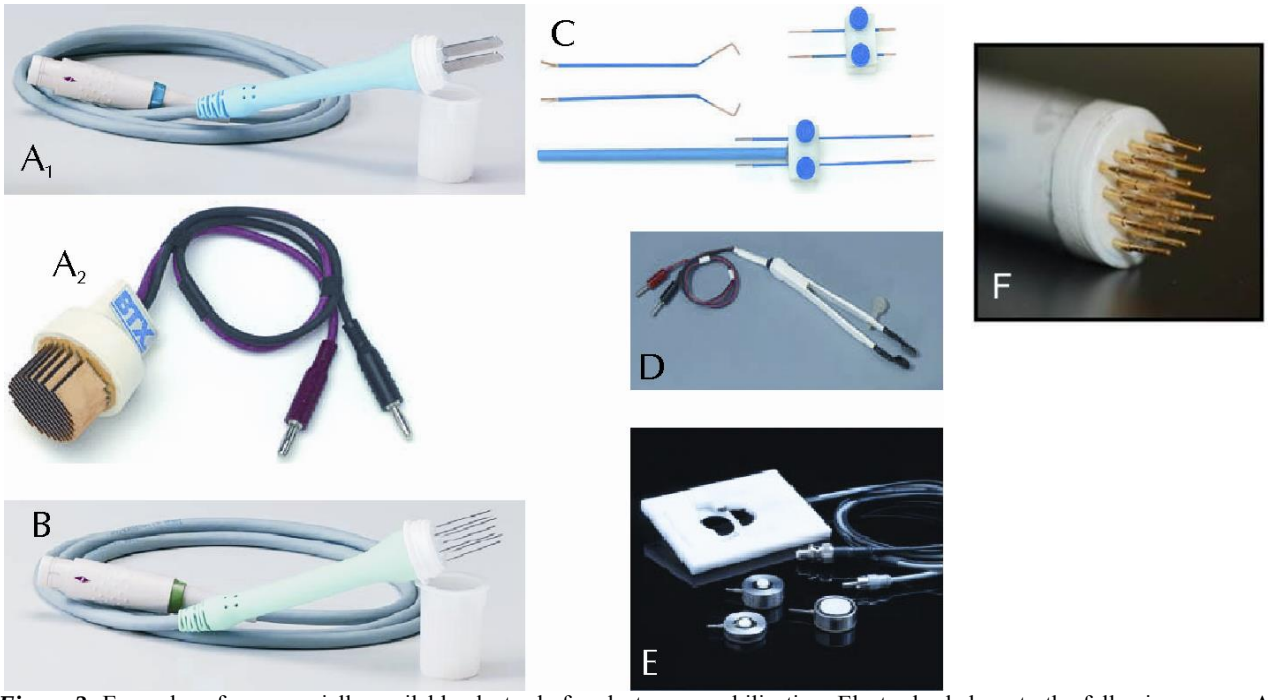

Figure 2: Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: A_1 and A_2 –parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes, E – coaxial electrodes and F – multiple electrodes array. Electrodes A_1 and B are produced by IGEA, Italy and are used for clinical applications of electrochemotherapy and electrotransfection. Electrodes A_2, C and E are used for different in vitro applications and are produced by: E – Cyto Pulse Sciences, U.S.A.; A_2, C and also D that are used for in vivo applications, are produced by BTX Hardware division, U.S.A, F are used for skin gene electrotransfer [19].

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% odf 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 [44], [45]. Shapes other than “rectangular” have been investigated with respect to electroporation efficiency [46]. 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 [47]. 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 tretaments 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 synchronization algorithms which minimasizes 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 $U = I \bullet Z$. 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 [48] and updated in 2010 [3] in a manuscripts that describe techniques of signal generation required for cell/tissue electropermeabilization.

To be sure the applied pulses are adequate we have to measure the applied voltage and current during the pulse delivery.

If rise time of the pulse is shorter than the electrical length between the source and the load the impedance of the load has to match the impedance of the generator, so that there are no strong pulse reflections and consequently pulse prolongations.

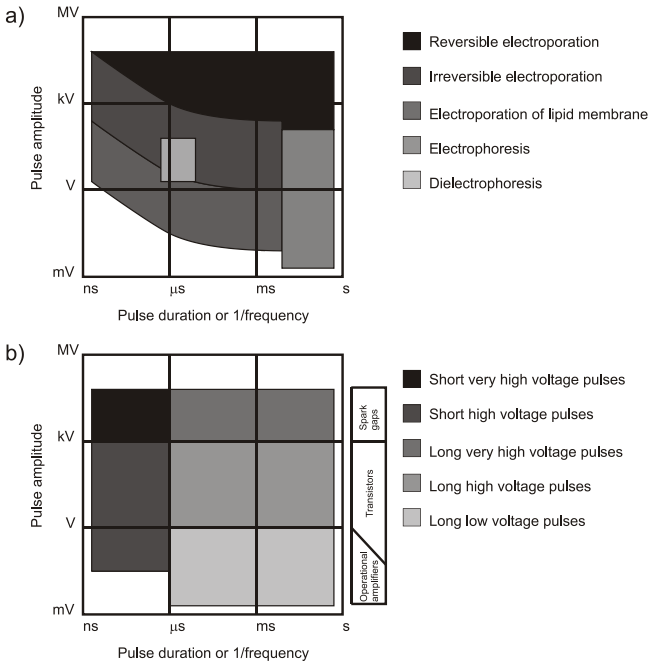

Figure 3: Areas of amplitude and duration of electrical pulses which are used in the research of electroporation and related effects (a). Five different areas of electroporation pulse generation (b). To amplify or to generate very-high-voltage electroporation pulses (over a few kV) spark gaps and similar elements are used, for high-voltage (a few V to a few kV) transistors and for low-voltage operational amplifiers are used. Nanosecond (short) pulses are generated with different techniques than pulses longer than 1 µs. Originally published in Advanced electroporation techniques in biology and medicine by Reberšek and Miklavčič 2010 [3].

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 [38], [40], [49].

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* situation. With a hold on viral vectors electroporation represents a viable non-viral alternative also for *in vivo* gene transfection. Clinical applications and expansion of electrochemotherapy and tissue ablation have been hindered by the lack of adequate electroporators and their certification in Europe (CE Medical Device) and limited approval by FDA in USA [47]. 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 purpuses: Cellectra, Elgen, Medpulser, Cliniporator VITAE, BetaTech, 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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electroporation devices and investigation of biological responses to nanosecond electrical pulses

NOTES

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].

Cell fusion results from a merging of the partner cell membranes followed by a cytoplasmic reorganization. A key process is the coalescence of the membrane. This results from a transient abolishment of the intermembrane repulsive forces.

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 P^{31} 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].

More recent investigations pointed out the role of the quality of cell-cell contacts [27]

PROTOCOLS

Electrofusion is always obtained under electric pulsing conditions inducing reversible electropermabilization. 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], attachement on dishes [24] or mild centrifugation [21] Pulse first fusion can only proceed by dielectrophoresis or centrifugation.

Dielectrophoresis appears as the most popular procedure to bring cells in contact but requests a non physiological buffer (very low ionic content). The pre-pulse contact phase was described in details and can control the contact between the partner membranes forcing them against each other [28, 29].

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].

More informations are clearly requested to understand the molecular processes involved in electrofusion. This may be obtained by the develent of approaches using microfluidic-based technologies [30].

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 dentritic and tumor cells (6).

ACKNOWLEDGEMENTS

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NOTES

NOTES

INVITED LECTURERS

Electrochemical Processes Occurring During Exposure of Cells by High-Voltage Pulsed Electric Fields and Their Consequences

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Abstract: During cell electromanipulation procedures, a cell suspension is exposed to high-voltage electric pulses and a strong electric current passes through the solution. At each electrode-solution interface, various electrochemical reactions occur. These may include the evolution of gas, the separation of substances, the dissolution of the electrode or the appearance of new substances in the solution. The processes of electrolysis lead to the changes of the temperature, pH, and the chemical composition of the experimental medium. Here, a short theoretical background of these electrochemical processes will be provided. The consequences of the primary and secondary electrochemical processes, which might be important for the understanding the results obtained as well as optimization of cell electromanipulation procedures, will be discussed to demonstrate that, when using high-voltage electrical pulses for electroporation of cells and tissues, scientists must keep in mind that the products generated due to electrochemical reactions can influence the biochemical processes taking part in their experimental systems.

INTRODUCTION

When a high-voltage is applied to the electrolyte solution, besides membrane permeabilization [1], various electrolysis reactions occur at the electrode-solution interfaces [2,3]. These may include evolution of gas, separation of substances, dissolution of the electrodes, and appearance of new substances [2-7]. Due to this, the efficiency of electroporation depends not only on the parameters of electric treatment but on the electrochemical processes as well. Studying electrochemical processes, which occur during high-voltage pulses, and their consequences is also necessary, as they are important for optimizing practical applications.

Here, the consequences of primary anodic and cathodic electrochemical reactions and secondary processes, which can take place during the treatment by high-voltage electrical pulses used in electroporation experiments, are reviewed.

PROCESSES OF ELECTROLYSIS

When a high-voltage is applied to the cell suspension a strong electric current passes through the solution. At each electrode-solution interface, the electrochemical reactions occur, that transfer electrons either to or from the electrode.

Primary electrochemical reactions

Several primary electrochemical half-reactions can take place at each electrode-solution interface. There are three possible anodic half-reactions [2]:
- the oxidation of water molecules

$$2H_2O \Rightarrow O_2(gas) + 4H^+(aq) + 4e^- \quad (1a)$$

- the oxidation of the anion of the solute

$$2Cl^-(aq) \Rightarrow Cl_2(gas) + 2e^- \quad (1b)$$

- the oxidation of the metal of the electrode, e.g.

$$2Al(solid) \Rightarrow 2Al^{3+}(aq) + 6e^- \quad (1c)$$

There are two possible cathodic half-reactions:
- the reduction of metal cations

$$K^+(aq) + e^- \Rightarrow K(solid) \quad (2a)$$

- the reduction of water molecules (or hydrogen ions)

$$2H_2O + 2e^- \Rightarrow H_2(gas) + 2OH^-(aq) \quad (2b)$$

Which of the anodic and cathodic half-reactions occur depends on the relative ease of each of competing reactions. However, when the anode and cathode potentials are sufficiently high, several reactions can proceed simultaneously.

Secondary chemical reactions

Besides the primary electrochemical reactions, secondary chemical reactions often take place in the solution after the electric pulse. What specific secondary reactions occur depends on composition of the solution, its pH, and the electrode material. Here are the examples of the secondary chemical reactions:

i) hydroxyl ions, which are generated as a result of the reduction of water molecules, (see Eq. (1b)), can attack chemically the aluminium cathode leading to its dissolution,

$$2Al + 6H_2O + 2OH^- \Rightarrow 2Al(OH)_4^- + 3H_2 \quad (3)$$

ii) Due to the reaction of chlorine (Cl_2) produced at the anode with water, hypochloric acid (HClO) can be formed [8]:

$$Cl_2 + H_2O \Leftrightarrow HClO + H^+ + Cl^- \quad (4)$$

iii) Metal ions released from the electrodes (e.g., Fe^{2+}, Fe^{3+}, Al^{3+}) can behave as a Lewis acid and hydrolyze water molecules:

$$Fe^{3+} + nH_2O \Leftrightarrow Fe(OH)_n^{3-n+} + nH^+ \quad (5)$$

iv) Iron ions released from the electrodes into solution, can act as a catalyst in the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^- \quad (6a)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^{\bullet} + H^+ \quad (6b)$$

Hydrogen peroxide can be produced by the reduction of dissolved oxygen:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (7)$$

or other chemical reactions.

v) The metal ions released from the electrodes, e.g. Fe^{2+}, Fe^{3+}, Al^{3+} or Cu^{2+}, can build complexes with the molecules, which are present in the solution, and subsequent adsorption of soluble or colloidal substances on these complexes can occur [9];

vi) The metal ions, which are released from the anode, can react with fluorescent molecules present in the solution [10].

Products of any of these secondary reactions can have an impact on the processes occurring after PEF treatment.

CONSEQUENCES OF ELECTROCHEMICAL PROCESSES

There are a variety of possible consequences of primary and secondary chemical reactions, which might be important for PEF treatment technology. Here are the examples of the main effects.

pH changes

There are several processes, as a result of which, medium pH can change. These include the oxidation of water molecules at the anode (Eq. (1a)); the reduction of water molecules (or hydrogen ions) at the cathode (Eq. (2b)), hydrolysis of water molecules by the metal ions released from the electrodes (e.g., Fe^{2+}, Fe^{3+}, Al^{3+}) (Eq. (5), and other ones (Eqs. (3), (4), (6), and (7).

The change of the pH of a solution occurring due to the exposure of an electrolyte solution to an electric pulse has been noticed many times [11-14]. A more detailed analysis of this effect was carried out recently [7,15]. It has been shown, that the increase in the pH value of electroporation solution of a whole chamber volume, caused by the application of electric field pulses, commonly used in cell electromanipulation and PEF treatment procedures, can exceed 1-2 pH units (Fig. 1) [7,15]. The pH shift was directly dependent on the intensity of electrotreatment (the greater the amount of electric charge that passes through the solution the greater ΔpH and was an inverse function of the buffer capacity (the greater the buffer capacity the lower the change of pH) and solution conductivity (the pH of a solution, in which sucrose was substituted for NaCl, changed about 5 times less) [7]. In addition, it has been shown that the cathode material can also be important in the degree of pH changes: the aluminium cathode gave approximately two-fold higher ΔpH in comparison with platinum, copper or stainless steel cathodes (Figs. 1) [7].

Gas evolution

One of the products of a water reduction half-reaction described by Eq. (2b) is hydrogen gas. The production of hydrogen gas at the cathode leads to formation of gas bubbles. At the opposite electrode (anode) O_2 or Cl_2 gas can be formed as a result of oxidation of water molecules of Cl^- ions (Eq. 1a and 1b).

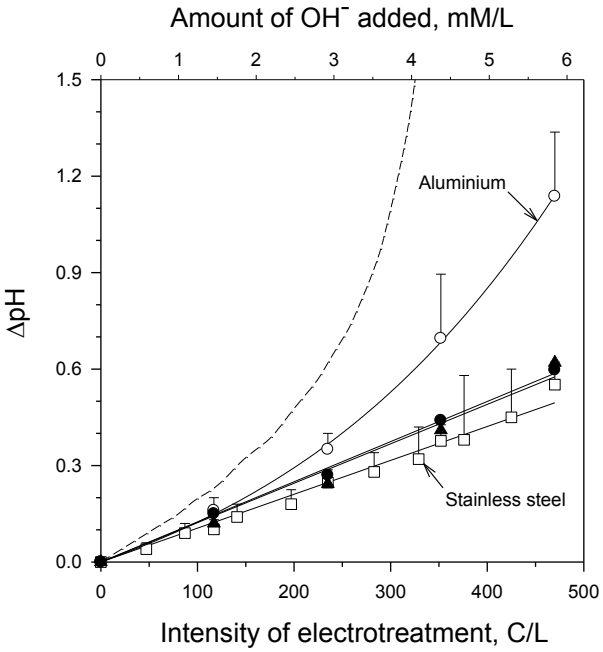

Figure 1: The change of the pH of a medium as a function of the intensity of electrotreatment for the 142.8 mM NaCl solution buffered with 11.2 mM of phosphates (Na_2HPO_4/NaH_2PO_4) for the cathodes made from various metals (aluminium, stainless steel, copper, and platinum (triangles) [7].

Reduction of the cell viability

The media treated by high-voltage electric pulses, which are usually utilized for cell electroporation or other electromanipulation (electrofusion, electroisertion, etc.) purposes, exert some cytotoxicity (Fig. 2) [16-19]. The cytotoxicity is attributed to the electrolytic production of free chlorine and oxygen, metal ions released from the electrodes, and possibly direct anodal oxidation of other substances [5,16,20], however, this effect still awaits more detailed analysis. For example, the reduction of the cell viability by the iron and aluminium ions released from the stainless-steel and aluminium anodes respectively has been reported (Fig. 3) [5,10,16].

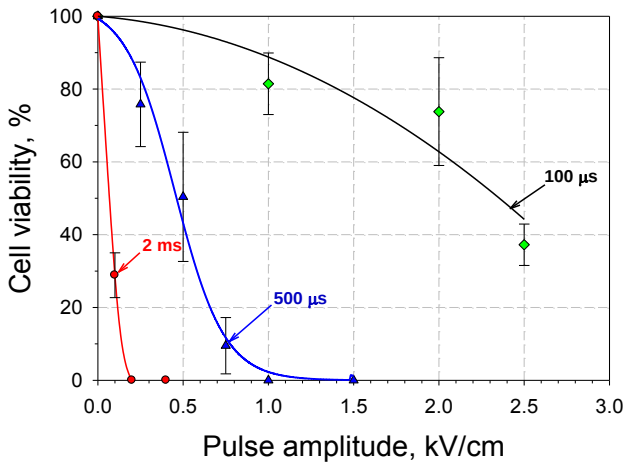

Figure 2: Viability of mouse hepatoma MH-22A cells in the culture medium pre-treated with a square-wave electric pulse with the duration of 0.1-2 ms [16].

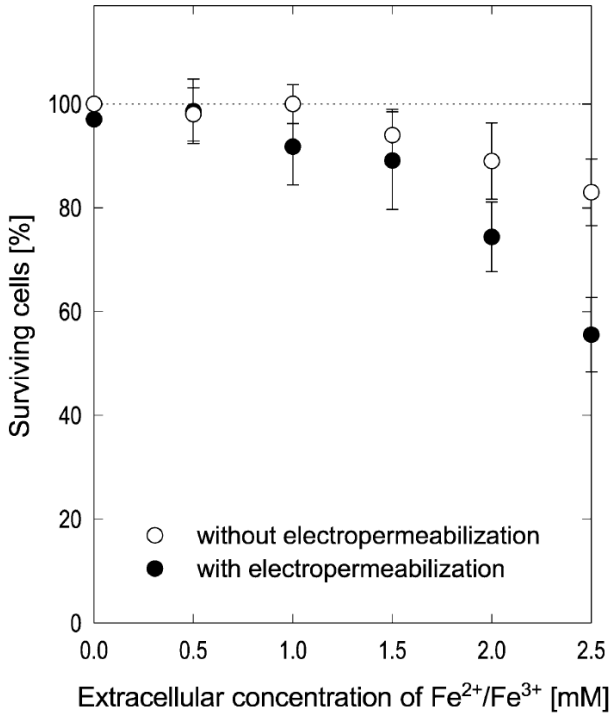

Figure 3: Cell survival (mean±S.D.) without (○) and with electropermeabilization (●) as the function of Fe^{2+}/Fe^{3+} concentration in the suspension. The cells were incubated for 1 h at room temperature. Electropermeabilization was performed at the beginning of the incubation using a train of eight unipolar rectangular pulses, each of 100 ms duration and 240 V amplitude (1200 V/cm voltage-to-distance ratio), delivered in 1 s intervals. (Figure from Kotnik et al. [5], reproduced with the kind permission of Elsevier).

Release of metal ions from electrodes

When a non-inert metal electrode is used, in most cases, the release of the metal ions from the electrode into the solution occurs, according to Eq. (1c). The release of Al^{3+} from the aluminium anode [4,5,7], Cu^{2+} from the copper anode [6] as well as Fe^{2+}/Fe^{3+}, chromium and manganese ions from the stainless steel anode has been observed during cell electroporation experiments [5,8-10,21].

For example, with increasing the amplitude of a 2-ms duration pulse the concentration of iron ions in the solution increased (Fig. 4) [10,16]. After the exposure of a 154 mM NaCl solution with a single square–wave pulse of the duration of 2 ms and with the amplitude of 240 V, which gives electric field strength of 1.2 kV/cm (current density of about $1.9 \cdot 10^5$ A/m^2), the concentration of iron ions released from the stainless-steel anode (Fe^{2+} & Fe^{3+}) exceeded 0.53 mM (27.9 mg/l) (Fig. 4) [10,16]. Similar results were reported by other groups [5,22].

