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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
Le Centre national de la recherche scientifique
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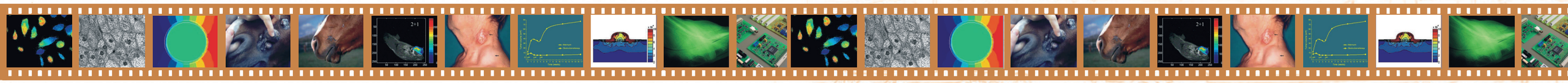

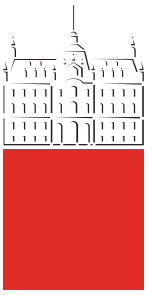

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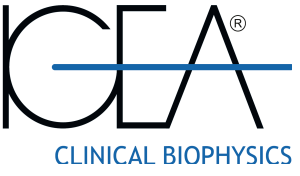

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Welcome note

Dear Colleagues,

Dear Students,

The idea of organizing the Workshop and Postgraduate Course on Electroporation Based Technologies and Treatments at the University of Ljubljana had been developing for several years. After preliminary discussions, the Workshop and Course was organised for the first time in 2003. In 2017 the Course is organised for the 11th time! In these fourteen years, the Course has been attended by 645 participants coming from 38 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 for their lectures and for the preparation of the practical trainings delivered during the course, to the agencies that have been sponsoring our research work for years, and to Slovenian Research Agency and Centre National de la Recherche Scientifique (CNRS), and to the Bioelectrochemical Society for supporting the Workshop and Course. We also would like to thank, Igea (Italy), Gorenje, Iskra Medical, Iskra PIO, Kemomed, Mettler Toledo, Mikro+Polo, Omega (Slovenia), and Leroy Biotech (France) whose financial support allowed us to assist many students participating in this Workshop and Course. The course is conducted in the scope of the LEA EBAM (European Associated Laboratory on the Pulsed Electric Fields Applications in Biology and Medicine).

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

Sincerely Yours,

Damijan Miklavčič and Lluis M. Mir

LECTURERS' ABSTRACTS

Cell in Electric Field – Induced Transmembrane Voltage

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

THE CELL AND ITS PLASMA MEMBRANE

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

From the electrical point of view, a cell can roughly be described as an electrolyte (the cytoplasm) surrounded by an electrically insulating shell (the plasma membrane). Physiologically, the exterior of the cell also resemble an electrolyte. If a cell is exposed to an external electric field under such conditions, in its very vicinity the field concentrates within the membrane. This results in an electric potential difference across the membrane, termed the *induced transmembrane voltage*, which superimposes onto the *resting transmembrane voltage* typically present under physiological conditions. Transmembrane voltage can affect the functioning of voltage-gated membrane channels, initiate the action potentials, stimulate cardiac cells, and when sufficiently large, it also leads to cell membrane electroporation, with the porated membrane regions closely correlated with the regions of the highest induced transmembrane voltage [1].

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

From the geometrical point of view, the cell can be characterized as a geometric body (the cytoplasm) surrounded by a shell of uniform thickness (the membrane). For suspended cells, the simplest model of the cell is a sphere surrounded by a spherical shell. For augmented generality, the sphere can be replaced by a spheroid (or an ellipsoid), but in this case, the requirement of uniform thickness complicates the description of the shell substantially. If its inner surface is a spheroid or an ellipsoid, its outer surface lacks a simple geometrical characterization, and vice versa.[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.

[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.

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 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.

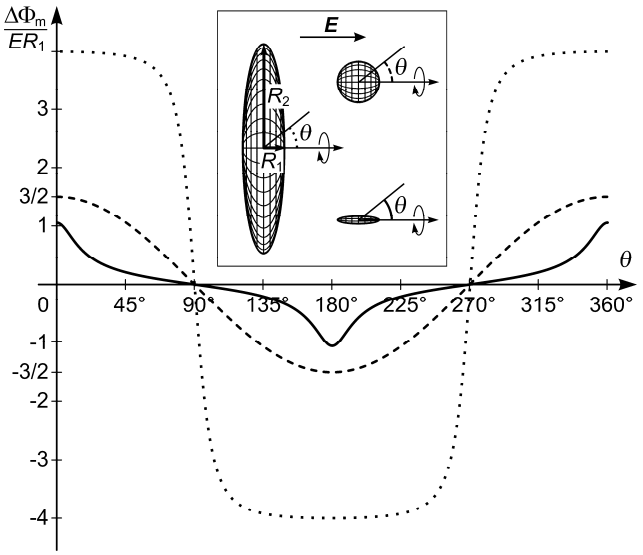

Figure 1: Normalized steady-state $\Delta\Phi_m$ as a function of the polar angle θ for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate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