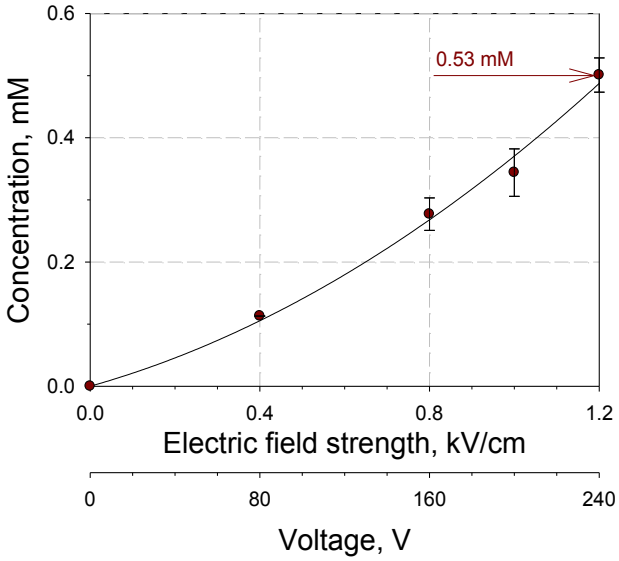

Figure 4: Dependence of the amount of Fe^{3+}/Fe^{2+} ions released from the stainless-steel anode on the amplitude of a single square-wave electric pulse with the duration of 2 ms [10].

It is supposed that aluminium cathode is attacked by hydroxyl ions generated during water reduction half-reaction (see Eqs. (2b) and (3)) [23]. This leads to a variety of secondary chemical reactions with different effects.

For example, when studying the changes of the pH value of electroporation solution, caused by the application of high-voltage electric pulses, the aluminum cathode gave approximately two-fold higher ΔpH, most likely due to secondary chemical reactions [7]. These secondary reactions might be responsible for anomalous heating of a solution in electroporation experiments with aluminium electrodes observed by Pliquett et al. [24].

Recently, a substantial release of the aluminium ions from the aluminium cathode caused by the application of electric field pulses, commonly used in cell electroporation experiments was observed [7]. The release of aluminium ions not only from the anode but also from the cathode, can also explain the fact, that aluminium electrodes caused almost exactly

two times greater precipitation of macromolecules (DNA, RNA and proteins) than stainless steel electrodes, reported by Stapulionis [6].

Complexation of the metal ions released from the electrodes with the molecules present in the solution

One more consequence of the dissolution of the electrodes is the complexation of the metal ions released from the electrodes with the molecules present in the solution [6,9,25], with aluminum electrodes causing about two times greater precipitation of macromolecules (DNA, RNA and proteins) than stainless steel electrodes [6].

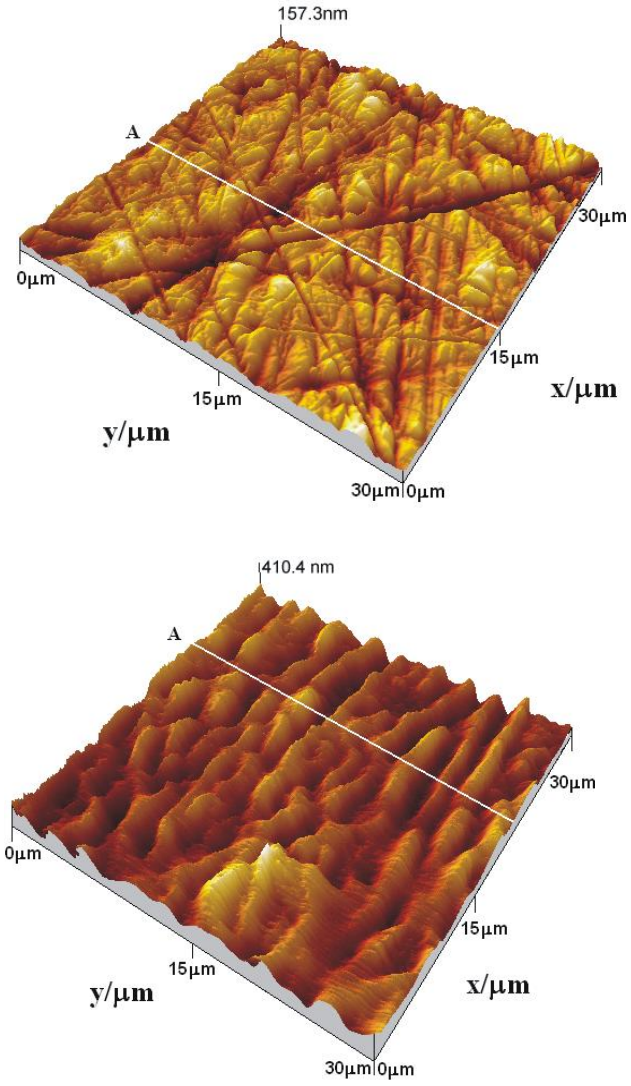

Figure 5: Typical three-dimensional AFM images of the surface of the stainless-steel anode: (A) polished anode prior to the exposure by high-voltage electric pulses and after the exposure to (B) 120 exponential pulses with the duration of about 20 µs (dissolution charge Q_{diss} = 0.24 As/cm^2 respectively). Scanning area 30 x 30 µm^2; *z* range: (A) 157 and (B) 410 nm [26].

Increase of the electrode surface roughness

The dissolution of the anode material can cause the increase of the roughness of the electrode surface. Recently, the changes of the surface topography of stainless-steel and aluminium electrodes occurring due to the action of electric pulses, which are commonly utilized in cell electroporation procedures, have been studied by using atomic force microscopy [26,27]. After the treatment of the chambers filled with 154 mM NaCl solution by a series of short (20–40 µs), high-voltage (4 kV) pulses with the total dissolution charge of 0.20–0.26 As/cm^2, the roughness of the surface of the electrodes has increased, depending on the total amount of the electric charge that has passed through the unit area of the electrode. Up to a three–fold increase of the surface roughness of the stainless-steel and aluminium anodes was observed due to the dissolution of the anode material (Fig. 5) [26,27].

Influence of metal ions on biochemical reactions

One of the first reports on such effects, the stimulation of the conversion of Ins(1,3,4,5)P_4 into Ins(1,4,5)P_3 by Al^{+3} ions released from the electrodes was noticed [28].

Changes of the medium conductivity

Due to anodic and cathodic half-reactions (1)–(2), changes in the electrolyte composition occur at each electrode. It can thus be expected that some changes in the conductivity of the solution treated by high-voltage pulse might occur. Recently, the changes of the electric conductivity of a minimum essential medium Eagle treated by a 2 ms-duration square-wave electric pulse of various amplitudes varied were reported. Albeit, conductivity changes were insignificant – within the range of 2–3% [10].

Quenching of fluorescence

Experimental data show that the medium treated with the electric pulse with the amplitude of 1.2 kV/cm and the duration of 100 and 500 µs almost completely quenched calcein fluorescence (Fig. 6) (Rodaite-Riseviciene et al., unpublished data). The metal ions, which are released from the electrodes, most likely are the reason of this effect as they can react with fluorescent molecules and decrease the intensity of their fluorescence. Experiments carried out by several groups have confirmed this assumption [10,20]. For example, ferric ions (Fe^{3+}) at the concentration of 1 mM totally suppressed the fluorescence of porphyrin-sulphonate and by 30% – the fluorescence of Adriamycin [10].

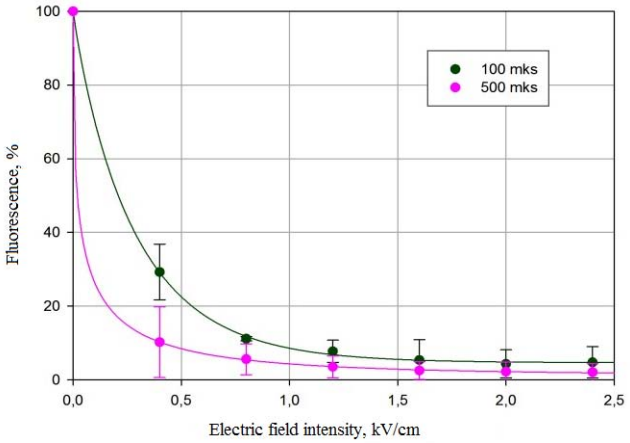

Figure 6: Quenching of calcein fluorescence by the medium treated with a single square-wave electric pulse with the duration of 100 and 500 μs.

Generation of reactive oxygen species

Due to the action of the electric current, various reactive oxygen species can be formed (Fig. 7) [29,30]. These include the highly reactive hydroxyl radical (•OH), the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) (Eq. (7)) and hypochlorous acid (HClO) (Eq. (4)).

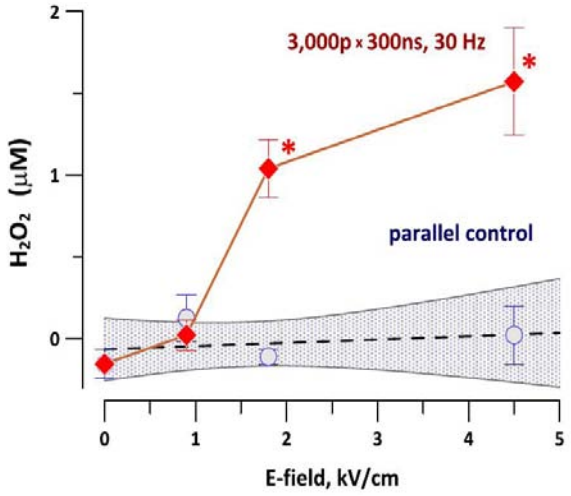

Figure 7: Yield of H_2O_2 in nsPEF-exposed phosphate buffered solution (PBS) as measured with Amplex Red reagent (mean +/- s.e., n = 3). Shaded area shows 95% confidence interval for control samples. *p < 0.05 compared to parallel control (two-tailed t test) [30].

WAYS TO REDUCE THE INTENSITY OF ELECTROCHEMICAL REACTIONS AND THEIR CONSEQUENCES

It is possible to reduce the intensity of electrochemical reactions, and, as a result, their undesirable consequences. The main ways are: i) reduction of the voltage used for PEF treatment by reducing the distance between the electrodes or local focusing of an electric field [31], ii) reduction of the pulse shape and/or duration [21], iii) reduction of the medium conductivity, and iv) utilization of bipolar pulses [5]. Each of these approaches has its advantages and limitations.

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Electroporation based technologies for Food and Biomass processing

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Abstract: This contribution reviews the modern state of the art in the field of PEF-assisted food and biomass processing of food plant materials, including pressing, solute extraction, drying, freezing, osmotic impregnation, and size reduction. It gives examples of applications and industrial perspective of PEF implementations.

INTRODUCTION

Application of electroporation in processing of foods and biomass transformation becomes more and more popular [1]. Electroporation can be induced by pulsed electric field (PEF), which is considered as a non-thermal treatment of very short duration (from several nanoseconds to several milliseconds) with pulse amplitude from 100-300 V/cm to 10-50 kV/cm. Electroporation can modify the mechanical and electro- physical properties of materials, and accelerate mass and heat transfer processes. Moreover, PEF-treatment allows avoidance of undesirable reactions in a biological material, which are typical for other techniques, such as thermal, chemical and enzymatic ones. The supplementary advantage of PEF treatment for food applications is related with a possibility of microbial killing.

Though the practical implementation of electricity for treatment of different biological tissues (of prunes, apples, grapes and sugar beets) was started in 1950th, number of problems still exist nowadays. The experimental works revealed that initiation of electroporation requires application of relatively high electric fields E~100 V/cm for plant cells and E~5-10 kV/cm for damage of microbial cells, and the effects are dependent on the treated material and details of pulse protocol [2]. Among numerous examples of PEF applications for intensification of food and biomass processing, pressing, solute extraction, drying, freezing, osmotic impregnation, size reduction may be mark out. However, the mechanisms of food plant and biomass modification due to PEF application are not well understood yet and it restrains the fast implementation of PEF-assisted extraction techniques.

This contribution reviews the modern state of the art in the field of PEF-assisted food and biomass processing of food plant materials, recent practical efforts, examples of applications, and industrial perspective of PEF implementations.

EXAMPLES OF SELECTIVE SEPARATION WITH PEF

Figure 1 presents the laboratory filter-press cell, equipped with an electrical treatment system. The frame was initially filled with the biological particles (sliced from fresh carrots, apples, and whole grapes and then was tightly closed from both sides by steel covers. The mobile electrode was attached to an elastic rubber diaphragm. The stationary wire gauze electrode was installed between the filter cloth and the layer of tissue slices. The electrodes of compression chamber were connected to the PEF generator. The constant pressure (5 bars) was applied to the layer

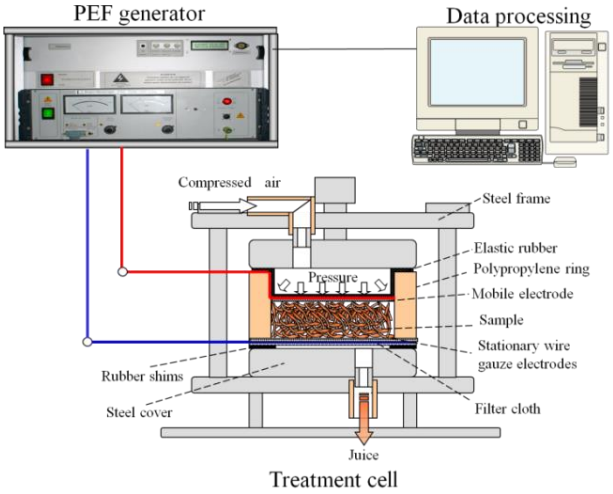

Figure 1: Experimental device for the pressing combined with PEF.

of fresh slices, grapes or wastes through the mobile electrode and elastic diaphragm. The PEF was applied after some time expression. Fig. 2 presents the curves of juice yield versus expression time t for grapes. The arrow show the moment of PEF application. As it can be seen, the PEF treatment enhances significantly the juice yield but it influences also the colour and dry matter of the expressed juices. The PEF application results in a considerable decrease of the absorbance and increase a dry matter content of juices immediately after the electrical treatment. It was also

confirmed for the different other biological press-cakes [1,2].

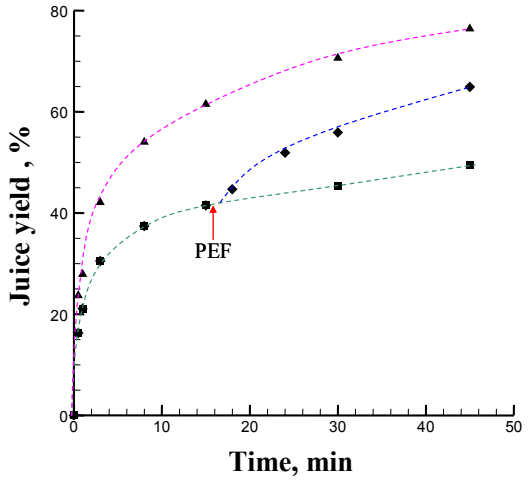

Figure 2: Juice yield expressed from grapes Muscadelle [3].

Another example of the juices obtained by PEF are presented in Fig. 4. It presents the juices obtained after PEF application to the Cabernet Sauvignon grapes in wine production permitting increase the wine color and the quantity of extracted anthocyanins [4].

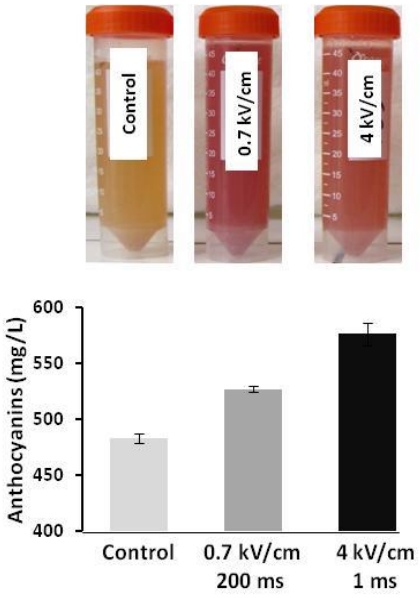

Figure 3: Juices obtained after PEF application to the Cabernet Sauvignon grapes in wine production [4].

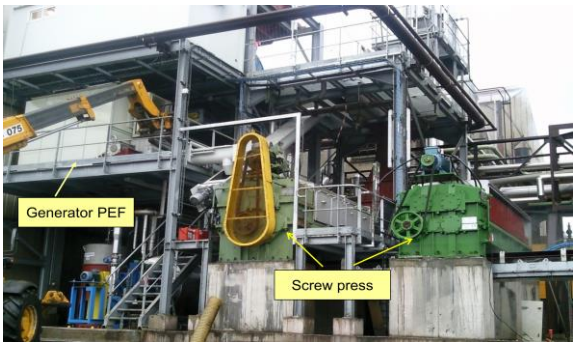

Figure 4: Industrial equipment for PEF treatment installed at the sugar factory.

The encouraging results, obtained by several research groups, revealed the industrial interest of PEF-pretreatment, and semi-industrial scale equipment was successfully tested for PEF-assisted sugar extraction by societies Maguin, Hazemeyer and the Technological University of Compiègne (Fig.4). Other examples were rapported for the PEF application in production of wines, starch, polyphenols, and proteins extraction.

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Expanding Electrochemotherapy from Local to Systemic Tumour Control

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Abstract: The interaction and relationship between cancer and the immune system is a complex one. The immune system can inhibit tumour growth by detecting abnormal antigens on these cells and eliminating them. This editing process however unless completely successful can ultimately result in the evolution of cancer cells that are considered weakly immunogenic and which can grow unchallenged by the immune system. In light of this, the focus of cancer treatment should be to enable immune detection of cancer cells and through the activation of an adaptive immune engagement ensure their systemic elimination. In this paper we will examine current and emerging understanding around the potential for electroporation and electrochemotherapy to induce Immunogenic Cell Death (ICD), a special type of cell death that allows for immune cell involvement and the generation of an anti-tumour specific immune response. When paired with immune modulating agents, capable of potentiating the immune response and reversing the immune-suppressive environment created by tumours, we may be looking at the future of anti-cancer therapy.

INTRODUCTION

The main function of the immune system is to protect the body against pathogens; it detects these pathogens via a set of pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs) [1]. PAMPs include viral RNA, the components of bacterial cells walls and when detected trigger the activation of the innate immune system to protect the host [2]. However not all threats come in the form of invading bacterial/viral organisms and so the immune system has developed the ability to identify and eliminate abnormal/cancerous cells. Two landmark murine studies demonstrated the importance of a functional immune system in preventing carcinogenesis; mice lacking IFN-γ responsiveness or specific immune cells (T cells, B cells and NK cells) were more susceptible to chemically induced tumour formation [3, 4]. Lung and kidney transplant patients are put on immunosuppressive drugs (cyclosporine A, corticosteroids etc.) to prevent against transplant rejection, the fact that these patients have been shown to have a higher chance of developing neoplastic malignancies, reinforces the protective importance of the immune system [5-10]. Subsequent work in the field has shown that 'immuno-surveillance' is only one aspect of the complex relationship between the immune system and cancer [11] and has led to formation of the 'cancer immuno-editing' hypothesis.

Cancer immuno-editing is a refinement on the original 'immuno-surveillance' idea and suggests that the immune system not only protects the host against cancer, but also shapes tumour immunogenicity (the ability for the tumour to provoke an immune response). Murine studies have shown that tumours that develop in immune-competent mice (deemed 'edited' tumours) often grow more easily than tumours that originate from immunocompromised mice ('unedited'), when transplanted into syngeneic immune-competent mice [4].

The innate immune system acts as our body's first line of defence and its main components are dendritic cells (DCs), macrophages and monocytes, neutrophils, natural killer (NK) cells, and natural killer T (NKT) cells. Dendritic cells (DCs) are often regarded as the most effective of the antigen presenting cells (APCs) and type I IFNs have important effects on DC differentiation and maturation [12, 13].

Adaptive immunity consists of T and B-lymphocytes and their respective mediators (cytokines and antibodies) and its ability to generate an immune 'memory'. It is the interplay and communication between both arms of the immune system that make it effective against cancer Functional cytotoxic $CD8^+$ (CTL) and helper $CD4^+$ T (T_H1) cells are key for the eradication of cancerous cells. The T cell receptor (TCR) of cytotoxic $CD8^+$ T cells is capable of binding with the MHC-1 molecule of abnormal/cancerous cells or antigen presenting cells (APC) and causes subsequent cellular lysis through the release of perforin, granzymes and granulysin.

Immune inhibitory receptors such as Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and Programmed Death 1 (PD-1), which are expressed on the surface of activated T cells, can effectively shut down T cell activity upon binding to their ligands CD80/CD86 and PD-L1/ PD-L2 respectively. The complex interplay between these stimulatory and inhibitory mechanisms has been shown to be crucial in the immune mediated elimination of cancer [14]. For example increased expression of PD-L1 is associated with decreased T cell activity, infiltration and poorer prognosis in many cancers [15-18]. Similar correlations have been found

in melanoma patients expressing higher levels of CTLA-4 receptor and its ligands [19]. Regulatory T cells (Tregs), a type of T cell that suppresses exaggerated and prolonged immune responses, have a very negative impact on tumour elimination by promoting an immunosuppressive environment [20, 21]. Increased Tregs at the tumour site correlates with more advanced disease and poorer prognosis [22-25] while low Treg percentage correlates with improved survival [25, 26].

THE ABSCOPAL EFFECT & IMMUNOGENIC CELL DEATH

In light of this complex relationship between the host immune system and cancer, an emphasis on maximizing 'Elimination' and preventing 'Escape' mechanisms is critical when applying interventions or developing new treatments. While most conventional treatment modalities (radiation, chemotherapy) were once thought to induce cancer cell death in an immune independent manner, however this is not the case.

The Abscopal Effect

There are a number of physical ablative modalities and chemotherapeutics which have caused regression in tumours that are distal to the primary site of treatment [27]. This has been observed in more than one type of cancer [28-30] and strongly indicates that immune mechanisms are involved. This phenomenon has been termed the 'Abscopal Effect' and while our understanding of the mechanism is still incomplete at best, it does have interesting parallels with Immunogenic Cell Death, a type of cell death that induces a positive immune response through the release of damage associated molecular proteins (DAMPS), [31]. Whether ICD plays a direct role in mediating the abscopal effect is yet to be determined but it could go a long way to explaining to the phenomenon.

Immunogenic Cell Death & DAMPs

The term Immunogenic Cell Death (ICD) was coined to describe cell death that stimulates immune cells in the tumour environment and results in a favourable anti-cancer host immune response. Whether a dying cell can be described as undergoing ICD, depends on a number of factors, the activation state of the cell is one such criteria [32]. The type of cellular stress is another differentiator, the DNA-damage-response and endoplasmic reticulum (ER) stress are two specific examples known to elicit immunogenic responses [33]. Caspase activation is also important as inhibiting caspase activity can prevent the mobilisation of immuno-epitopes within the cell also inhibits certain signals, key to the ICD response [34]. However the release of DAMPs from dying cells is probably the most important factor characterising ICD as DAMPs are the key mediator in causing the ICD related immune response generated by stressed or dying cancerous cells [35].

Many different DAMPs have been identified and their presentation/exposure to the immune system depends on the cancer treatment used and the mechanism of cancer cell death involved. Some of the key DAMPs involved in immunogenic cell death and how they interact with immune cells, are visually outlined in Figure 1.

ELECTROCHEMOTHERAPY, ICD AND DAMP RELEASE

With recent research highlighting the importance of DAMP release and ICD in immune mediated clearance of cancer, focus now needs to shift towards developing clinical interventions that harness this potential. **Figure 2** illustrates that the types of DAMPs released from dying cells depends on the cause of cell death and below we will look at some of the established and emerging ablative technologies known to induce ICD and/or DAMP release.

Electrochemotherapy and ICD:

A number of commonly used chemotherapeutic agents have been shown to cause ICD. Platinum based drugs, such as oxaliplatin, and various anthracyclines (doxorubicin) have been shown to induce the release of many DAMPs. These DAMPs, as previously discussed are critical to inducing ICD. In fact the anti-cancer abilities of oxaliplatin and anthracyclines are better in immune-competent mice, than in immune-deficient athymic mice, highlighting the importance the immune system plays in their mechanisms [36-38].

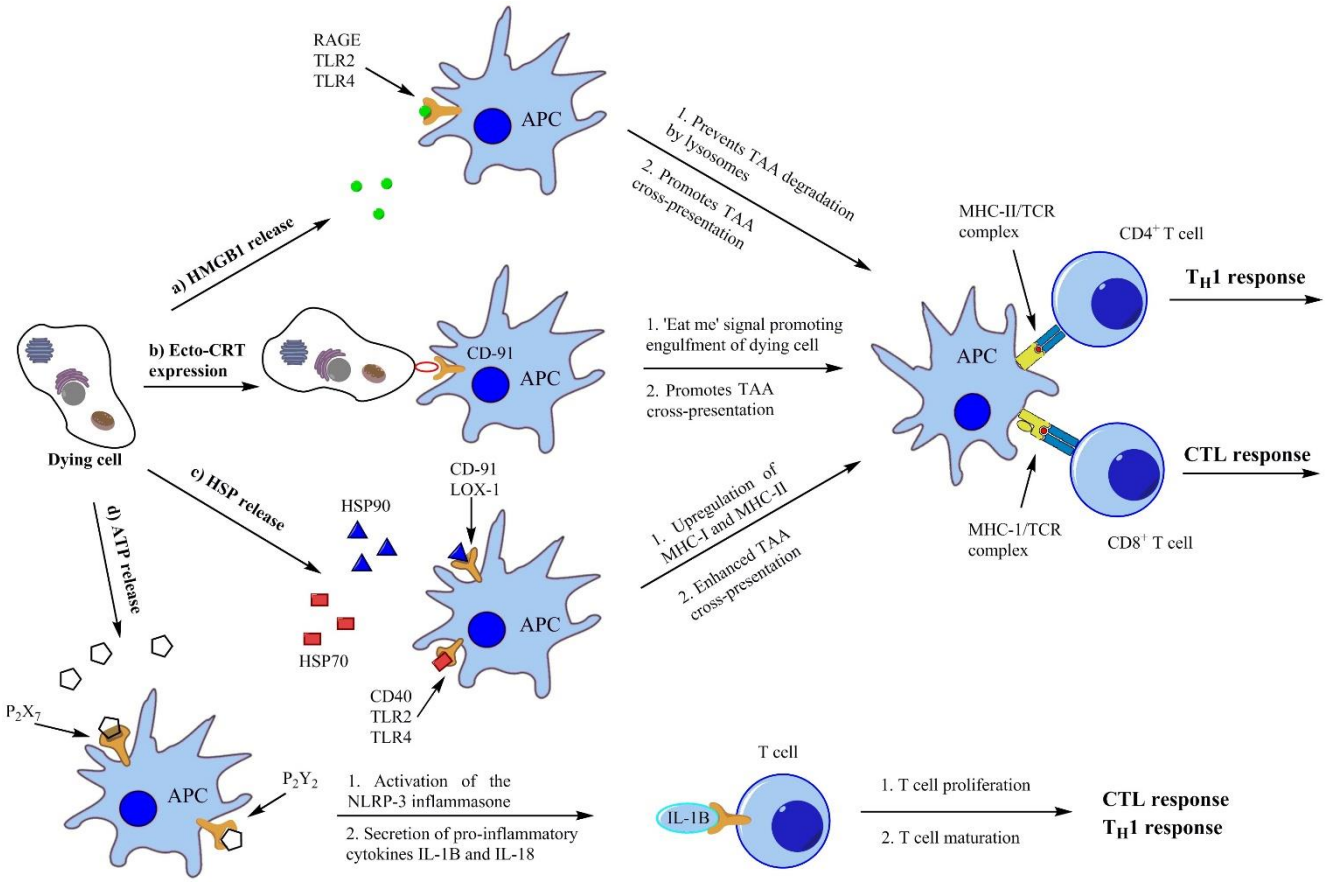

Figure 1: *Interaction between the cells of the immune system and DAMPs released from dying tumour cells.* a) HMGB-1 released from dying cell has the ability to bind to RAGE, TLR2 and TLR4 receptors on APCs, this increases the amount of TAAs processed and presented to T cells by preventing the degradation of TAAs in the lysosomes of APCs. b) The expression of ecto-CRT on dying cells effectively marks them for engulfment by APCs, the contents of the tumour cell (including TAAs) are then processed and presented to T cells. c) HSPs can bind to many surface receptors on APCs, including endocytic receptors and immune signalling receptors. This leads to increased tumour cell uptake and up regulation of the antigen presenting molecules MHC-1 and MHC-II. d) Extracellular ATP bind to purigenic receptors on APCs, activating the NLRP-3 inflammasome and causing the secretion of pro-inflammatory cytokines to promote T cell activity.

These capabilities become even more valuable when adding the potential of Electrochemotherapy (ECT) to the treatment. Given the ability of platinum based drugs to induce ICD through the release of DAMPs, the increase in cytotoxicity acheived with electroporation is very desireable. Another compound frequently used as part of ECT, bleomycin, has displayed the ability to induce ICD *in vitro*. Bleomycin as a stand-alone agent has caused ICD in a murine model [39], however when combined with EP, as part of an ECT regime, the activity of bleomycin is increased 700 fold [40], with bleomycin causes the release of HMGB1 from dying cells [41], and interestingly, the same study showed that application of electric pulses alone was enough to cause ATP release and extracellular CRT expression on treated cells [41]. Therefore ECT can be thought of as a tool to enhance the effect of other ICD inducers, facilitating their access into tumour cells in much higher concentrations. Unlike other ablative methods, ECT is non-destructive to healthy tissues surrounding treated tumour sites and because it requires much lower doses of chemotherapy drugs to achieve greater results it has an incredibly low side effect profile, making it a very attractive method of causing ICD [42].

IMMUNE MODULATION & BIOLOGICAL INTERVENTION

While ablative technologies are certainly effective at causing ICD and the generation of anti-tumour specific immune response, they are often not enough to cause total cancer elimination on their own, especially in cases of metastatic disease. To potentiate their effects, the combination of an immune modulating agent with an ablative modality, is the logical step forward. A summary of current and ongoing clinical trials combining ablative methods with immune modulators can be found in **Table 1**. The immune inhibitory receptors that have generated the most interest in cancer therapy are CTLA-4 and

PD-1, and the development of checkpoint blocking antibodies has been largely focused on these two receptors.

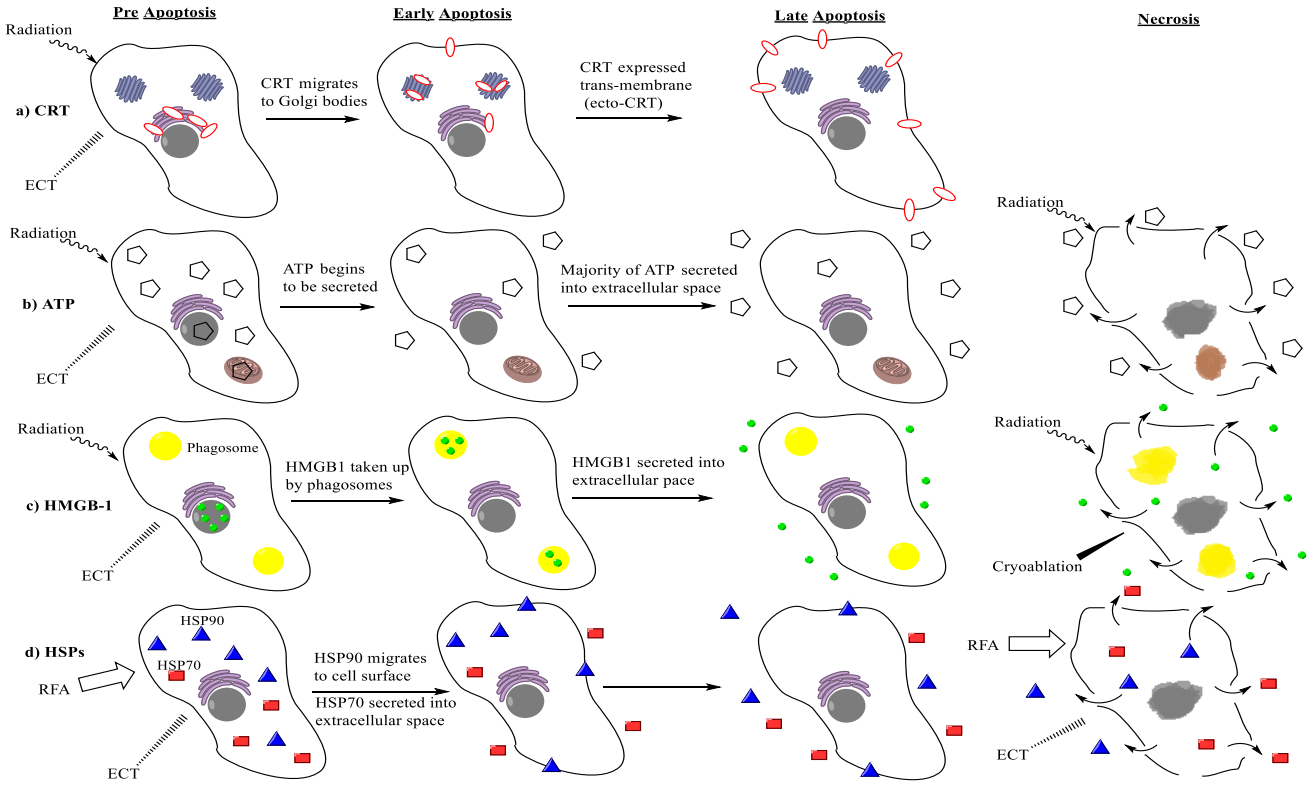

Figure 2: *Immunogenic cell death (ICD) caused by ablative modalities and subsequent DAMP release.* a) Cellular stress causes CRT to migrate from ER to Golgi bodies and as apoptosis progresses; CRT is expressed on the plasma membrane (ecto-CRT). This can be induced by radiation and ECT. b) During cellular apoptosis, ATP is actively secreted from dying cells. The loss of membrane integrity during necrosis allows leakage of ATP into the extracellular space. Radiation and ECT have been proven to cause this ATP release. c) HMGB-1 is typically located within the nucleus but during apoptosis it relocates to phagosomes in the cytoplasm, late stages of apoptosis and necrosis sees it expelled in the extracellular space. Ablative techniques that cause this include cryoablation, radiation and ECT. d) Heat shock proteins move from intracellular compartments to the extracellular space and form complexes on the plasma membrane as the stages of apoptosis advance. Cellular necrosis, as caused by RFA and in some cases ECT, lead to passive diffusion of HSPs into the extracellular space.

CONCLUSION

Developments recently have improved our understanding of the complex relationship between the immune system and cancer. We now have a better understanding of the mechanisms used by the immune system to detect and eliminate cancer, as well as the ways in which cancer cells can evade and manipulate it. The emergence of ICD and DAMPs has created awareness that causing cancer cell death alone is insufficient, the type of cell death and the method by which it is achieved is crucial if we are to get the best responses. Conventional cancer therapies tend to focus on either causing maximal cancer cell death or modulating the immunological response of the body to the cancer. For improved clinical outcomes, these two strategies must be married together, developing treatment regimens that maximise cancer cell death in an immunogenic manner and harness the significant capabilities of the immune system thus leading to improved patient survival. Initial data from current clinical trials, combining these two modalities, is very encouraging and indicated the opportunties that exist for expanded clinical investigations in this space. Treatments such as ECT show great promise given its low side effect profile and its ability to generate ICD, further work combining ECT and immune modulators will likely yield very positive outcomes in the clinic.

Table 1: Table of all current clinical trials combining immune modulators and ablative modalities.

Biological Agent	*Ablative Intervention*	*Chemical Agent*	*Cancer Type*	*Study Size*	*Outcome*	*Ref*
Ipilimumab	-	-	Metastatic Melanoma	Phase III -167 patients	10.1 month median overall survival	161
Ipilimumab	-	Carboplatin, Dacarbazine	Small Cell Lung Cancer (SCLC)	Phase II -130 patients	irPFS of 6.2 month and median overall survival of 12.9 months	162
Ipilimumab	-	w/wo dacarbazine	Advanced melanoma	Phase II -72 patients	14.3 and 11.4 month median overall survival repectively	163
Ipilimumab	-	dicarbazine	Metastatic Melanoma	PhaseIII -251 patients	11.2 month median overall survival	164
Ipilimumab	Radiotherapy	-	Prostate Cancer	Phase I/II - 33 patients	PSA decline ≥ 50% and 21% postive response	165
Ipilimumab	Radiotherapy	-	Prostate Cancer	Phase II - 45 patients	PSA decline ≥ 50% and median postive response of 23 weeks	166
Ipilimumab	Cryoablation	-	Breast Cancer	Pilot - 18 patients	Study ongoing	170
Ipilimumab	ECT	Bleomycin	Advanced melanoma	Pilot - 15 patients	67% local regression, 47% distal regression and median survival of 12.4 months	171
Nivolumab	-	-	NSCLC, melanoma, renal cell carcinoma	Phase I - 296 patients	NSCLC: 18% Melanoma: 28% Renal cell carcinoma: 27% Durable respones lasting over 12 months	183
Nivolumab	-	-	Advanced melanoma	Phase I - 107 patients	31% respone rate and improved median survival of 17 months	184
CT-011	-	Gemcitabine	Pancreatic Cancer	Phase II - 39 patients	Study ongoing	NCT01313416
CP-870,893	-	-	Advanced Solid Malignancies	Phase I - 29 patients	14% overal postitive response; 27% positive response in melanoma patient sub group	216
CP-870,893		Carboplatin and paclitaxel	Advanced Solid Malignancies	Phase I - 32 patients	20% positive partial responses obsevred, with another 40% diplaying stable disease	220
SGN-40	-	-	Non Hodgkins Lymphoma	Phase I - 35 patients	37.5 % postitive objective response rate and overal survival monitoring still ongoing	219

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Electrochemotherapy of the colorectal liver metastases – has the time come?

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Abstract: Patients with colorectal liver metastases belong to the stage IV (the highest) according to the AJCC (American Joint Committee on Cancer). The best treatment option for these patients is surgical resection with or without systemic treatment. Complete surgical resection of these metastases, however, is not always possible. In such cases, there are alternative, so-called ablative techniques which destroy metastases and surrounding tissue using very high or very low temperatures, usually generated by high frequency current or cooling devices. Survival after such procedures is not high. Electrochemotherapy is a new, non-thermal procedure, which combines reversible electroporation and cytotoxic drugs. A prospective study was designed to evaluate the feasibility, safety and efficacy of intraoperative electrochemotherapy in the treatment of colorectal liver metastases. This study radiologically showed 85% complete and 15% partial responses. Histological analysis showed significantly less viable tumor tissue ($p = 0.001$) in metastases treated with electrochemotherapy. The efficacy of electrochemotherapy was almost the same on metastases located distally or proximally to the major, which is not the case using convention ablative procedure. Electrochemotherapy of the colorectal liver metastases proved to be feasible, safe and efficient treatment modality. It was shown to have a specific place in difficult to treat metastases, located in the vicinity of major hepatic vessels, not amenable to surgery or radiofrequency ablation.

CONVENTIONAL TREATMENT OF THE COLORECTAL LIVER METASTASES

Treatment of the patients with resectable colorectal liver metastases with best chances to high survival is surgical, but many patients are presented with unresectable metastases, due to their size, location, and inadequate remnant liver volume, or they are inoperable due to the extent of the disease and comorbidities. In such unresectable or inoperable cases, several so-called ablation techniques are used, such as radiofrequency ablation [1], microwave ablation [2,3], laser ablation [4], cryosurgical ablation [5], alcohol ablation [6] as well as other procedures using interventional radiologists approach. Radiofrequency ablation (RFA) is by far mostly used thermal method of ablation, demanding delivery of at least 60°C in the target tissue to make a cell kill effect. Therefore, its efficacy is lower in the vicinity of big vessels due to heat sink effect [7]. RFA has also additional side effects such as hyper-thermic effect on healthy liver tissue surrounding metastases by destruction of important liver structures affected by high temperature and releasing various tissue factors.

ELECTROCHEMOTHERAPY

Electrochemotherapy (ECT) is a new, non-thermal local treatment modality that combines the use of poorly or non-permeant chemotherapeutics, such as bleomycin or cisplatin, with electroporation, which facilitates drug diffusion into the cells, thus increasing their cytotoxicity [8,9]. Electrochemotherapy has proven efficacy on different superficial tumors, predominantly in melanoma [10,11]. Local tumor control of skin metastases up to 3 cm in diameter is 84%, but larger ones that have not responded completely can be effectively re-treated [11–14]. The treatment is now widely used in Europe, as standard treatment for in transit melanoma metastases [15]. With the aim of broadening indications for electrochemotherapy, new technological developments have been made, which have enabled the treatment of tumors located in the colon and rectum, esophagus, and various internal or deep seated tumors [16].

In order to explore the feasibility, safety and efficacy of this treatment approach for liver tumors, we conducted a prospective clinical trial for the treatment of colorectal liver metastases. In the study, long needle electrodes as well as electrodes with fixed geometry were inserted into the tumor and around it to cover the tumor, including a margin of normal tissue, with a sufficiently high electric field [17], as described previously in the treatment of liver metastasis in a clinical case [18]. The aim of this prospective study reported here was to provide the evidence of the feasibility, safety and efficacy of this approach, on 16 patients on whom 29 colorectal liver metastases were treated by electrochemotherapy.

CLINICAL TRIAL

Trial design

The study was a prospective pilot study, conducted at the Institute of Oncology Ljubljana, Ljubljana, Slovenia. Regulatory approvals from the Institutional Board, as well as from the National Medical Ethics

Committee (# 45/09/08) were obtained. The study was registered at ClinicalTrials.gov: NCT01264952.