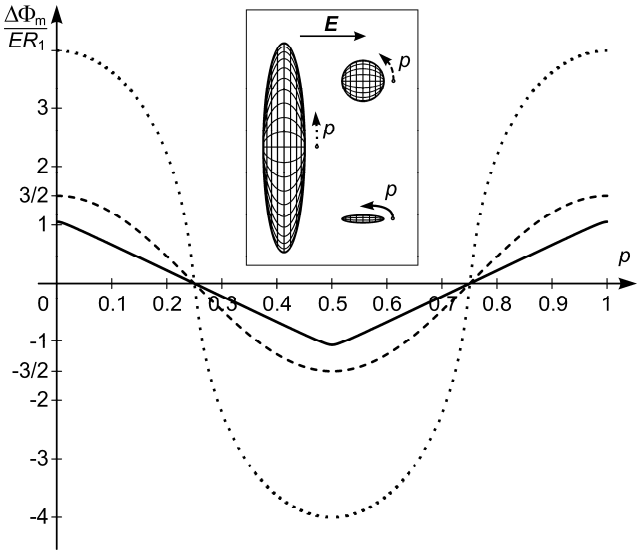

Figure 2: Normalized steady-state $\Delta\Phi_m$ as a function of the normalized arc length *p* for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: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

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

[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.

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.

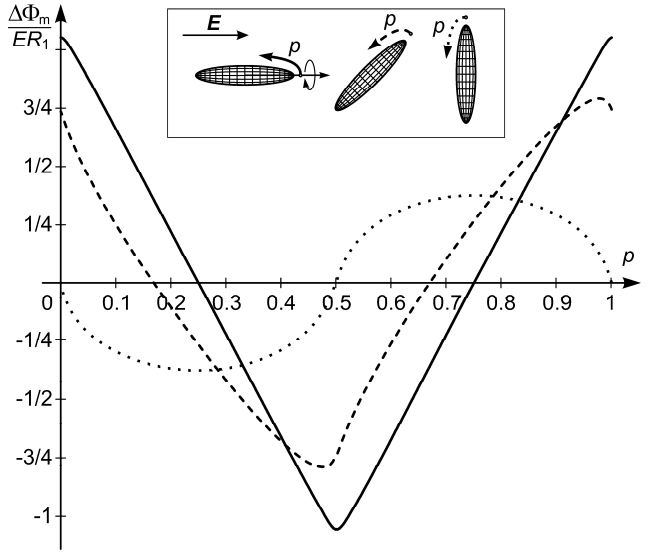

Figure 3: Normalized steady-state $\Delta\Phi_m(p)$ for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

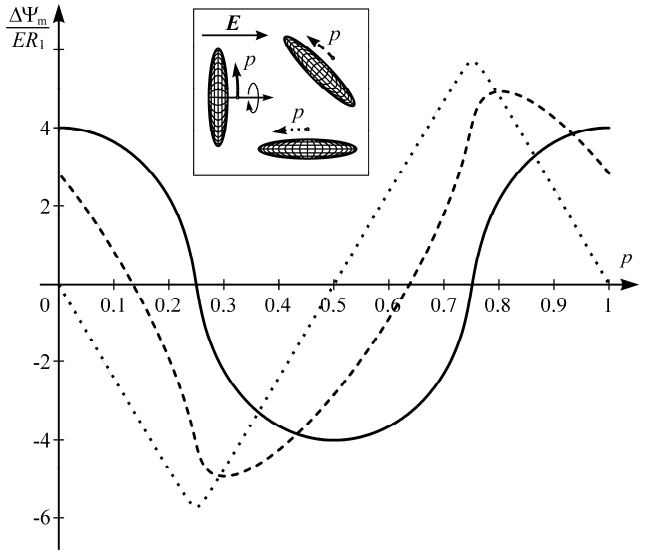

Figure 4: Normalized steady-state $\Delta\Phi_m(p)$ for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

The second complication of generalizing the cell shape from a sphere to a spheroid or an ellipsoid is that the induced voltage now also becomes dependent on the orientation of the cell with respect to the electric field. To deal with this, one decomposes the field vector into the components parallel to the axes of the spheroid or the ellipsoid, and writes the induced voltage as a corresponding linear combination of the voltages induced for each of the three coaxial orientations [11,12]. Figs. 3 and 4 show the effect of rotation of two different spheroids with respect to the direction of the field.