The primary objective of the study was evaluation of the feasibility and safety of intraoperative electrochemotherapy of colorectal liver metastases. The secondary objective was to determine the efficacy of the treatment based on histological analysis of the treated metastases and radiological evaluation of the electrochemotherapy treated metastases.

Patients

Three groups of patients with colorectal liver metastases were included in the study:

The first group included patients with bilateral, multiple, metachronous metastases who would anyway had two-stage surgery, due to the extent of the disease and/or their general condition. During the first operation, some metastases on the right side were treated by electrochemotherapy and some were not, and the right portal vein ligated. Metastases on the left side were excised or ablated with radiofrequency ablation. During the second operation, both treated and non-treated metastases on the right side were removed with liver resection.

The second group included patients with synchronous metastases but their general condition and extent of the disease would not allow simultaneous removal of the primary tumor and metastases. During the first operation, the primary tumor was removed (colorectal resection) and some of the liver metastases treated by electrochemotherapy. About 6 weeks later, during the second operation for liver metastases, both treated and non-treated metastases were removed with liver resection.

The third group included patients with up to 3 metachronous liver metastases that were unresectable, demanding too excessive resection or were untreatable by standard the [19] ablative methods due to the close proximity of the major blood vessels.

Treatment procedure

The treatment of colorectal liver metastases was performed during open surgery, as described previously (13). Briefly, based on computed tomography or magnetic resonance scans, target lesions (up to 3 cm in the largest diameter) were identified and serial images were used for the preparation of the treatment plan to assure coverage of the tumor with a sufficiently high electric field, based on numerical modeling [17,20,21]. Several different electrode configurations were designed in consultation with the surgeon, to identify the most accessible route of electrode insertion. Using an optimization algorithm coupled with a finite-element model of electroporation, the minimum required voltage for each electrode pair was computed, to guarantee adequate electric field distribution in the tumor [21]. Long needle electrodes with variable geometry (1.2 mm in diameter with 3 or 4 cm non-insulated tip) were inserted into the tumor and around it in the normal liver tissue (Figure 1) according to the individual treatment plan which included also calculated voltage between each neighboring pair of the electrodes. When smaller (< 2 cm in diameter) not more than 3 cm deep metastases were treated, hexagonal needle electrodes with fixed geometry were (Fig 1) used (17 mm in diameter, 30 mm long) with fixed voltage of 730 V between each neighboring pair of electrodes. Regardless of the type of the electrodes, 8 100 µs pulses were delivered between each neighboring pair of needles.

Electrodes and the electric pulse generator Cliniporator VITAE were produced by the same producer (IGEA SpA, Carpi, Italy).

Figure 1. Long needle electrodes (left) with variable geometry and electrodes with fixed geometry (right)

Electrodes were inserted during open surgery under ultrasound (US) guidance, following the treatment plan. When the entire set up was prepared and the electrodes were connected to the generator, the amplitudes of electric pulses and the sequence of delivering electric pulses between the electrode pairs were set according to the treatment plan. Electric pulses were delivered during an interval of 8-28 minutes after the intravenous injection of bleomycin 15,000 IU/m^2 in bolus (Heinrich Mack Nachf. GmbH & CO. KG, Illertissen, Germany). Sets of 8 electric pulses (each pulse 100 µs long) were delivered between each pair of electrodes, consecutively [18].

To maximize the safety of the patient, the delivery of electric pulses was synchronized with the absolute refractory period of the heart to prevent the electric pulses from being delivered during the vulnerable period of the ventricles [22–24].

RESULTS

In total, 29 metastases (from 16 pts) were treated by electrochemotherapy. The median diameter of the

treated metastases, measured radiologically on transversal planes, was 18 mm (range 6 - 29 mm). Of 27 evaluable metastases, 6 were treated by electrodes with fixed geometry and 21 by electrodes with variable geometry, providing a variable geometry of electrode positioning.

Pathologic analysis was performed on metastases resected at the second operation, after the first operation when electrochemotherapy was performed. Altogether, 35 colorectal liver metastases were microscopically analyzed, 13 of them being treated by electrochemotherapy. Pathological analysis revealed that non-electrochemotherapy treated metastases were associated with a significantly higher percentage of residual vital tumor tissue than electrochemotherapy treated metastases. On average, electrochemotherapy treated metastases had 9.9 ± 12.2 % (AM±SD) viable tissue, and those without electrochemotherapy 34.1 ± 22.5 % (p = 0.001, two tailed t-test). This data is already published [25]. When patients were stratified according to the magnitude of tumor viability (0-<1%, 1-25%, 26-50%, >51%), a statistically significantly higher proportion of patients treated by electrochemotherapy had a complete or major pathologic response, compared to patients that received no electrochemotherapy (77.0% vs. 45.4%; p = 0.006, Chi-square test) (Table 1).

	Pathological response				
Group	Complete (%)	Major (%)	Medium (%)	Minor (%)	∑
Control	1 (4.5)	9 (40.9)	7 (31.9)	5 (22.7)	22
ECT	7 (54.0)	3 (23.0)	3 (23.0)	0 (0.0)	13
∑	8	12	10	5	35

Table 1. Pathological changes in electrochemotherapy treated and non-electrochemotherapy treated metastases, evaluated 4-6 weeks after the treatment

All patients except one (who progressed after the ECT) were radiologically evaluated after ECT. The median interval between the treatment and evaluation was 33 days (range 14-76 days). Twenty seven metastases were evaluated with complete response (CR) observed in 23 (85%) (Figure 2). In 4 metastases (15%) some enhancement of the treated lesion was seen, in both phases of liver enhancement, and they were evaluated as a partial response (PR) or local tumor progression.

In the group of 8 patients with a single stage operation, 14 metastases were treated by electrochemotherapy. On the first follow up examination at a median of 33 days (range- 14-59 days) after electrochemotherapy, a complete response was seen in 12 metastases (86%). In the second follow-up, median 147 days after electrochemotherapy, 10 (71%) metastases were still in complete response, while the other 4 progressed. More details can be found in [25].

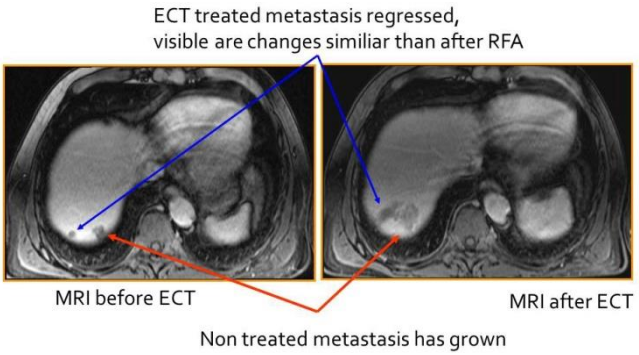

Figure 2.Difference between treated and non-treated metastasis (ECT – electrochemotherapy)

Out of 27 evaluable metastases, 6 of them were treated by electrodes with fixed geometry and 21 by electrodes with variable geometry. In the group of 6 metastases treated by electrodes with fixed geometry, there were 4 metastases (66.6%) with CR and 2 metastases (33.3%) with PR after the first radiological evaluation. In the group of 21 metastases treated by electrodes with variable geometry, there were 19 metastases (90%) with CR and 2 metastases (10%) with PR. There was no statistically significant difference in efficacy between electrodes with variable geometry and electrodes with fixed geometry (P = 0.1477, Chi square test).

Out of these 27 metastases, 13 were located close to or on the major vessels and 14 were located more peripherally. 10/13 (77%) metastases located close to the major vessels radiologically completely responded as well as 13/14 (93%) located more peripherally. There was no statistically significant difference between these two groups (p=0.244, Chi square test)

DISCUSSION

In our study, we treated 27 evaluable colorectal liver metastases in different anatomical locations of the liver, including 13 metastases in the vicinity of major hepatic vessels. All treatments were feasible, as long as the electrodes could be precisely placed into and around the metastases. Based on the carefully prepared treatment plan, also difficult approaches to the tumors, not only from the shortest distance from the surface, were possible, by using long needle electrodes that have an non-insulated part of 3 or 4 cm in length. For smaller metastases (< 2 cm) on the surface of the liver, not deeper than 3 cm, electrodes with fixed geometry can be used, which do not require a treatment plan.

We observed higher rate of CR (90%) in the metastases treated with electrodes with variable

geometry compared to metastases treated with the electrodes with fixed geometry (66.6% CR). This difference is not statistically significant; however, it suggests the treatment by electrodes with variable geometry is at least as effective as treatment by electrodes with fixed geometry.

It is very well known that metastases adjacent to the major vessels do not respond very well to the RFA due to the heat sink effect. These metastases are often also non-resectable, so actually there is no effective treatment for these metastases. In our trial, both metastases located more peripherally and adjacent to the major vessels responded very well without statistically significant difference between the two groups. This data suggest that electrochemotherapy may be used *a priori* for metastases located adjacent to the major vessels. This; however, should be confirmed with extended phase II clinical trial before becoming a part of clinical guidelines.

After second radiological follow-up, 4 metastases from group III progressed. The most probable reason for their progression is inaccurate introduction of the electrodes into the liver and consequently inadequate distribution of the electric field. However also other possible reasons such as poor vascularization of tumor and consequently insufficient drug concentration must not be excluded. The technology for treatment of liver metastases using electrochemotherapy has been proved to be feasible; however, there is still room for improvements.

CONCLUSSION

Electrochemotherapy is safe and efficient way for treatment of liver metastases. It is an evolving procedure which currently may be used only within clinical trials. With available data, we can be almost certain that it will inevitably become a part of our clinical guidelines.

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Monitoring of electric field distribution during electroporation pulse delivery by means of MREIT: from concept to first in vivo results

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Abstract: Monitoring of electroporation process presents one of the most important aspects towards safe and efficient use of electroporation in clinical procedures such as electrochemotherapy and irreversible electroporation tissue ablation. Various methods of monitoring electroporation process were already suggested, particularly of irreversible electroporation where changes of tissue properties can be detected quite easily. However, monitoring of reversible electroporation is more demanding task since there are almost no immediate visible physical changes in the treated tissue. As accurate coverage of the tissue with a sufficiently large electric field presents one of the most important conditions for successful electroporation, we proposed a method for determining electric field distribution during electroporation based on magnetic resonance electrical impedance tomography (MREIT). MREIT proved to be able to determine electric field distribution during electroporation in agar phantoms, ex vivo tissues and in vivo. Here, the concept of monitoring electric field during electroporation is briefly explained and its feasibility is demonstrated.

MONITORING OF ELECTROPORATION

Monitoring of electroporation process presents one of the most important aspects towards safe and efficient use of electroporation in clinical procedures such as electrochemotherapy [1,2] and irreversible electroporation tissue ablation [3,4]. Different approaches have already been suggested: electric conductivity measurement with electric impedance tomography [5,6], voltage and current measurements of delivered pulses [7], magnetic resonance (MR) imaging [8, 9], and ultrasonography [4,10]. However, they are mostly limited solely to irreversible electroporation applications [4,10], are unable to be used to monitor the process during pulse delivery [8,11], and can lead to inaccurate results [7], thus preventing control of the procedure and not allowing potential corrective intervention. Monitoring of reversible electroporation is even more demanding task since there are almost no immediate visible physical changes in treated tissue. Practical and reliable method of monitoring electroporation process is therefore needed. A method capable of determining electric field distribution during the pulse delivery seems to be useful as electroporation depends on local electric field [12]. This would enable detection of insufficient electric field coverage before the end of either reversible or irreversible electroporation treatment, thus increasing and assuring its effectiveness. As the electric field distribution inside the treated tissue cannot be measured directly, an indirect approach was proposed and its feasibility evaluated.

Magnetic resonance electrical impedance tomography (MREIT) is a prime candidate for determining electric field distribution during electroporation [13]. The method enables reconstruction of electric field distribution by measurement of the electric current density distribution [14] and electrical conductivity of the treated subject during application of electric pulses using MRI and numerical algorithms. This enables determination of electric field distribution *in situ* while taking into account changes that occur in the tissue due to electroporation. Feasibility of this method was demonstrated by determining electric field distribution in an agar phantom [13], in liver tissue *ex vivo* [15,16], *in silico* [15] and recently during electroporation of mouse tumor *in vivo* [17].

MAGNETIC RESONANCE ELECTRICAL IMPEDANCE TOMOGRAPHY

Electric field distribution **E** can be calculated using Ohm's law when an electric current density **J** and electrical conductivity σ are obtained:

$$\mathbf{E} = \frac{\mathbf{J}}{\sigma} \qquad (1)$$

The electric current density inside a conductive tissue can be obtained by MRI using current density imaging technique (CDI) by measuring magnetic field changes caused by applied current [18,19]. MREIT is relatively new medical imaging modality based on CDI for visualizing electrical conductivity distribution inside a conductive sample [20]. Typical MREIT generator for applying electric pulses to establish a current density inside an imaging object is based on a current source [21]. It should be noted that typical MREIT setup for delivering electric pulses differs greatly from the typical electroporation setup which

employs voltage sources and sometimes even needle electrodes if required (Figure 1).

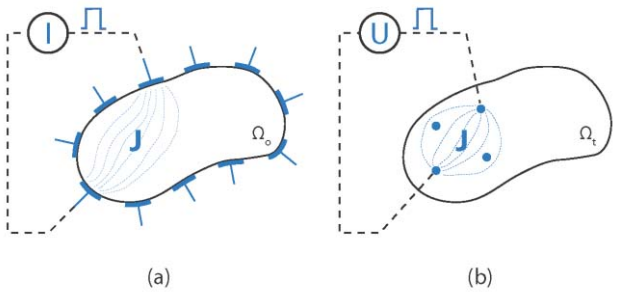

***Figure 1*:** Typical MREIT setup for establishing current density distribution in the imaging object Ω_o using current source and surface electrodes (a). Electroporation setup for delivering electric pulses in treated tissue Ω_t using voltage source and needle electrodes (b).

MREIT J-substitution algorithm [22,23] was applied for reconstruction of electrical conductivity in the measurements objects in all described experiments. The algorithm is based on solving Laplace's equation inside mathematical model of the measurement object Ω_M:

$$\nabla \cdot \sigma_M \nabla u = 0 \text{ in } \Omega_M \quad (2)$$

where σ_M is electrical conductivity of the measurement object. Equation 2 is solved iteratively using finite element method. After each iteration the solution u is used in

$$\sigma_M^* = \frac{|\mathbf{J}_{CDI}|}{|\nabla u|} \quad (3)$$

where σ_M* is the new conductivity and $\mathbf{J}_{CDI}$ is the current density obtained by the CDI method. The iterative scheme lasts until the relative difference between two successive σ_M is negligible.

All experiments were performed using 2.35-T Oxford MR imager (100-MHz proton nuclear MR frequency) equipped with Bruker microimaging accessories with maximum gradients of 250 mT/m.

RESULTS

The method of determining electric field distribution during application of electric pulses was demonstrated in agar phantoms, in tissues *ex vivo*, *in silico* and *in vivo* during electroporation of mouse tumor.

Agar phantoms

Determination of electric field distribution during tissue electroporation was first demonstrated experimentally and compared to numerical results on homogeneous and heterogeneous agar phantom with electrical properties similar to human tumor and surrounding tissue [13].

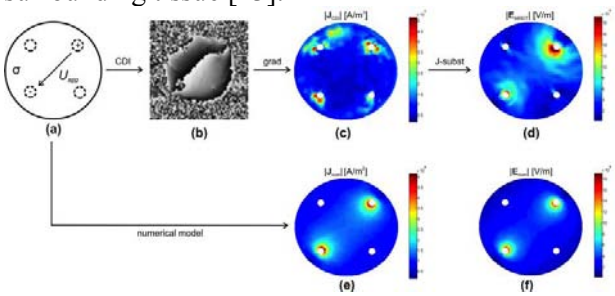

***Figure 2*:** Homogeneous phantom exposed to four high voltage pulses of 1000 V delivered between diagonal electrodes (a). The initial phase image (b) was acquired by the two-shot RARE based CDI sequence. The current density distribution (c) in the phantom was calculated from the phase image using (3). Finally, the electric field distribution (d) was calculated using J-substitution algorithm from the current density distribution. Numerical model results of current density distribution (e) and electric field distribution (f) in the phantom are shown below the corresponding experimental results

A good agreement between experimental and numerical results was obtained for different pulse sequences, i.e. for different number and amplitude of pulses, suggesting that MREIT can be used to determine electric field distribution during electroporation.

Ex vivo tissue

As shown on Figure 3 determination of electric field distribution during tissue electroporation was also successfully demonstrated on *ex vivo* chicken liver tissue [15].

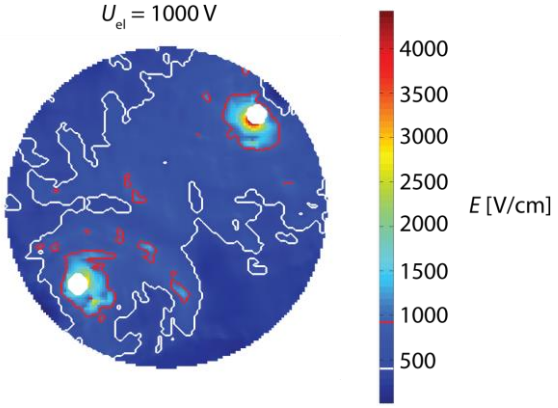

***Figure 3*:** Electric field distribution in the liver tissue exposed to sequence of four electric pulses of amplitude 1000 V obtained by means of MREIT. Pulses were delivered between two needle electrodes marked with + and -. A white and red line enclose an area exposed to an electric field strength above reversible (400 V/cm) and irreversible (900 V/cm) electroporation threshold value, respectively.

Furthermore, experimental and numerical investigation on the anisotropy ratio of *ex vivo* tissue

was also performed [16]. Alteration of anisotropy ratio of the conductivity tensor was detected when reversible electroporation threshold was exceeded. Experimental results agreed with numerical and were also consistent with experimental investigations performed by other research groups [24].

In silico evaluation

A concern whether proposed method for determination of electric field distribution can be implemented in electroporation applications was addressed by a simulation in the case of a 3-D numerical model designed for the purpose of ECT treatment of deep-seated liver tumors [15]. As shown on Figure 4, it is possible to obtain sufficiently accurate information on electric field distribution in the targeted and surrounding tissue by measuring only one component of magnetic flux density.

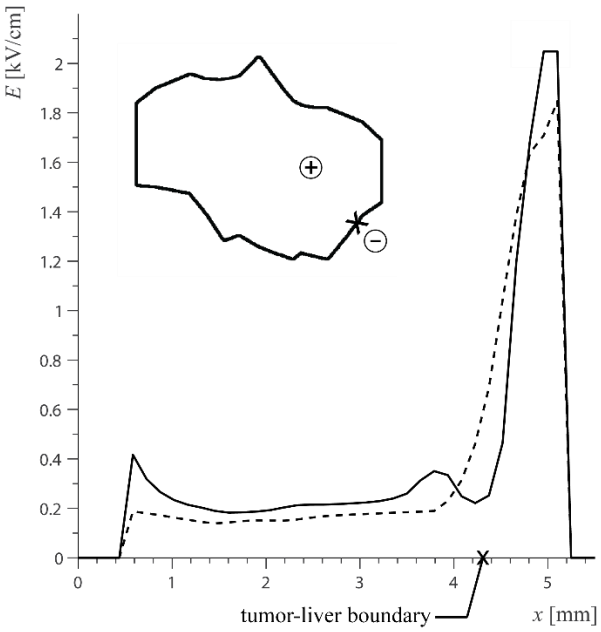

***Figure 4*:** An electric field distribution across the tumor-liver region obtained by means of the MREIT algorithm using only one magnetic field component (solid line) and the corresponding true electric field calculated by the numerical model (dashed line). Electric pulses were applied between two electrodes (see an insert of a figure); one was inserted in the tumor (marked with~+) while the other in the liver (marked with -). Tumor-liver boundary is marked with x.

In vivo determination of electric field distribution

Investigation of the feasibility of MREIT for *in situ* monitoring of the electric field distribution during *in vivo* reversible electroporation was performed in mouse tumors [17]. Mice were also injected with contrast agent Gd-DOTA for assessment of reversibly electroporated areas in the tumor by means of MR imaging [25].

Electric field distribution was successfully obtained in all treated tumors (Figure 5). Correlation was evaluated between coverage of tumors with electric field that leads to reversible electroporation of tumor cells obtained with MREIT and tumor fractions with entrapped Gd-DOTA with linear Pearson correlation analysis. Coverage of tumors with electric field in the range of 400–900 V/cm, where reversible electroporation is expected, had good correlation with Gd-DOTA cell entrapment ($r = 0.956$, $P = .005$).

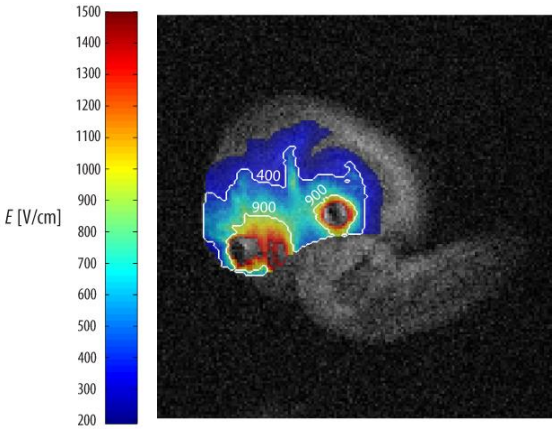

***Figure 5*:** The electric field distribution in the tumor, obtained with MREIT superimposed onto the T1-wighted image acquired before the application of electric pulses. A white contour line encloses an area exposed to an electric field strength between reversible (400 V/cm) and irreversible (900 V/cm) electroporation threshold values. Tumor cells located outside the area are either irreversibly electroporated (the area close to the electrodes) or remain unelectroporated (the area toward the tumor boundary).

DISCUSSION

Feasibility to determine electric field distribution using MREIT was first evaluated by performing experiment on agar phantoms [13]. Different number of pulses and of their amplitudes was used in separate experiments to evaluate feasibility of the method to determine electric field distribution during the electroporation. Experimental results were evaluated by comparison to the simulation result obtained by the numerical model. As expected, the experiments showed that the electric field intensity increases with applied electric pulse amplitude and that electric field intensity and distribution are both independent of the number of pulses. The main limitation of the agar phantoms study was the phantom substance – agar, which became deteriorated after long exposure to high-voltage pulses resulting in measurement failures. Nevertheless, the results confirmed that MREIT can be used to determine electric field distribution during electroporation and next step toward *ex vivo* experiments was designed.

When examining results of *ex vivo* evaluation and comparing them to previous findings on agar phantoms, considerable differences were observed. While dielectric properties of agar remained unchanged in spite of applied high electric field,

presumably due absence of a cell structure in the agar gel, a considerable increase of conductivity (by a factor ~1.5) was measured in liver tissue exposed to pulses of different amplitudes.

At this point, there is a lack of knowledge on the anisotropy properties of electrical conductivity in biological tissues and there are only few reported experimental studies of permittivity and electrical tensors of biological tissues [24], especially in the case when they are exposed to electroporation pulses [26]. As described in [16], successful experimental and numerical investigation on the anisotropy ratio of biological tissue was performed using MREIT, sugesting that described approach could be used in future studies on anisotropy properties of electrical conductivity in biological tissues.

A major concern of the MREIT, namely, whether it is possible to determine an accurate electric field distribution from only one component of the magnetic field, was addressed *in silico* by using a 3-D numerical model built for the purpose of ECT treatment of deep-seated tumors in liver. Electric field in the tumor region and in the tumor-liver region was successfully obtained. Since tumor-liver region consisted of two different tissues with different conductivities we would need to apply more than one sequence of electric pulses in at least two directions in order to obtain unique solution [27]. However, this cannot be done in electroporation applications where high electric pulses alter electrical conductivity after each pulse sequence. Nevertheless, *in silico* results showed that a single pulse sequence enables sufficiently accurate determination of electric field distribution [15].

Finally, electric field distribution was successfully determined *in situ* during *in vivo* electroporation of the tumor [17]. As accurate coverage of treated tissue with a sufficiently large electric field represents one of the most important conditions for successful electroporation, electric field distribution determined by means of MREIT could be used as a predictive factor for electrochemotherapy and irreversible electroporation tissue ablation outcome. This method would be helpful in particular for electrochemotherapy, a procedure that is currently lacking real-time monitoring.

The main difficulty of using MREIT to determine electric field distribution during electroporation in a clinical environment is associated with the limited capability of MRI scanners for their use in interventional procedures. Although, a recent report on MREIT with an open MRI scanner [28] makes implementation of MREIT feasible in the near future. Conductivity changes that occur during the pulse are at the moment also too demanding to asses with MREIT as a function of time. Even though, it is important to be aware that with the CDI technique the accumulative effect of electric current on the MRI signal phase is measured. Therefore, this technique yields a current density distribution, which is a time average of its altering time course so that all the consequences of conductivity alteration, which affect electric current, are not neglected within this distribution.

Future studies should focus on implementation of MREIT in electroporation based clinical applications, especially electrochemotherapy and irreversible electroporation tissue ablation, where it may be applied for corrective interventions before the end of the procedure and, thus, additionally improve the treatment outcome.

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

NOTES

NOTES

SHORT PRESENTATIONS

Comparison of sinusoidal versus square wave pulses for electroporation

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INTRODUCTION

Electroporation offers a number of applications in biology, oncology, immunology, and biotechnology [1]. However, when cell suspension is exposed to high–voltage electric pulses, various electrochemical reactions occur at each metal–electrolyte interface [2]. These may include evolution of gas, dissolution of the electrodes, pH changes, formation of reactive oxygen species (ROS), etc. ROS, such as superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radical are well known to be cytotoxic and have been implicated of a wide array of human diseases, including cancer [3]. Studying of the electrochemical processes occurring during high–voltage pulses, and their consequences is necessary for optimizing practical applications.

MATERIALS AND METHODS

Chemicals and Solutions: hightly buffered solution HB1, horseradish peroxidase (HRP) (Frederick, USA), Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich Chemie, Steinheim, Germany), PBS (Phosphate buffered saline) (Sigma-Aldrich Chemie).

Detection of H_2O_2: AmplexRed® (10-Acetyl-3,7-dihydroxyphenoxazine) (Molecular Probes, Eugene, Oregon, USA) reagent has a great specificity, stability, and selectivity. In the presence of horseradish peroxidase, AmplexRed is nonfluorescent until it reacts with hydrogen peroxide to produce highly fluorescent resorufin. 50 μM of the AmplexRed dye were added to a cell-free medium and treated with high-voltage pulses. Fluorescence intensity in a solution was measured using TECAN GeniosPro

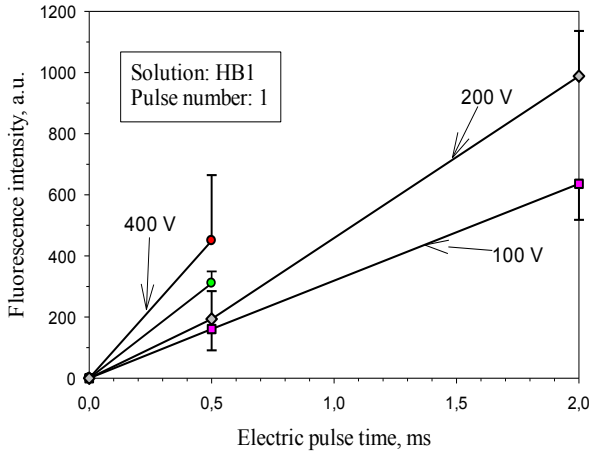

Figure 1: AmplexRed fluorescence intensity as a function of the amplitude of a single square-wave electric pulse.

RESULTS AND DISCUSSION

Treatment of HB1 medium supplemented with 50 μM AmplexRed® dye with a single square-wave electric pulse with the duration of 0.5–2 ms and the amplitude of 100–400V significantly increased dye emission (Figure 1). The AmplexRed fluorescence intensity was also dependent on the number of pulses, the conductivity and composition of the medium (PBS, H_2O, DMEM, HB1) (Figure 2).

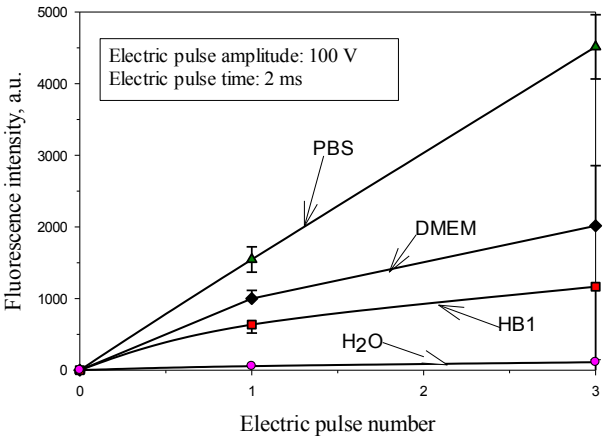

Figure 2: The dependence of AmplexRed fluorescence intensity on the number of square-wave electric pulses for different media.

CONCLUSIONS

It can be concluded, that during high–voltage electric pulses, hydrogen peroxide is generated in cell–free media. Pulses of micro–millisecond duration increased fluorescence of hydrogen peroxide indicator AmplexRed depending on the duration, amplitude, and/or number of square-wave electric pulses as well as the conductivity and composition of the medium.

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Fluorescence quenching by the cell culture medium treated with a high-voltage pulses and metal ions released from the stainless-steel anode

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INTRODUCTION

Cell electroporation is widely used in cell biology, biotechnology, and medicine [1,2]. To study cell membrane permeabilization, visualization of the entrance into or efflux out of the cell of fluorescent dyes is often used [3]. When a high-voltage pulse is applied to the electrolyte solution, besides cell membrane permeabilization, a variety of electrolysis reactions occur at the metal-solution interfaces [4]. Metal ions, released from the electrodes during high-voltage pulse, can react with the fluorescent dyes and quench their fluorescence. This may have an impact when estimating the efficiency of electropermeabilization.

In this study, influence of the medium treated with high-voltage pulse and the metal ions released from the stainless-steel anode on the fluorescence of the fluorescent dyes was studied.

MATERIALS AND METHODS

Cell culture medium consisted of Dulbecco‘s modified Eagle‘s medium supplemented with 9% fetal bovine serum and 1% L-glutamine solution (all Sigma–Aldrich Chemie, Steinheim, Germany). 50 µl of the culture medium or a solution of a fluorescent dye was treated with a square-wave electric pulse with the duration of 0.1–2 ms and the amplitude 0.2–2.4 kV/cm.

The influence of the medium treated with high-voltage pulse and the ions of Ni^{2+}, Fe^{3+}, Fe^{2+}, Mn^{7+}, and Cr^{6+} on the fluorescence of meso-tetrakis (4-sulfonatophenyl) porphyrin (TPPS4), calcein, and doxorubicin (Adriamycin) was studied. The fluorescence was measured at room temperature using Tecan spectrofluorimeter (Tecan Group, Männedorf, Switzerland).

RESULTS AND DISCUSSIONS

Here, influence of the medium treated with high-voltage pulse and the metal ions released from the stainless–steel anode on the fluorescence of the calcein dye, which is used in studying cell electroporation, was studied. Data obtained show that the medium pretreated with the electric pulse with the amplitude of 1.2 kV/cm and the duration of 100, 500, and 2000 µs almost completely quenched calcein fluorescence (Fig. 1). As it was expected calcein fluorescence quenching was caused by the metal ions.

One of the most popular materials utilized for electrodes to electroporate the cells, besides aluminium is stainless–steel. Stainless–steel is an alloy, which typically consists of iron (60–70%), chromium (11–25%), nickel (0.24–20%), and manganese (1–10%) [5]. So, the influence of the metal ions (Fe^{2+}, Fe^{3+}, Cr^{6+}, Ni^{2+}, and Mn^{7+}) on the calcein dye fluorescence in solution was studied. It was observed, that when the concentration of the metal ions in solution was increased, the intensity of calcein fluorescence decreased significantly. The fluorescence of calcein was almost completely quenched in the presence of 1 mM of Fe^{2+} ions in the medium. 1 mM of Fe^{3+} or Ni^{2+} ions suppressed fluorescence of calcein by 15 and 79% respectively.

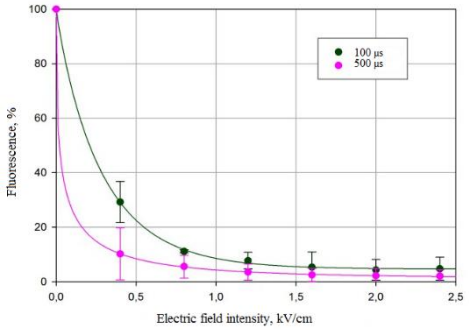

Figure 1: Quenching of calcein fluorescence by the medium treated with a single square-wave electric pulse with the duration of 100 and 500 µs.

CONCLUSIONS

The cell culture medium pretreated with high-voltage pulse quenches the fluorescence of the fluorescent molecules, mainly due to the metal ions released from the stainless-steel anode.

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Statistical analysis of current signal recorded during electroporation of cell suspension

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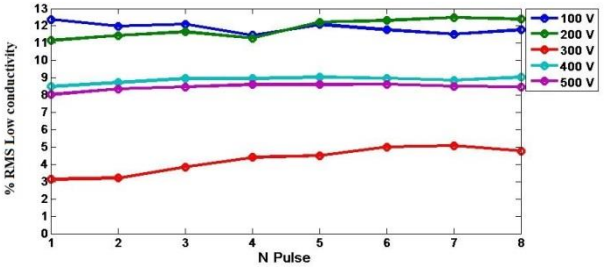
Electroporation of cells is being successfully used in biology, biotechnology and medicine. Practical problems still arise in electroporation of cells in suspension. Conductivity measurement was suggested to monitor membrane permeabilization, however caution is needed when using this approach. The conductivity signal exhibits various interferences due to reactance and resistance dispersion, electrochemical reactions and interfaces [1, 2]. The goal of this work was to analyze conductivity signal recorded during electroporation of cell suspension. The analysis is based on statistical analysis used in signal processing to determine and extract noise (interference phenomena).

In our current study, the analysis of the current signal of the medium free of cells and the cell suspension are performed using MATLAB programs. Both signals recorded from low conductivity and high conductivity buffers were analysed. Using MATLAB functions, statistical features (median, RMS, mode, mean, variation, kurtosis, etc.) are extracted for different spectra. 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 applied, and cuvettes of 2 mm distance between aluminium electrodes 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.

Before feature extraction, the conductivity signals are calculated from measured current and voltage signals, and then values of buffer contribution are eliminated. The analysis is carried out on the whole pulses and then each pulse was divided on small intervals to study the signal dynamics during the pulse.

Figure 1 shows RMS of the conductivity signal determined for low and high conductivity cell suspension.



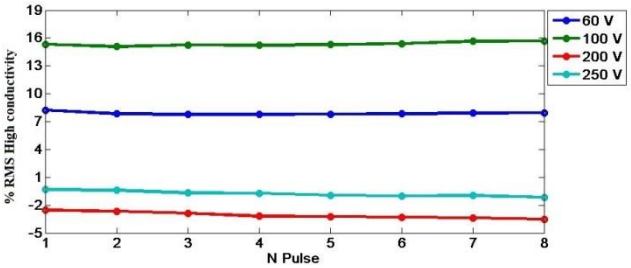


Figure1. RMS of the conductivity signal

The values of the statistical indicators (RMS, Mode, Mean, and Median) are almost the same and do not excess 13%, and decrease till around 4% at 300 V. At 400 V reach another maximum around 9%, which we found already in Impedance measurements (not presented here) of low conductivity medium. From the four indicators of amplitude, we can see clearly that the conductivity of the suspension is increased compared to the solution which is due to the leakage of intracellular liquid. From the first pulse to the last pulse the conductivity increase is low and almost constant after the 3 pulse. Very important to notice that the high values of conductivity difference (really due to cell suspension→electroporation) was at 100 V, this can be only explained by the fact that the solution at higher voltage contribute to the conductivity changes. In other term, at low voltage all membrane changes are detected since the solution conductivity is low and does not change too much, at high voltage both cells and solution contribute to the conductivity increase.

Statistical analysis of the conductivity signal shows clear contribution of other phenomena accruing in solution free of cells to the measured signal and help for better understanding of the electroporation phenomenon.

ACKNOWLEDGEMENTS:
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ELECTROPORATION AS A TOOL FOR STUDYING THE ROLE OF PLASMA MEMBRANE IN THE MECHANISM OF CYTOTOXICITY

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INTRODUCTION

One of the main functions of the cell plasma membrane is the maintenance of a barrier between the cytoplasm and extracellular medium. It is a challenge for molecules to overcome this barrier and reach the cell cytosol and organelles. In the case of anticancerous treatment, one of the main approaches is to induce deregulation in the cell homeostasis, reaching in this way severe cytotoxicity resulting in cell death [1,2].

However, even very potent drugs can be eliminated from future research, due to their lack of ability to penetrate the plasma membrane or penetrate only in insufficient amounts [3]. Unfortunately, it is not always clear what role of the cell plasma membrane is playing in the cytotoxicity caused by a particular substance. This role can be determined by comparing the cytotoxic action of drugs/substances on cells with intact plasma membrane and cells with the membrane permeable to that particular substance.

Permeability of the cell plasma membrane can be increased by applying pulses of strong electric field. In this way, electroporation can be used as a tool for evaluating contribution of the cell plasma membrane in the drug-induced cytotoxicity.

METHODS

Here, the following cytotoxic agents were used: vitamin C (ascorbate), vitamin K_3 (menadione), bisphosphonates pamidronate and ibandronate. Single square-wave 100 μs in duration electric pulse was applied between two plate stainless-steel electrodes (2-mm gap) and monitored with oscilloscope [4]. Mouse hepatoma MH-22A and Chinese hamster ovary cells were grown in monolayer culture in CO_2 incubator in standard conditions *in vitro* and were used as a research object. Cell viability was evaluated by means of a colony-forming assay.

RESULTS

Vitamin K_3 is cytotoxic to MH-22A cells (Figure 1) in investigated concentration range. Combination of vitamin K_3 and electroporation do not change cell viability significantly. Results with vitamin C are similar (data not shown).

Ibandronate's (Figure 2) effect on MH-22A cells viability is low in investigated concentration range for intacted cells. Combination of ibandronate with electroporation does not reduce cell viability significantly. Results with pamidronate are similar (data not shown).

CONCLUSIONS

Data obtained here show that the cell plasma membrane does not play significant role in cytotoxicity caused by vitamins C and K_3. Cell plasma membrane also does not represent a barrier for bisphosphonates pamidronate and ibandronate.

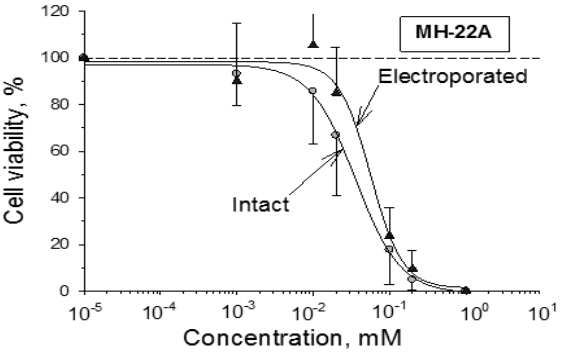

Figure 1: Cytotoxicity of vitamin K_3 alone and in combination with electroporation (single, 2 kV/cm, 100 μs) on MH-22A cell line.

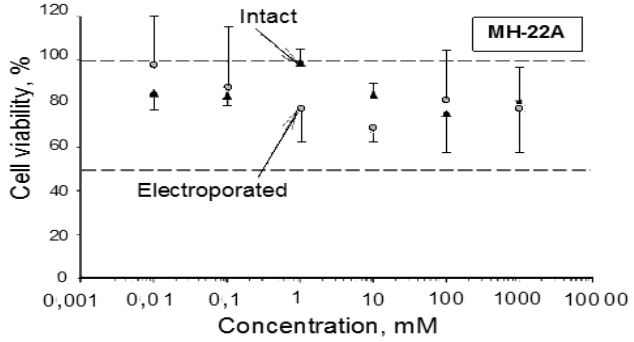

Figure 2: Cytotoxicity of ibandronate alone and in combination with electroporation (single, 2 kV/cm, 100 μs) on MH-22A cell line.

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The effects of a pulsed electric field on interactions between *Saccharomyces cerevisiae* cells

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INTRODUCTION

Saccharomyces cerevisiae is one of the most studied and widely used eukaryotic microorganisms and is ideal experimental model for investigation of abiotic factors. Many biotechnological applications require yeast cells to be transformed in order to give them desirable traits. It is known that exposure to electric field can improve membrane permeability, therefore, increase transformation efficiency of yeast cells. This method is widely used in various laboratories, however, there are still gaps in the knowledge about the mechanism and the effect of different electric field properties on other cell characteristics.

Often in order to affect permeability or viability of yeast cells, high frequency or multiple high voltage electric field (HVEF) pulses are used. However, there is not much information about single electrical pulse effect on yeast cell viability and interaction between cells.