Irregularly shaped cells

For a cell having an irregular shape, the induced transmembrane voltage cannot be determined exactly, as for such a geometry Laplace's equation is not solvable analytically. Using modern computers and finite-elements tools such as COMSOL Multiphysics, the voltage induced on a given irregular cell can still be determined numerically, as described in detail in [13,14]. While the results obtained in this manner are quite accurate, they are only applicable to the particular cell shape for which they were computed. Fig. 5 shows examples of two cells growing in a Petri dish and the voltages induced on their membranes.

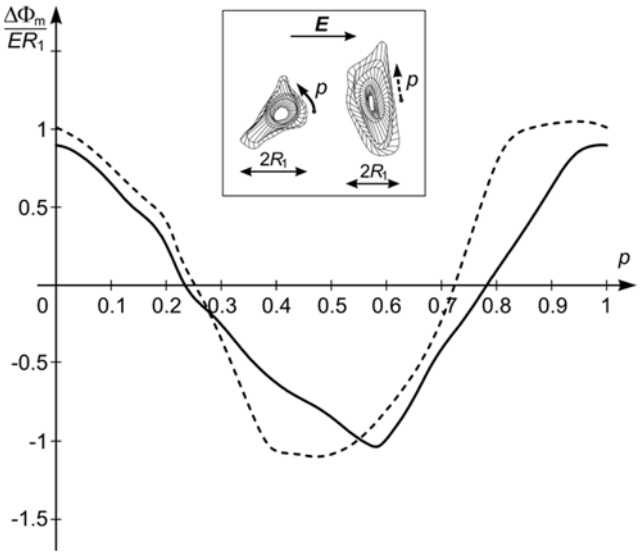

Figure 5: Normalized steady-state $\Delta\Phi_m(p)$ for two irregularly shaped cells growing on the flat surface of a Petri dish.

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.

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.

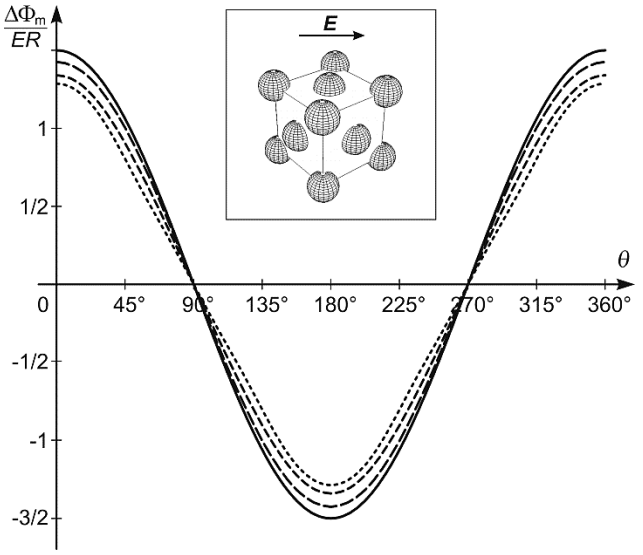

Figure 6: Normalized steady-state $\Delta\Phi_m(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by Eq. (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (with decreasing dash size) 10%, 30%, and 50% of the total suspension volume.

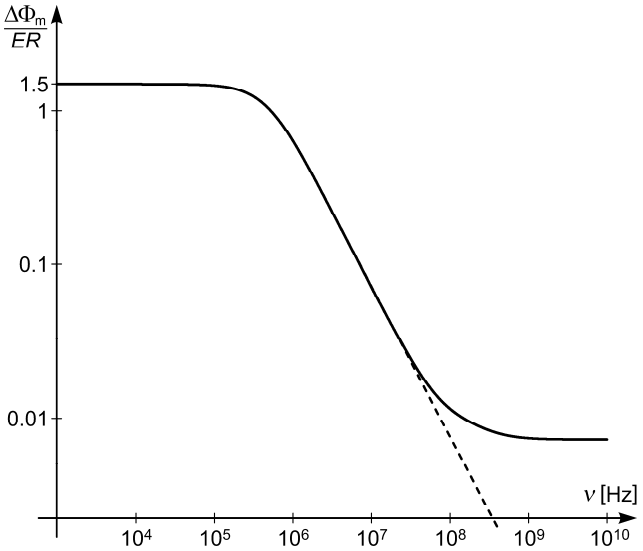

Figure 7: The amplitude of normalized steady-state $\Delta\Phi_m$ as a function of the frequency of the AC field. The dashed curve shows the first-order, and the solid curve the second-order Schwan's equation. Note that both axes are logarithmic.