In this study, the yeast cells were exposed to single high power electric field pulse of microsecond duration (τ). The aim was to detect effect of single HVEF pulse on viability and interaction between the yeast cells.

MATERIALS AND METHODS

The yeast *Saccharomyces cerevisiae* strains Y00000 (WT), Y07034 (Δ*MNN4*) and Y02775 (Δ*MNN6)*, phenotypically diverse by the composition of the cell wall, suspended in the electroporation buffer containing 1 M sorbitol, 20 mM Tris-HCl buffer, pH 7,4 were used.

High-voltage electric pulse generator, generating square shaped pulses was used for the treatment of the yeast cell suspension. The device was designed in our laboratory [1].

After exposure to HVEF (τ= 5 μs – 300 μs), cells were plated on solid YPD medium. Size of colonies was measured with Totallab Quant Colony Counting software (Totallab, DB).

Viability of cells was estimated with Microbial Viability Assay Kit-WST (Dojindo, Japan). Cells were diluted 9 times with physiological peptone solution prior to viability estimation.

RESULTS

Investigation of yeast cell viability showed that single HVEF pulse with duration of microseconds affects cell proliferation. Average size (Z) and number (N) of colonies grown on solid media were also affected.

Data of colony sizes and numbers is presented as percentages, where 100% is a respective parameter of a colony grown from untreated cell suspension. Exposure of yeast suspension to HVEF pulse revealed the following effects on colonies formed. Largest colonies grew from cell suspension treated with 3.22-4.88 kV/cm electric field: Z_{wt}= 197 ± 41 %, Z_{MNN4}= 164 ± 49 %, Z_{MNN6}= 131 ± 6 % (Figure 1). Smallest colonies grew from suspension treated when E= 6.84-8.41 kV/cm, τ= 300 μs: Z_{wt}= 67 ± 24 %, Z_{MNN4}= 100 ± 42 %, Z_{MNN6}= 72 ± 6 %. When E= 0.42-4.88 kV/cm, both increase and decrease of yeast colony size were observed. Effects were different between strains and were dependent on the width of the pulse. Lowest values of average colony number were observed when high in amplitude and long pulses were used (E >4.88 kV/cm, τ= 300 μs): N_{wt}= 46 ± 25 %, N_{MNN4}= 30 ± 15 %, N_{MNN6}= 64 ± 16 %.

CONCLUSIONS

It was observed that when electric field is from 0.42 kV/cm, to 3.22 kV/cm, it induces dispersion of natural yeast aggregates. In the electric field range from 3.22 kV/cm to 4.88 kV/cm formation of aggregates with slight decrease in viability were detected. It was found that the electric field with strength exceeding 4.88 kV/cm significantly decreases the size, number of colonies and viability of cells.

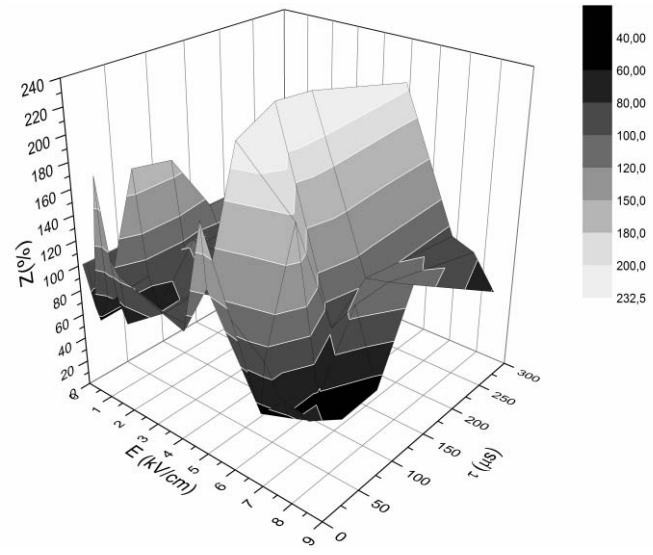

Figure 1: Size of WT strain colonies, formed after exposure to single electric field pulse. Z – average size of colonies where 100% is size of colonies formed from untreated cell suspension.

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New micro-devices, using electromagnetic sensor, dedicated for analysis of cells subjected to ElectroChemoTherapy ECT

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INTRODUCTION

Electrochemotherapy (ECT) is a modality of treatment that combines the administration of chemotherapeutic agents having a low cell membrane penetration rate, with the application of electric pulses to facilitate the delivery of drugs into the cancer cells [1]. Despite progress in this field, enlarging our knowledge about the mechanism of cell electroporation (EP) and kinetics associated with ECT remains a pressing need.

On the other hand, we find ourselves in the era of development of miniaturized systems providing analysis until now not accessible with conventional techniques. Micro and Nano technologies together with innovative biosensing technique, using electro-magnetic waves combined with microfluidics functionalities [2] could enlarge and enrich our vision of biology.

MATERIALS AND METHODS

Macro-metric scale: Electric pulses are delivered to the cells using a Betatech pulse generator with two stainless steel electrodes with distance of 4 mm. Electrical parameters that are used: 8 pulses of 320 V at pulse duration of 100 µs with a frequency of 1 Hz. Cell viability was estimated by counting, 24 hours after the treatment.

Micrometric scale: In the cleanroom of LAAS-CNRS, two types of micro-devices are developed in order to further analyze cells subjected to ECT. The first device, with an interdigitated capacitor IDC of 150×150 µm^2 (Fig.1, a), is dedicated to the study of a group of adherent cells. The second device (Fig.1, b), is dedicated to single cell analysis. They both present (1) a microfluidic channel that conveys cells to the sensing zone, (2) micro-electrodes for the application of EP electric field and (3) a bio-sensor providing real-time monitoring of the effects of ECT on the cell studied. This last ability is based on the interaction between electromagnetic fields with the biological sample which reflects the dielectric properties of cells and makes it possible to assess its state in real time [3].

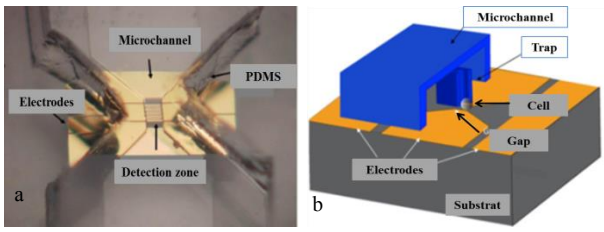

Figure 1: (a) photo of the micro-device with IDC, (b) schematic view of the single cell micro-device.

CONCEPT

In this context, a collaboration between a team of IPBS working on electropermeabilization and its various applications, and a team from LAAS working on the development of electronic microdevices for biological analysis was initiated to develop a method studying cells subjected to ECT by a micro-sensor using electromagnetic waves.

RESULTS

Cells were treated with 100 µM of Cisplatin, exposed to electric pulses or not. We assessed the viability of the cells 24h post-treatment (Fig.2): the presence of the electric field combined with the Cisplatin, enhances its activity, thus reducing cell viability to almost 40%.

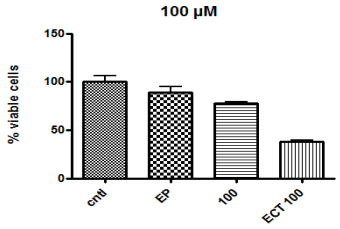

Figure 2: Cell viability post-treatment

Two preliminary results, obtained with our microscale device presented in fig. 1(a), were achieved. The first one is a successful on-chip electroporation, performed by planar micro-electrodes (Fig.3, a). The second one is an electromagnetic real-time sensing of cells submitted to ECT (Fig.3, b): once electric pulses are applied, electromagnetic cells' response occurs. The remaining question we are addressing now: what does this kinetics response reflect on the cell basis?

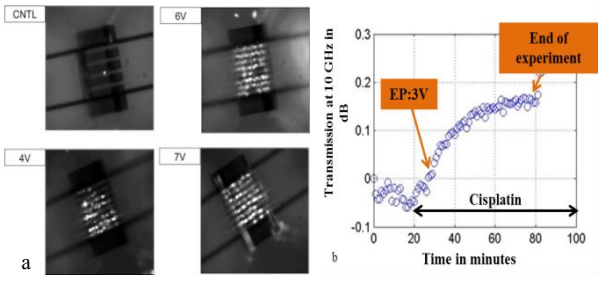

Figure 3: (a) Electropermeabilized cells on micro-device, (b) Cells bio-sensing post-ECT

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Microalgal cell's electroporation for lipid extraction: a real time study in a microfluidic device

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INTRODUCTION

Although biofuel production from microalgae is considered as a serious alternative to classic petrochemistry, costs remain a big issue of competitiveness. New technologies must be developed in order to overcome this difficulty. One of them is the use of Pulse Electric Field (PEF) applied for lipid extraction. Optimized process parameters (pulses intensity, duration, rise time, bipolar pulses etc...), could lead to reversible membrane permeability. Fatty acids, located in the cytoplasm as lipid droplets, could be more easily extracted from the cell by a "green" solvent. In order to optimize and model this complex process, a microfluidic device of electroporation was designed to monitor the effects of PEF on cells and lipids extraction.

Experimentations

Several algal strains which morphology and lipid content differs are cultivated and conditioned to accumulate neutral lipids as oil droplets. Cells are subjected to an electric field in a microfluidic chip. The first aim is to characterize the behaviour of the strains under these various fields. Some strains possess a very thick cellulosic cell wall, which others are much more vulnerable when facing a field gradient. In this study, we investigate (1) the behaviour of cells under pulse electric fields (Goettel et al. 2013; Sheng et al. 2011), (2) Dielelectrophoresis response of cells or/and their eventual permeation when submitted to such stress (Bahi et al. 2011), and possibly (3) response to electrorotating field which is a relevant mean to monitor dielectric properties (Mu 1998).

A study of this electrical process on a chip enables visualisation and recording, at single cell scale, of the morphological changes. Microscopic observation can be in widefield, phase contrasts, epifluorescence, of confocal laser. Images recording can be performed *via* colour, high speed or sensitive camera, or with a photomultiplier tubes (PMT) in the case of confocal microscopy. Different molecular probes could highlight the process of algal electroporation: cellulosic wall staining, permeability staining, enzymatic activity probe, or neutral lipid staining.

In addition to the visualisation benefits provided by chip technology, it is easy to model electric fields distribution and side effects like heat and streams.

A deep insight of the electroporation process of complex cells such as microalgae is necessary to target and optimize key parameters and to pursue towards the extraction of metabolites.

Results

First results have shown strong sensitivity differences of strains with respect to required amplitude of electric field for membrane permeabilisation. Tests on the selected strains are in progress.

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Effect of Pulsed Electric Field applied in the millisecond and microsecond range on the permeabilization and carotenoid extraction from *Chlorella vulgaris*

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INTRODUCTION

Several studies have demonstrated that electroporation of cells for improving extraction can be achieved with trains of electrical pulses with duration in the range of milliseconds (ms) or in the range of microseconds (µs) [1-2]. However, it is unclear which type of pulses is more effective for carotenoid extraction. The aim of this study was to compare the efficacy of ms and µs, pulses on the extraction of carotenoids from the microalgae *Chlorella vulgaris*.

MATERIAL AND METHODS

C. vulgaris was cultivated in BG-11 medium at 30°C in cycles of light: dark (12:12). Once the culture achieved the stationary phase (10 days), a suspension at concentration of $2x10^8$ cells/ml was PEF treated in batch. In the ms range, 10, 20, 40 and 60 pulses of 1 ms were applied at electric field strengths of 3.5, 4, 4.5 and 5 kV/cm and frequency of 0.25 Hz. In the µs range 5, 10, 25 and 50 pulses of 3 µs were applied at electric field strengths of 10, 15, 20 and 25 kV/cm and frequency of Hz.

Staining with propidium iodide (PI) before and after PEF-treatment was used to monitor permeabilization of algal cells upon their exposure to PEF. Extraction from control and PEF-treated cells was performed in ethanol (96%) for 20 minutes just after the treatment and after 1 hour minutes of incubation in the treatment medium.

RESULTS

Independently of the type of pulses applied, carotenoid extraction yield (CEY) increased with the electric field strength and treatment time (Fig. 1). The maximum increment in the CEY obtained in the PEF treated cells as compare with the control cells was 70%. This increment was similar for both ms and µs treatments. The lower PEF treatment conditions (4.5 kV/cm-40 ms) required to obtain the maximum irreversible permeabilization of the *C. vulgaris* cells corresponded with the highest CEY when ms pulses were used. In the case of µs pulses, to obtain the highest CEY more intense PEF-treatment conditions were required (20 kV/cm-75 µs) that those necessary to obtain the maximum irreversible electroporation (15 kV/cm-75 µs).

CEY extracted from PEF treated *C.vulgaris* cells after 1 h of the application of the PEF-treatment significantly increased when electroporation was performed with µs pulses, however, these improvement was not observed when ms pulses were used. The minimum total specific energy required to obtain the highest CEY was higher using pulses in the ms (121.5 kJ/ml) that in the µs range (40.2 kJ/ml).

The lower energetic requirements and the improvement in the CEY observed when extraction was performed 1 h after the application of the PEF treatment indicates that pulses in the µs range are more effective for improving extraction of carotenoids from the microalgae *Chlorella vulgaris*.

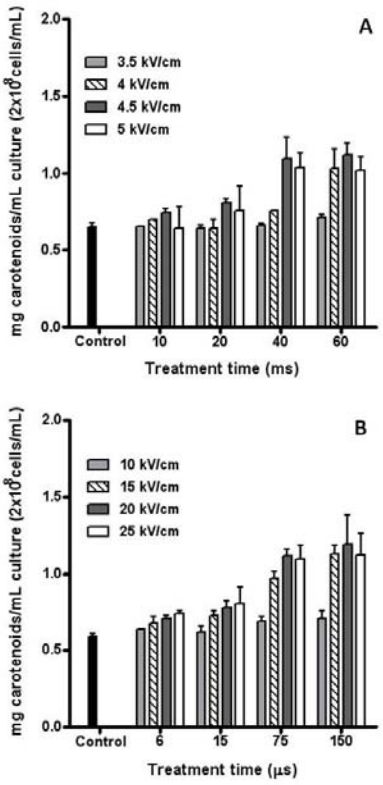

Figure 1: Influence of treatment time on CEY at several electric field intensities when PEF-treatments were applied in mµ (A) and µs (B) range.

Acknowdeledgments: E.L gratefully acknowledge the financial support for the STSM mission provided by COST TD1104

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The dual-porosity approach to mass transport and filtration-consolidation behaviour modelling of electroporated biological tissue

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INTRODUCTION

We recently proposed a new approach in modelling mass transport by diffusion [1] and juice expression [2] from electroporated plant tissue – *the dual-porosity model*.

Model is based on two assumptions. Primarily, tissue comprises two domains: the *intracellular* and *extracellular*. A permeability or porosity is attributed to each domain, so that extracellular porosity corresponds to the porosity of the cell wall matrix, while membrane permeability determines porosity of the intracellular domain. Secondly, we write the mass conservation laws for the two domains, with concentration and pressure gradients as energy sources that promote mass transport and liquid expression, respectively.

We present the mathematically equivalent models, quantities, and parameters for the two physically different transport phenomena, and show how diffusion or expression kinetics from laboratory-scale experiments are well described by the proposed model.

THEORY

The two sets of partial differential equations (1–2 and 3–4) follow from mass conservation laws. Boundary and initial conditions (omitted) depend on the particular system.

For diffusion, we have

$$\frac{\partial c_e(z,t)}{\partial t} - D_e \frac{\partial^2 c_e(z,t)}{\partial z^2} - \frac{1-\varepsilon}{\varepsilon}\delta\cdot\left[c_i(z,t)-c_e(z,t)\right]=0 \quad (1)$$

$$\frac{\partial c_i(z,t)}{\partial t} + \delta\cdot\left[c_i(z,t)-c_e(z,t)\right]=0 \quad (2)$$

where c is the solute concentration (directly measurable quantity), with indices e and i corresponding to extracellular and intracellular domain, respectively; D_e is the diffusion rate of solute in the extracellular domain; ε tissue porosity (1 – cell volume fraction); and δ is the key electroporation-dependent parameter determining transmembrane diffusion.

Table 1: Mathematically equivalent parameters

diffusion		pressing	
concentration	c [mol.m^{-3}]	liquid presure	p [Pa]
diffusion coef.	D_e [m^2.s^{-1}]	fluid mobility	k_e/μ [m^2.Pa^{-1}.s^{-1}]
diffusion rate*	δ [s^{-1}]	flow rate* per unit pressure	α/μ [Pa^{-1}.s^{-1}]
tissue porosity	ε [-]	-	-
-	-	compressibility	G_e, G_i [Pa]

*transmembrane

In liquid expression, fundamental model equations are

$$\frac{1}{G_e}\frac{\partial p_e(z,t)}{\partial t} - \frac{k_e}{\mu}\frac{\partial^2 p_e(z,t)}{\partial z^2} - \frac{\alpha}{\mu}\left[p_i(z,t)-p_e(z,t)\right]=0 \quad (3)$$

$$\frac{1}{G_i}\frac{\partial p_i(z,t)}{\partial t} + \frac{\alpha}{\mu}\left[p_i(z,t)-p_e(z,t)\right]=0 \quad (4)$$

where p is the liquid pressure (indirectly measurable); k_e the extracellular hydraulic permeability; μ liquid viscosity; G_e and G_i compressibility moduli; and α the key parameter describing membrane hydraulic permeability.

RESULTS

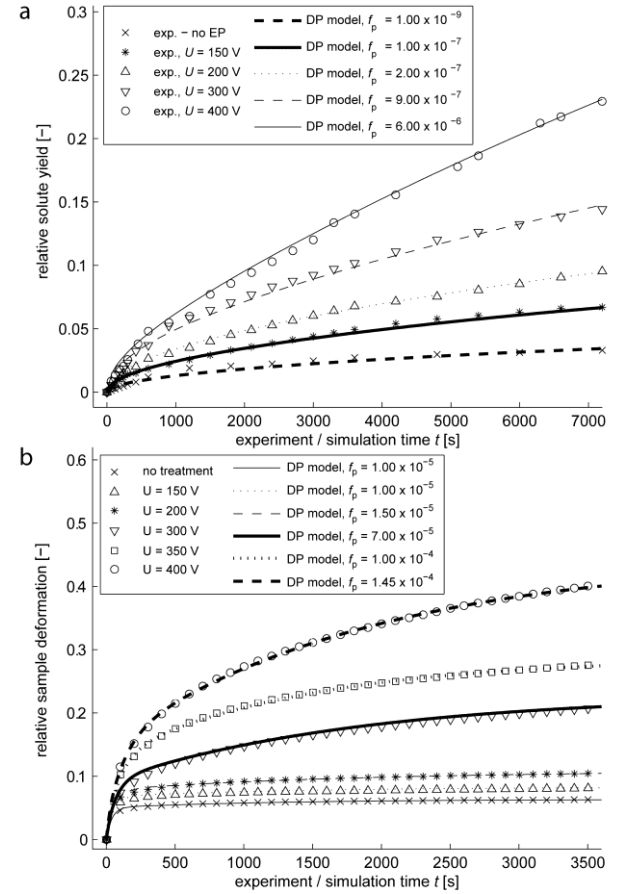

Figure 1: Sugar beet experimental results and model fitting for (a) diffusion and (b) pressing.

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Evaluation of cell viability after cell electrochemotherapy using different cell viability tests

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INTRODUCTION

Electroporation is a well-established method that employs the use high voltage (HV) electric pulses to facilitate intracellular delivery of various molecules and ions [1,2]. This approach was applied for antitumor electrochemotherapy, that combines administration of anticancer drug bleomycin and tumor treatment with HV pulses [3]. Nevertheless the dynamics of cell viability after cell treatment with bleomycin and HV pulses is not well described and therefore can be confusing. In this study we aimed to compare two cell viability tests using crystal violet and MTT with cell colony formation test.

METHODS

All experiments were performed on CHO cells *in vitro*. After cell treatment with one HV electric pulse of 1200 V/cm pulse strength and 100 µs pulse duration in the presence of bleomycin, the cell viability was evaluated using two cell viability tests, namely MTT and crystal violet. MTT dye after getting into cells by diffusion is converted into formazan by glutathione S-transferase. Cell viability was evaluated by measuring OD at 492 nm and 612 nm at certain time points (from 6 to 72 h) after experiment. Crystal violet method is based on diffusion of the dye into the cell. After calibration, the number of viable cells was evaluated by measuring OD at 612 nm, and at the same time points as using for MTT test.

RESULTS AND CONCLUSIONS

It is well known that bleomycin transfer into cells using electric pulses results in cell death, which depends both on the number of permeabilized cells and bleomycin concentrations. Different research groups evaluate this cell death using either cell colony formation test or propidium iodide. Some others use MTT test and also crystal violet test.

First of all we evaluated cell viability after cell treatment with HV pulse (1200 V/cm, 100 µs) in the presence of bleomycin (20 nM) using cell colony formation test. This resulted in 17±5 % viable cells. In the following experiments these results were compared with cell viability using MTT and crystal violet tests. Cell viability using these tests was evaluated at different time points after the experiment (Figure 1).

As it is seen from the Figure 1, 6 hours after the experiment, the cell viability was 100 % both using MTT and crystal violet test. Nevertheless, 24 hours after the experiment the results were strikingly different: while MTT test showed cell viability close to 100 %, it dropped to 25 % using crystal violet test. At 48 and 72 hours after the experiment cell viability was 79 and 59 % using MTT test and 19 and 9 % using crystal violet test.

These results clearly show that within 24-48 hours, the results of crystal violet test are comparable with cell colony formation test. On the other hand, the results show that MTT test is not suitable to evaluate cell viability after cell electrochemotherapy. Indeed, since efficiency of MTT conversion to formazan depends on cell metabolic activity, the measured optical density value reflects the cell metabolic activity rather than total number of viable cells.

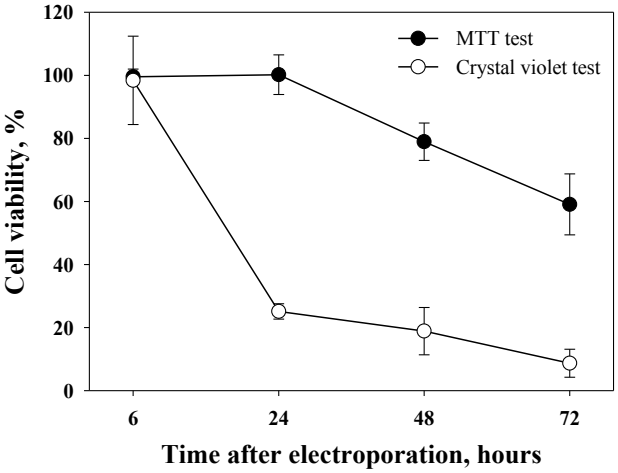

Figure 1: Dynamics of cell viability after cell treatment with a single HV pulse (1200 V/cm, 100 µs) in the presence of bleomycin (20 nM) assessed by MTT and crystal violet tests. Each experimental point in the figure represents the mean± SEM (n=3).

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Mathematical modelling of cell membrane permeabilization and cell survival

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INTRODUCTION

When treating tumours with electrochemotherapy or non-thermal irreversible electroporaton as a method of tissue ablation we first prepare treatment plan where we predict the extent of destroyed tissue. Currently we use a specific value of electric field (*E*) for prediction. We can make predictions more realistically using mathematical models of permeabilization and survival. Using these models the percentage of destroyed tissue takes all the values between 0% and 100% and not just 0% or 100% [1].

METHODS AND MATERIALS

Using non-linear least squares method we fit mathematical models of permeabilization (symmetrical and asymmetrical sigmoid, hyperbolic tangent, Gompertz curve) and survival (first-order kinetics, Hülsheger, Weibull, Gompertz, Geeraerd, Peleg-Fermi, biphasic, and logistic model) [2]. We evaluated goodness-of-fit using a coefficient of determination (R^2). We exposed cells grown in a monolayer to a homogeneous *E*-field and used experimental values to acquire optimized models of permeabilization. We then used these optimized models of permeabilization in numerically calculated inhomogeneous *E*-field around the needle electrodes. Next we sought for a relation between electric pulse parameters and parameters of mathematical models. We did experiments on cell suspension. For each fit we calculated optimized values of parameters and R^2. We used propidium iodide to detect permeabilization and MTS assay to detect survival.

RESULTS AND DISCUSSION

We identified Gompertz curve as the most suitable for modelling cell permeabilization as it had the highest R^2 [3] and there was a relation between parameters of electric pulses and values of model's parameters. We found density of the cell monolayer to be the most important factor for a correct prediction of permeabilized cells (Fig. 1). We found Peleg-Fermi and logistic mathematical model [4] to be the most suitable for modelling cell survival in dependence on *E*-field (Fig. 2).

CONCLUSION

Logistic model seems the most promising for modelling cell survival while Gompertz curve seems the most suitable for cell permeabilization. We need however to perform more experiments in order to establish the connection between the values of parameters of mathematical models and electric pulse parameters. Mathematical models of survival should be validated in a similar way as with mathematical models of permeabilization. So far it seems possible to use mathematical models in treatment planning but before their use *in vivo* validation is needed.

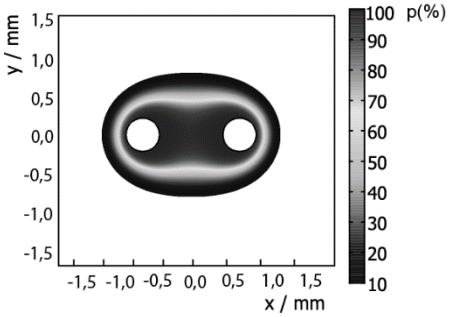

Figure 1: Predicted percentage of permeabilized cells in inhomogeneous *E*-field.

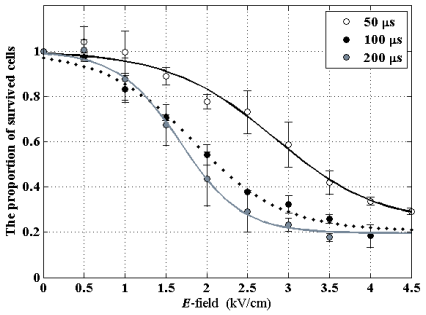

Figure 2: Logistic model fitted to results of cell survival, applied 8 pulses of 50 µs, 100 µs or 200 µs at 1 Hz.

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ACKNOWLEDGEMENTS

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Dynamic effects of point source electroporation on the rat brain tissue

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INTRODUCTION

Despite aggressive therapy, existing treatments offer poor prognosis for glioblastoma multiforme (GBM) due to tumor infiltration into the surrounding brain as well as poor blood-brain barrier (BBB) penetration of most therapeutic agents. Due to the extreme adaptability of GBM cells, the infiltrating zone surrounding the tumor must also be treated efficiently.

In order to meet these challenges a combined approach, consisting of inducing significant/rapid necrosis in the tumor mass in parallel to delivery of high chemotherapy doses to the residual tumor and infiltrating zone is suggested. Since GBMs are highly vascularized tumors, the tumors' own vasculature may be used for delivering the drug to the tumor, while disrupting the BBB in its local vicinity.

For this purpose we have developed a strategy to apply both irreversible EP (IRE) and reversible EP (RE) in the same treatment by a single insulated intracranial needle electrode with a 1mm exposed tip placed in the brain tumor tissue and an external surface electrode pressed against the skin. The electric field produced by this electrode configuration is highest at the exposed electrode tip tissue interface and then decays in an exponential fashion. Therefore, the electric fields surrounding the electrode tip induce irreversible effects (thus necrosis in the tumor mass) that gradually taper down to reversible EP effects, inducing BBB disruption in the residual tumor and surrounding infiltration zone.

METHODS

2 sets of experiments were conducted using 98 naïve rats: (1) 7 groups of naïve rats (n=3-6 rats each) were treated with 10-540 pulses at 600V, 50µsec pulses and 1sec interval. (2) 5 groups of naïve rats (n=3-6 rats) were treated with 90 pulses at 250-1000V. Gd-based contrast agent was injected systemically prior to treatment. Rats were scanned by MRI immediately post treatment and periodically thereafter. BBB disruption volumes (BDVs) were calculated from T1-MRIs acquired 30 min post treatment and irreversible tissue damage volumes (IRVs) from T2-MRIs acquired after 2 weeks.

RESULTS

Clear enhancement on T1-MRIs acquired 30 min post treatment was observed. This effect is attributed to BBB disruption. Early treatment effects (ETVs) on the brain tissue (including reversible/irreversible effects) were depicted on the T2-MRIs acquired immediately post treatment. Late irreversible damage was clearly depicted on T2-MRIs acquired on day 14.

The average volumes of the permanent tissue damage were significantly smaller ($p<0.0001$) than those calculated from the enhancing volumes on T2-MRIs acquired on the treatment day which was significantly smaller than the average volumes of BBB disruption ($p<0.0001$). Significant correlation ($r^2=0.94$, $p<0.0002$, $r^2=0.99$, $p<0.001$, for experiments 1 & 2 respectively) was found between BBB disruption volumes and permanent damage volumes.

The dependence of BDVs, ETVs and IRVs on the number of pulses can be described by: $Vol=Vol(ss)\cdot(1-\exp^{(-n\_p/k\_p)})$, where Vol(ss) is the stationary volume, n_p is the num of pulses and k_p the pulse num coefficient. The calculated stationary volumes were found to be $Vol(ss)=132.8\pm6.7mm^3$, $80.7\pm10.5mm^3$ and $65.5\pm16.5mm^3$ for BDVs, ETVs and IRVs respectively; The pulse num coefficient was found to be $k\_p=109\pm12$, 83.6 ± 22.3 and 311.2 ± 6.7 for BDVs, ETVs and IRVs respectively; Goodness of fit: $r^2=0.93$, $p<0.0003$, RMSE=1.36; $r^2=0.96$, $p<0.0001$, RMSE=0.94; $r^2=0.93$, $p<0.0003$, RMSE=0.9 for BDVs, ETVs and IRVs respectively.

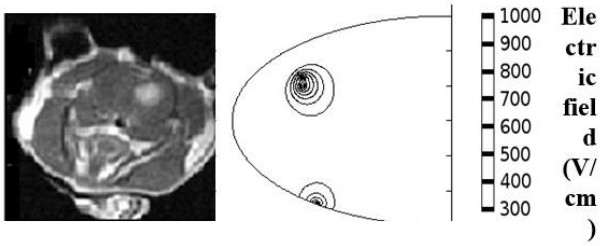

Figure 1: A) T1-MRI obtained 30 min post EP. B) Electric field distribution (simulation) using the single electrode setup.

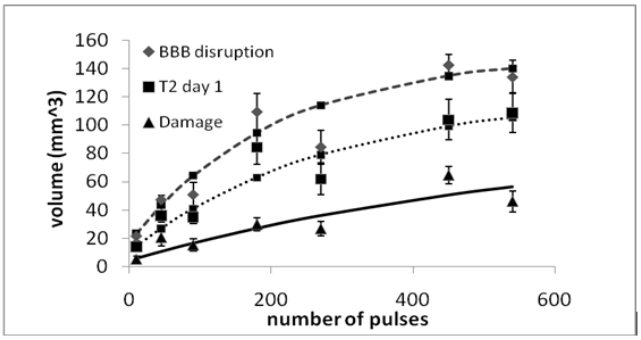

Figure 2: Average BDVs, ETVs and IRVs as a function of the num of pulses. The exponential dependence on the num of pulses suggests an accumulative effect reaching saturation.

These results indicate that EP may be applied for a combined treatment of point source EP located in the tumor in parallel to systemic chemotherapy. Point source EP will induce local tumor destruction by IRE while chemotherapy is efficiently delivered to the surrounding infiltrated tissue due to the larger coverage of temporary BBB disruption by RE.

Nanochannel Electroporation for Delivery of Biomolecules

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INTRODUCTION

In order to perform successful gene delivery to the cell, it is required to obtain complete control of the electroporation process of the cell membrane. Electroporation can induce pore formation by the use of an applied electric field. Nanochannel electroporation (NEP) can deliver precise amounts of a variety of transfection agents into living cells. By precisely controlling pore formation at the membrane, DNA can be delivered safely to the living cells [1]. Moreover, dose control is achieved by adjusting the duration and number of pulses. Therefore, the nanochannel electroporation device is expected to have high-throughput delivery applications.

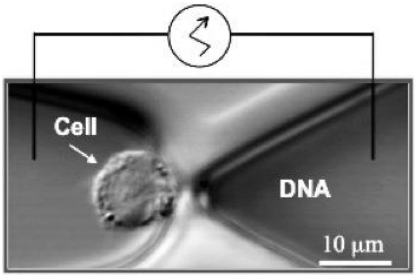

Figure 1: A microscopic image of the nanochannel electroporation device.

METHODOLOGY

The nanochannel electroporation device is fabricated by an established DNA combining and imprinting (DCI) [2]. One side of the devise is used as a reservoir for the transfection material, and the other side for the cell. Both reservoirs contain an electrode. The experimental setup of the nanochannel device is shown in figure 1 [3].

In order to visualize the permeability of the cell membrane, propidium iodide (PI) dye is used to deliver to K562 cells. For all cases, bulk electroporation (BEP), microchannel electroporation (MEP) and NEP.

RESULTS

The PI dye uptake for BEP, MEP and NEP are shown in figure 2. For conventional BEP the PI fluorescence increases gradually in a period of approximately 150 seconds. A similar increasing fluorescence signal is observed when confining the electric field to a microchannel. Interestingly, the uptake profile on NEP is instant and directly in the middle of the cell. Therefore, it seems like a different delivery mechanism takes place then for BEP and MEP.

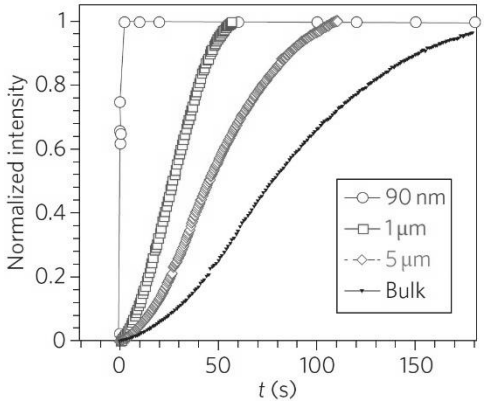

Figure 2: The duration of the PI dye uptake for all different mechanisms: NEP (180V/2mm), MEP-1μm (150V/2mm), MEP-5μm (60V/2mm) and BEP (70V/mm).

Table 1: Results of the finite-element simulations for the PI dye in the different sized channels.

	d = 90 nm	d = 1 μm	d = 5 μm
Length (μm)	3	5	5
Voltage (V)	180	150	60
Max (E) (Vm^{-1})	5.8×10^7	1.6×10^7	1.1×10^6
Exit velocity (m/s)	0.44	0.056	0.00011
Travelling time(μs)	5.52	32	630

Additionally, to show the versatility of the device, several agents are delivered successfully to Jurkat cells, such as oligodeoxynucleotide and quantum dots.

FUTURE PERSPECTIVE

NEP is shown to be an efficient and controlled technique to deliver several transfection agents to the cell. It appears that a confinement of the electric field to the nanoscale changes the delivery mechanism. In order to optimize this technique for the use of gene delivery, we aim to fully understand this mechanism.

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2D/3D models to study the effects of pulsed electric field on extracellular matrix and intercellular junctions

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INTRODUCTION

The electroporation results in transiently permeabilizing cell membranes by applying electrical pulses (1). The mechanisms remain a fragmented knowledge in vivo (2). Indeed, although electrostranfer of genes is highly effective on cells grown in 2D *in vitro*, it is much less efficient *in vivo*, particularly in solid tumors (3). Tissue organization is much more complicated than *in vitro*, since cells develop numerous intercellular junctions and secrete extracellular matrix. These structures may limit movement and distribution of DNA plasmids (4).

We aimed at characterizing modifications of the extracellular matrix and the intercellular junctions induced by application of electric field. To achieve this, we have developed different models *in vitro*, healthy and cancerous, increasing the degree of complexity. By using Matrigel to mimic the basal lamina and collagen gels to mimic the dermal compartment, rich in collagen I and III, we can study direct effects of electrophoretic forces. For cellular models we use fibroblast sheets, spheroids and substitutes skin to study the effect of pulsed electric field in a 3D context. Study with models of increasing complexity will unravel the potential effect of cells pulsed on the extracellular matrix and intercellular junctions on gene electrotransfer. Finally, we validated the results obtained in our 2D/3D models by working on samples of native human skin.

METHODS

The HCT-116 cell line originates from a human colorectal carcinoma was chosen for its ability to form multicellular tumor spheroids. Human Dermal Fibroblast-neonate (HDF-n) cell line was selected for its ability to produce collagen and make cellular sheets.

Spheroids were generated using the "non- adherent" technique. Ultra-low attachment 96-well plates were used by deposing 5000 or 10000 cells into each well. The plate is centrifuged to accelerate cell sedimentation. To induce the production of extracellular matrix 50 µg/ml ascorbic acid is added to the medium.

Fibroblast sheets were prepared by seeding fibroblasts in six wells plates for 4 weeks in DMEM supplemented with ascorbic acid in order to form sheet of extracellular matrix amenable to manipulation.

Second harmonic was observed by biphoton imaging (Multiphoton FLIM Zeiss 7MP).

RESULTS

Fibroblast sheets show that there is no fibrillar collagen observed without ascorbic acid. The sheets treated with ascorbic acid present second harmonic where the collagen fibers are organized. Figure 2 shows that there is very few fibrillar collagens in fibroblast spheroids treated with ascorbic acid. For spheroids which have not received ascorbic acid there is no trace of second harmonic generation, thus no fibrillar collagen

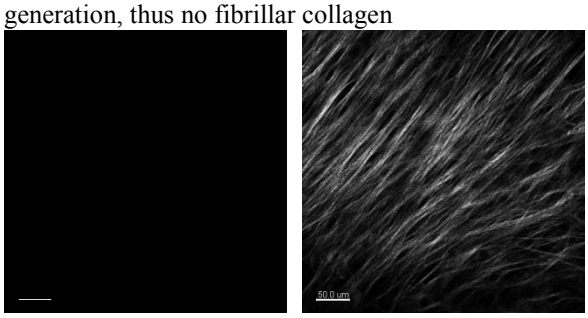

Figure 1: Second harmonic generation (SHG) fibroblast sheet, without (left) and with ascorbic acid (right)

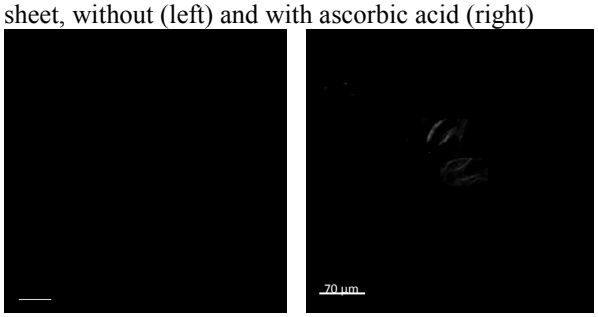

Figure 2: Second harmonic generation fibroblast spheroid, without (left) and with ascorbic acid (right)

CONCLUSIONS

Our preliminary result on generation of models show that adding acid ascorbic in cell culture, fibroblast cells produce organized fibrillar collagen that we can observe with generation of second harmonic in fibroblast sheet. Spheroid with the same cell line does not produce organized collagen. These first results show that 2D and 3D cultures modify the architecture and organization of collagen produce by cells. This confirms our choice to work on different models to unravel the effects of pulsed electric field on extracellular matrix and intercellular junctions. This work is still under development.

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Research was conducted in the scope of the EBAM European Associated Laboratory.

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TARGET CANCER THERAPY BY NOTCH AND WNT INHIBITORS- CANCER CURRENT APPROACH AND FUTURE PERSPECTIVE

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INTRODUCTION

NOTCH signalling pathway is important for communication between cells, which includes gene regulatory mechanisms that control cellular processes during embryonic life. It takes part in neuronal development and function, stabilization of arterial endothelial and angiogenesis, communication between endocardium and myocardium during ventricular development and differentiation. NOTCH signal pathway is involved in growth of some cancer cells, migration, invasion and angiogenesis. Its contribution to carcinogenesis is through inhibition of differentiation, inhibition of apoptosis and stimulation of proliferation (1).

WNT signalling plays a central role in embryonic development, tissue regeneration, protein phosphorylation, osteoblast differentiation and signal transduction. Its involvement in carcinogenesis results in increased growth of tumour cells, cell migration and formation of metastasis.

Inhibitors of NOTCH and WNT fall into these categories: small molecule drugs, peptides and blocking antibodies. Here we present currently used molecules targeting NOTCH and WNT signalling pathways and discuss its potential.