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].

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Tadej Kotnik is the first author of 22 articles in SCI-ranked journals cited over 850 times excluding self-citations, and a co-author of additional 27 such articles cited over 650 times excluding self-citations. His h-index is 24. In 2001 he received the Galvani Prize of the Bioelectrochemical Society, and for the year 2014 he was among the recipients of the annual award “Ten Most Prominent Research Achievements of the University of Ljubljana”.

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 conductivity and relative permittivity 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. At low frequency electric conductivity is determined by extracellular ion concentration and their mobility.

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 divided 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 are thus 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).

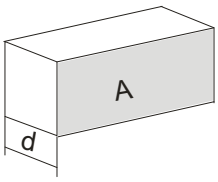

Figure 1: A considered theoretical small part of a material.

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

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

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]. 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.

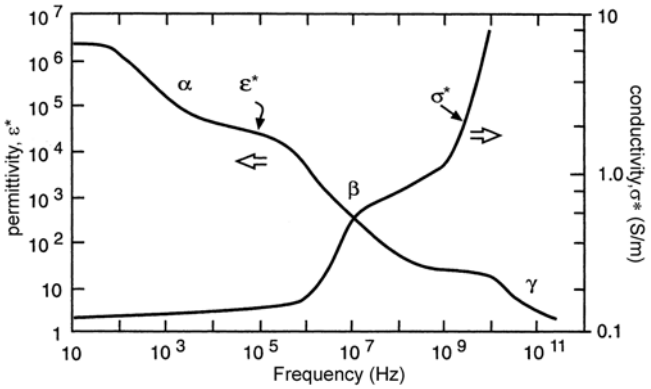

Figure 2: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.

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 (if not impossible) 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 [8]. 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. The drawback is that measurement results are interpreted based on the assumption of tissue being homogeneous in the entire region where measurement is performed.

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).

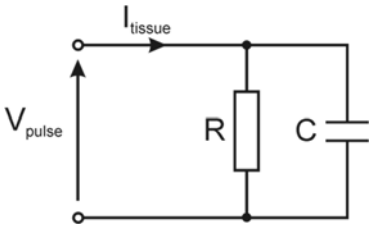

Figure 3: Parallel RC circuit: a theoretical representation of tissue response to electic pulses.

The complications arise from the facts that i) the pulse parameters (the pulse duration, the rise and the fall time) determine the content 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) [9]. 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 [9], [10].

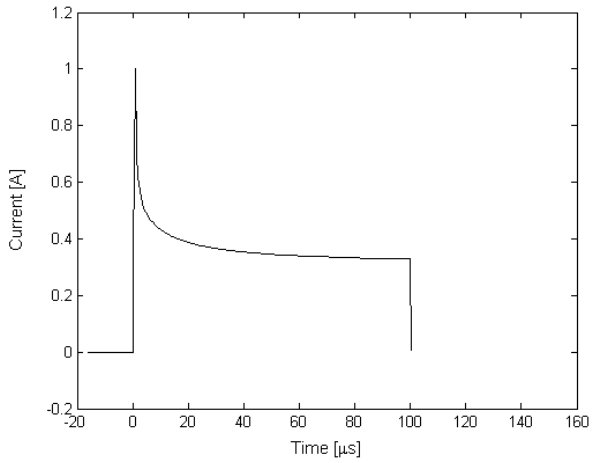

Figure 4: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 µs having the rise time of 1 µs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

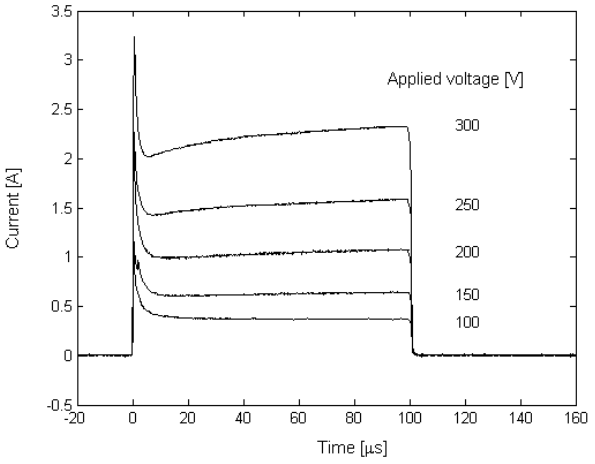

Figure 5: Measured tissue response during delivery of 100 µs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [9]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

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 [11]-[15]. In measuring ex vivo tissue and phantom tissue made of gel like material [16] 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 [17].

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 [18]. 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 [19]. 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.

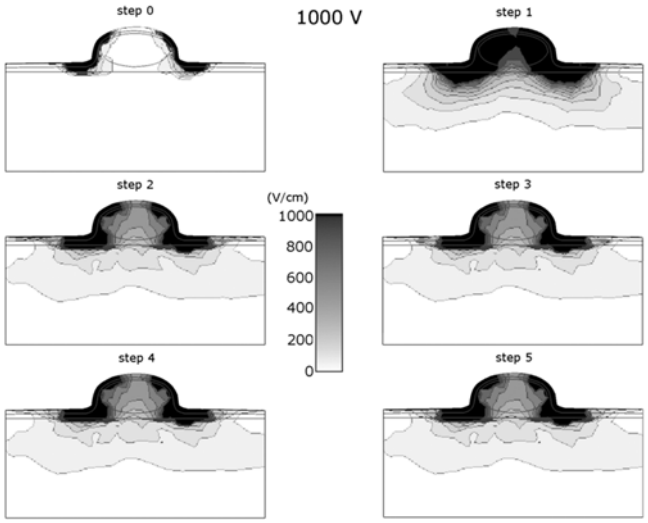

Figure 6: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [19]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact 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 [20]. 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 [21].

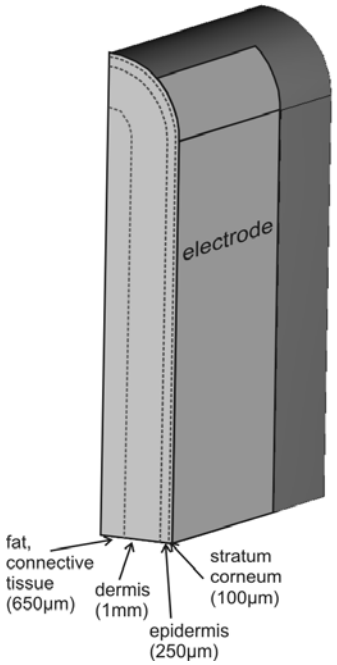

Figure 7: Schematics of a skinfold as described in a numerical model. Only one quarter of the skinfold is presented here.

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 [22]-[24]. Therefore, due effort needs to be invested into measurements of tissue electrical properties and their changes during electroporation [25].

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 [26], therefore thermal aspect in treatment planning and theoretical analysis of specific applications of electroporation-based treatments should be considered [27]. 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 [28]-[31].

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NOTES

In vitro Cell Electropermeabilization

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Abstract: Electropulsation (delivery of short lived electric pulses) is one of the most successful non-viral methods to introduce foreign molecules in living cells *in vitro*. This lecture describes the factors controlling electropermeabilization to small molecules (< 4 kDa). Pulse durations are selected from submicroseconds to a few milliseconds. 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. The membrane events appear to be controlled by the cellular metabolism.

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].

The present lecture is reporting what is called "classical electropermeabilization". This meant that it is relevant of the effect of field pulses lasting from μs to several ms, with a rising time of a few hundreds of ns. In this time domain, dielectric spectroscopy of a cell shows that the membrane can be considered as a non conductive insulator (indeed some active leaks may be present). The physics of the process was part of Prof. Kotnik lecture.

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 main target of cell electropermeabilization is the cell membrane, more precisely the plasma membrane. Organelles may be affected when they are shielded by the plasma membrane or by a back effect of the transport linked to the plasma membrane permeabilization (uptake of ions, leakage of secondary metabolites. In many approaches such as molecular dynamics simulations, 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. Spontaneous transverse movements are energy driven or result from membrane traffic related events (endocytosis, exocytosis). 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. The mechanical signals are transduced by the membrane. 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 (using lipid vesicles, 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 (TMP) 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]. More locally on the cell surface, it is affected by the local curvature and the associated defects in packing.This description is valid with dilute cell suspensions. In dense systems, self shielding in the cell population affects the local field distribution and reduces the local (effective) field distribution [7]. Stronger field intensities are needed to get the same induced potential. Another factor affecting the induced potential differences is the shape of the cells and their relative orientation to the field lines. When the resulting transmembrane potential difference ΔΨ (i.e. the sum between the resting value of cell membrane ΔΨo and the electroinduced value ΔΨi) reaches locally 250 mV, that part of the membrane becomes highly permeable for small charged molecules and transport is detected [3, 8].