NOTCH SIGNALING PATHWAY

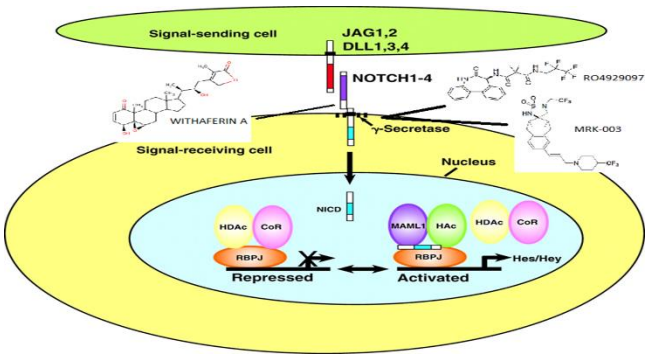

Figure 1. NOTCH signaling pathway. Ligand-receptor complex allows signal transmission between neighboring cells and generate NICD (NOTCH intracellular domain), required for gene expression of key factors. NOTCH inhibitors as target use DLL4, NOTCH receptors, γ-secretase.

Table 1. NOTCH inhibitors

Name of inhibitor	Tipe of molecule	Target
MRK-003	small molecule	γ-secretase
MK-0752	small molecule	γ-secretase
R04929097	small molecule	γ-secretase
DAPT	small molecule	γ-secretase
DEMCIZUMAB	bloking antibody	DLL4
WITAFERIN A	natural component	Notch1

WNT SIGNALING PATHWAY

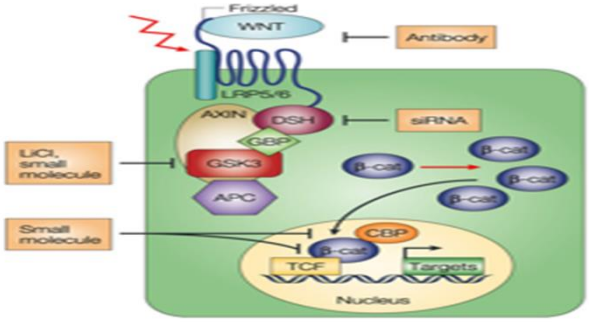

Figure 2 WNT signaling pathway. The absence of WNT signals result in degradation of β-catenin, while the presence of WNT signals are responsible for increased levels of β-catenin in cell.

Table 2. WNT inhibitors

Name of inhibitor	Tipe of molecule	Target
IWR-1	small molecule	PORCN
IWR-2	small molecule	PORCN
C-59	small molecule	PORCN
LGK974	small molecule	PORCN
XAV939	small molecule	Tankyrase
VANTICTUMAB	bloking antibody	Frizzled

Inhibition of the PORCN acyltransferase prevents growth of WNT-driven mammary cancer. Phase 1 study for VANTICTUMAB demonstrates pharmacodynamics modulation of the WNT pathway in patients with advanced solid tumours (2).

FUTURE PERSPECTIVES

Because of the importance and involvement of these two signaling pathways in a number of cancers, further research should focus on developing drugs that will use the NICD from NOTCH signaling pathway and APC (adenomatous polyposis coli protein) segment of WNT signaling pathway as a target.

NOTCH and WNT inhbitors such as DEMCIZUMAB and VANTICTUMAB, are large molecules and their entry into cells may be difficult. Electroporation will allow entry into the cytosol of cells and easy access to their targets.

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Differential transfection of skin or subcutaneous tissues by specific gene electrotransfer protocol

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INTRODUCTION

Easy accessibility for treatment and immunological properties of the skin make it interesting target for gene transfections and vaccination. Gene electrotransfer is one of the most efficient and safest non-viral methods for delivery of genes into target tissue. Since the skin is very complex tissue that consists of different cell types, each cell type can be targeted and transfected by specific gene electrotransfer protocol and other experimental conditions [1]. Therefore the aim of our study was to control the depth and duration of the transfection by gene electrotransfer into the skin or subcutaneous tissue by varying the electric pulse parameters.

METHODS

The study was performed with approval from the Ministry of the Agriculture and the Environment of the Republic of Slovenia (permission 34401-4/2012/4) in accordance with the guidelines for animal experiments of the EU directive. Balb/c female mice were shaved on the left and right flank a day before the experiment. 3-5 mice were assigned per experimental group. The animals were anesthetized in the induction chamber using 2% isoflurane in oxygen and remained under anaesthesia during the procedure. Animals received an injection of pCMV DsRed-Express2 (Clontech) plasmid DNA encoding red fluorescent reporter protein DsRed (2mg/mL) in the single volume of 50 µL subcutaneously or 4 intradermal injections of 20 µL around an area of 6 mm in diameter. Electric pulses were applied immediately after plasmid administration. Two different electric pulse protocols were used: high voltage pulses at 570V with duration of 100 µs and low voltage pulses at 60V with duration of 150 ms. The non-invasive multi-array electrodes, consisting of 7 spring loaded pins arranged on hexagonal mesh and spaced 3.5 mm between each other (Iskra Medical, Slovenia), were used. A total of 24 electric pulses were delivered during the treatment [2]. To determine the transfection efficiency, fluorescence of DsRed protein was observed under fluorescence stereomicroscope Zeiss Stereo Lumar.V12 (Zeiss, Germany). Fluorescence intensity and fluorescence area were analysed (AxioVision, Zeiss) and statistical analysis was performed using Sigma Plot software.

RESULTS

DsRed expression after gene electrotransfer to skin with intradermal plasmid injection was followed by intensity and duration of its expression in the skin. Using high voltage electric pulses the expression peaked at 2 days after the treatment, and then started to decline for next two weeks. The onset of gene expression was similar with low voltage electric pulses, but after two days the fluorescence started to blur and spread, indicateive of transfection of deeper levels in the skin (Figure 1). Fluorescence was detected for up to 5 days longer than under high voltage conditions.

The shape of expression area and the longer duration of the expression indicate that with low voltages pulses the transfection of muscle cells was achieved (panniculus carnosus), while high voltage pulses promote transfection in upper layers of the skin (dermis and epidermis). This is furthermore supported by the observation that after the subcutaneous injection of plasmid DNA followed by 570 V/100 µs pulses no expression was observed, while significant fluorescence signal was detected after administration of 60 V/150 ms pulses. To further validate these results histology analysis will be performed.

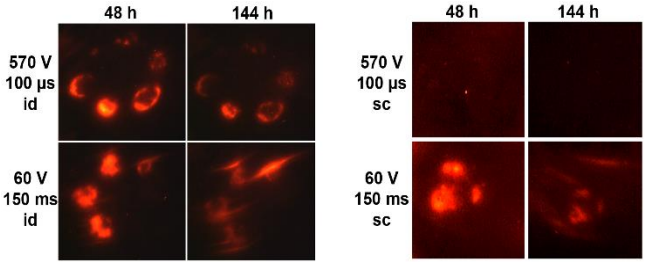

Figure 1: Expression of red fluorescent protein after 4 x 20 µL intradermal (id) injection (left) and after 1 x 50 µL subcutaneous (sc) injection of pCMV DsRed (right). Images were taken at day 2 and 6 post-treatment.

CONCLUSIONS

Our results indicate that different gene electrotransfer protocols affect the transfection of skin or subcutaneous tissue. This has a great potential to adjust the duration of gene therapy and differentiate between the local and systemic effects.

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Evaluation of gene electrotransfer efficiency in canine malignant melanoma cells

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INTRODUCTION

Spontaneous tumors in pet animals represent a very good model for human tumor. Specifically, spontaneous canine melanomas serve as an excellent model for human melanomas as they appear at the same localizations and most of canine melanoma tumors are homologous to human rare morphological melanoma types (1). Therefore, studies using canine tumor cells and spontaneous tumors are becoming more and more valued and represent an important translational bridge to human medicine. Gene electrotransfer studies have not been performed so far in canine cell lines, therefore, the aim of our study was to determine the transfection efficiency of plasmid DNA encoding green fluorescence protein (GFP) in canine melanoma cell lines.

METHODS

Two cell lines CMeC-1, isolated from spontaneous canine melanoma, and CMeC-2, isolated from CMeC-1 metastasis (kind gift from prof. dr. T. Nakagawa, University of Tokyo) (2), and human skin malignant melanoma SKMel28 cell line (ATCC), were transfected with plasmid pEGFP-N1, encoding GFP using electroporation. Cells in exponential growth phase were prepared at a concentration (2.5×10^7 cells/ml) in electroporation buffer (125 mM sucrose, 10 mM K_2HPO_4, 2.5 mM KH_2PO_4, 2 mM $MgCl_2\times6\ H_2O$). $1x10^6$ of cells were mixed with plasmid DNA (10 µg) and exposed to electric pulses (two stainless steel electrodes 2 mm apart, 8 square-wave electric pulses, amplitude per distance ratio 450 V/cm (SKMel28) or 500 V/cm (CMeC-1 and CMeC-2), 5 ms duration time, and 1 Hz pulse repetition frequency) generated by electroporator GT1 (University of Ljubljana, Faculty of Electrical Engineering). Then, the cells were incubated for 5 min at a room temperature and placed into cell culture media for 2 days. Cell survival was determined with cell viability indicator PrestoBlue (Invitrogen). Transfection efficiency was determined by flow cytometry, determining the number of fluorescent cells and their fluorescence intensity.

RESULTS

Survival of the CMeC-1 cells after gene electrotransfer was significantly higher compared to CMeC-2 cells and SkMel28 (Fig.1A). The survival of CMeC-2 and SKMel28 was very low (~10%) at the conditions tested and indicate on high sensitivity of these cells to electroporation. Transfection of CMeC-1 with EGFP-N1 plasmid was also higher compared to other two cell lines (Fig.1B, Fig.2), concerning the number of GFP positive cells (Fig.2), while fluorescence intensity per cell (Fig.1B) did not differ between the tested cell lines.

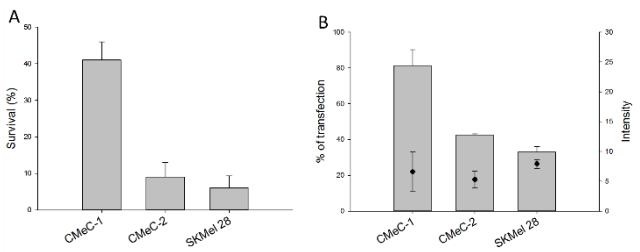

Figure 1: Cell survival 2 days after gene electrotransfer (A) and % of transfected cells (bar plot) with fluorescence intensity (scatter plot) (B).

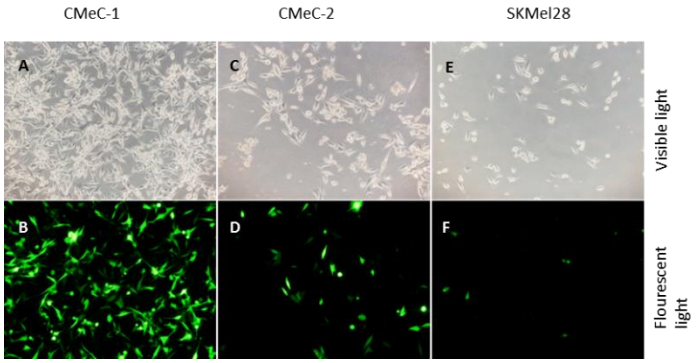

Figure 2: Images of cells transfected with plasmid DNA, taken under visible (A, C, E) and fluorescent (B, D, F) light. The images were acquired 48 h after plasmid DNA electrotransfer.

CONCLUSIONS

Our results demonstrated higher transfection and better survival of CMeC-1 cells compared to CMeC-2 cells. Therefore, CMeC-1 cell line seems more suitable for further research for in vitro and in vivo gene therapy studies using electrotransfer.

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The authors acknowledge the financial support from the state budget by the Slovenian Research Agency (program no. P3-0003). The research was conducted in the scope of LEA EBAM (French-Slovenian European Associated Laboratory: Pulsed Electric Fields Applications in Biology and Medicine) and is a result of networking efforts within COST TD1104 Action.

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SHORT PRESENTATIONS

LATE SUBMISSIONS

On The Characteristics Of Pores Created By High Voltages, Recent MD Investigations

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INTRODUCTION

A current goal in improving our understanding of electroporation (EP) is the development of a comprehensive microscopic description of the phenomenon, not an easy task due to the nanoscale dimensions of the lipid electropore and the short time scale (nanoseconds) of pore creation, which present challenges to direct experimental observations. For these reasons, molecular dynamics (MD) simulations have become a useful tool for the study of EP in atomic detail. In the last decade, several MD simulations have hence been conducted in order to model the effect of electric fields on membranes, providing perhaps the most complete molecular model of the EP process of lipid bilayers [1-6].

Here we extend these investigations to study the characteristics of the pores formed under a constant voltage. In particular, we present a new protocol allowing for the first time to perform simulations of a single bilayer under constant voltages that is suitable for comparison to experiments undertaken under similar conditions (patch clamp experiments). Using this new protocol, in contrast to previous simulations [3,5,6], the pores created at a given transmembrane voltage (TMV) are stable in size. This allows one to evaluate the structural (e.g. pore radius) and the electrical properties of the pore (e.g. conductance) providing information inaccessible by experiments.

METHODS

Systems: Two systems were studied. A bilayer composed of 256 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) lipids, and a bilayer composed by 1024 POPC lipids, both fully hydrated and at a 0.5 M NaCl salt concentration. The lipid topology and force field were derived from CHARMM36 all-atom parameters. The TIP3P water model was used. Bond lengths were constrained using the LINCS algorithm. Short-range electrostatic and Lennard-Jones interactions were cut off at 1.0 nm. Long-range electrostatic interactions were calculated by the PME algorithm, with a direct space sum tolerance of 10-6 and a spherical truncation of 1.2 nm.

All simulations were performed using GROMACS.4.6 on the GENCI-CINES High Performance Computing Linux cluster. The systems were first equilibrated at constant pressure (1 atm) and constant temperature (300 K) for at least 100 ns. They were then extended to study the effects of the charge imbalance imposed at constant surface tension and constant temperature (300 K).

MD protocol: The charge imbalance method used to mimic *in silico* the effect of millisecond EP (msEP) on planar bilayers, and it consists in imposing a net charge imbalance (ions) across the membrane [3]. This protocol suffers from one serious shortcoming. The charge imbalance that is set at the initial stage of the simulations decreases after the pore is formed, since ions translocation occurs from few tens to few hundred picoseconds. A drop in the value of the TM voltage follows with a consequent collapse of the pore. Here the charge imbalance is maintained constant by "swapping" charges between the electrolytes each time a ion goes through the pore.

RESULTS AND CONCLUSIONS

This protocol enable one to measure the pore radius, the conductance, the selectivity of a single pore created under different constant TMVs. Our first results from extensive on-going investigations given in Table 1, clearly show a size effect on the characteristics of the pores created by EP.

Table 1: Effect size and pore characteristics.

	TMV[V]	radius [nm]	G [nS]
256 POPC	0.5±0.2	1	12±1
1024 POPC	0.5±0.1	2.6	98±16

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Light-triggered Release of Lapatinib from Gold (Au) Nanoshells for Cancer Treatment

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INTRODUCTION

Brain metastases from primary breast cancer are a critical clinical challenge. Between 5 and 15% of all breast cancer patients have Central Nervous System (CNS) metastases,[1] The current mainstays of treatment for metastatic brain tumors are whole brain radiation therapy (WBRT), surgery, stereotactic radiosurgery or a combination of these modalities. However, significant neurotoxicity has been reported with the use of WBRT, resulting in endocrine dysfunction, significant memory loss and dementia.[2,3] Drug uptake into the brain is limited by numerous factors, such as the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (blood-CSF) barrier, and a substrate's affinity for specific transport systems located at these interfaces.[4,5] However, new expectation is that Lapatinib as a breast cancer drug, is a dual tyrosine kinase inhibitor which can effectively interrupt the HER2 and epidermal growth factor receptor (EGFR) pathways. Besides, more than two decades ago, Fidler and colleagues provided evidence that macrophages, originating as blood monocytes, can infiltrate brain metastases while the blood brain barrier is intact.[6]

Our interest is to utilize the Au nanoshell for drug delivery vehicle, since it is sensitive to NIR light, cover the surface by dsDNA which form a dense monolayer. As a host, it can be loaded with a large variety of molecules, such as fluorescent imaging agents or therapeutics. The whole nanocomplex will be uptake by the macrophage before injecting into the system circulation. As macrophage can infiltrate brain metastases while the blood brain barrier is intact, we shine NIR light outside with proper power to release the drug lapatinib to cure the brain metastases.

PRELIMINARY RESULTS

Light-induced release of DAPI (4',6-diamidino-2-phenylindole), a blue fluorescent molecule used to stain cell nuclei, within H1299 lung cancer cells is evidenced. (a). Top: Schematic of nanocomplexes in a general intracellular environment before and after near-IR illumination, triggering release. Bottom: schematic of nanocomplex consisting of a Au/SiO2 nanoshell and a dsDNA monolayer coating loaded with DAPI intercalant molecules. The DAPI is released when the dsDNA is dehybridized. (b) Left: H1299 lung cancer cells following 12 hour incubation with DAPI-laden nanocomplexes, before illumination; Right: post-illumination (1 W/cm2, 800 nm wavelength, 5 minute irradiation time). Flow cytometry cytotoxicity assay revealed no significant increase in cytotoxicity due to treatment protocol. (Figure 1).

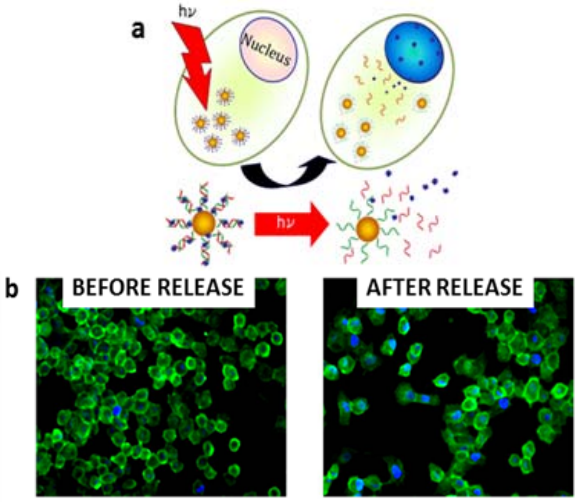

Figure 1: Evidence of light-induced release of DAPI

PERSPECTIVES

NIR light induced drug release seems promising, and we will continue exploring more details like how to synthesize nanocomplexes, quantify nanocomplex uptake by macrophage and assess cytotoxicity, determine the laser power can be delivered, and if lapatinib can be successfully delivered to brain metastases.

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Electrochemical study of the electroporation of liposomes encapsulating redox molecules

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INTRODUCTION

Chemotherapy is to this day the most promising way to deal with cancer. Nevertheless the undesirable side effects make researchers look for improvement. Electrochemotherapy might be one; by applying an electric field on a cell, its membrane is then permeabilized enabling more anti-cancer drugs to go in the tumorous cell. However, it is still fairly recent, thus the kinetics and dynamics of the electroporation phenomenon are still unknown..

DEVICE REALISATION

Using liposomes as a model for cell, we hope to fill this knowledge gap. Here liposomes will contain redox molecules. Their study of electrochemical signature will give us information concerning the pores that will appear in the liposome's membrane layer. Having a working device to realize this experiment is another main objective. Due to the small size (micro metric scale) of our devices, verifying their properties compared to regular electrodes is the final step of this work.

RESULTS

We have been able to show that working devices at this scale can be achieved and that macro liposomes can be synthesized and are stable. The main result is the fact that our devices are fully working and can both be used to do electroporation and to measure its electrochemical consequences. However no liposome electroporation took place because of technological barriers such as thin layer electric isolation or carbon electrode high linear resistance (300Ω.m-1).

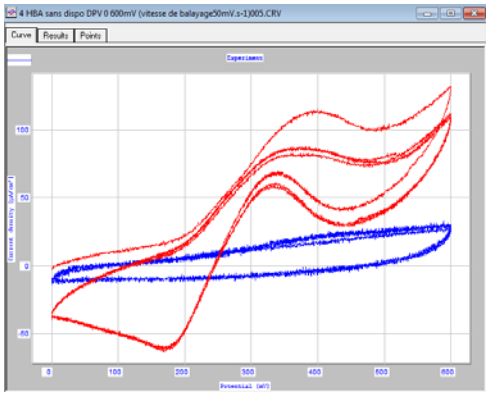

Figure 1: 1 Cyclic voltammetry 0mV 600mV of 4-HBA and 3,4 DHBA in an electrochemical cell.

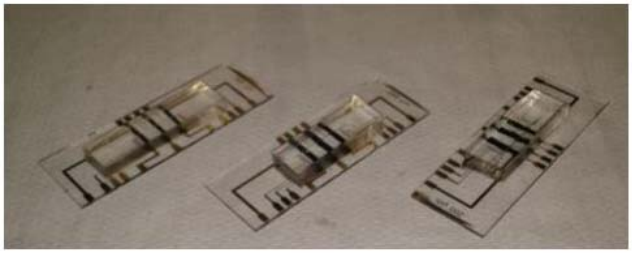

Figure 2: Three complete working devices.

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