One more parameter is that as the plasma membrane must be considered as a capacitor, there is a membrane charging time that may affect the magnitude of the TMP when the pulse duration is short (submicrosecond) or in poorly conducting pulsing buffers.

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] in a population 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 detected on a restricted cap at 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 by signal transduction.

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. 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.

Unpublished results using CARS (coherent anti Stokes raman spectroscopy) are indicative of an alteration of the interfacial water molecules.

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]. Concentration driven 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. X is reporting the density of conducting defects in the field affected cap on the cell surface. Ep depends on r (size). The delay between pulses is clearly playing a role in the definition of X but this remains to be investigated in details. Characterization of electropermeabilization is clearly dependent on the transport of S through Ps and the sensibility of its detection. 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] due to the induced osmotic swelling bringing hemolysis.

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). Biochemical modifications such as lipid oxidation may be present as suggested by membrane blebbings formed just after the pulse delivery [30, 31]. 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) [32, 9]. SiRNA are only transferred by the electrophoretic drag present during the pulse [33]. 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 [34, 35]

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APPENDIX - Transmembrane transport

Introduction

A Membrane transport complies with basic thermodynamic principles. A general principle of thermodynamics that governs the transfer of substances through membranes and other surfaces is that the exchange of free energy, ΔG, for the transport of a substance of concentration C_1 to another compartment where it is present at C_2 is:

$$\Delta G = RT \log \frac{C_2}{C_1}$$

(T, temperature; R, gas constant *i.e.* 8.3145 J/mol·K.)

When C_2 is less than C_1, ΔG is negative, and the process is thermodynamically favorable. As the energy is transferred from one compartment to another, an equilibrium will be reached where $C_2=C_1$ ($\Delta G=0$). However, there are circumstances, relevant for the in vivo functioning of biological membranes, under which this equilibrium will not be reached. A membrane electrical potential can exist which can influence ion distribution. For example, for the transport of ions from the exterior to the interior of a cell,

$$\Delta G = RT \log \frac{C_{inside}}{C_{outside}} + ZF\Delta P \qquad (1)$$

Where F is Faraday's constant, Z the charge of the ion and ΔP the transmembrane potential. If ΔP is negative and Z is positive, the contribution of the term $ZF\Delta P$ to ΔG will be negative, that is, it will favor the transport of cations out of the cell. So, if the potential difference is maintained, the equilibrium state ΔG=0 will not correspond to an equimolar concentration of ions on both sides of the membrane.

Passive diffusion

Simple diffusion and osmosis are in some ways similar. Simple diffusion is the passive movement of solute from a high concentration to a lower concentration until the concentration of the solute is uniform throughout and reaches equilibrium. Osmosis is much like simple diffusion but it specifically describes the movement of water (not the solute) across a permeable membrane until there is an equal concentration of solute on both sides of the membrane. Simple diffusion and osmosis are both forms of passive transport

$$J = -D\frac{dC}{dx} \qquad (2)$$

Electrophoretic drift

When an external electric field *E* is present, it has an action on molecules in the buffer. If the molecule is charged (nucleic acids, ions, dyes), it will migrate in an electric field to the electrode of opposite charge.

Consider the simple case of a charged particle (+*Q*) moving in an electric field (*E*) in a poorly conducting medium, such as water. If the particle is moving at a constant velocity toward the cathode (- electrode), the net force Ftot on the particle is 0 (since *F*=*ma*, and the acceleration (*a*) of the particle is 0 at constant velocity). Two forces are exerted on the particle, the force exerted on the charged particle by the field F_e, which is in the direction of the motion (toward the cathode), and the frictional force on the charged particle, F_f, which retards its motion toward the cathode.

Therefore:

$$F_{tot} = F_e + F_f = 0, \qquad (3)$$

where $F_e = QE$ (the electric force) and $F_f = -fv$ (the frictional force),

where *v* is the velocity of the particle, and *f* is a constant called the frictional coefficient. The last equation shows that the force F_f hindering motion toward the cathode is proportional to the velocity of the particle (Note: in the case of negatively charged particles such as nucleic acids, the directions of the forces are inverted and the direction of motion as well).

The frictional coefficient depends on the size and shape of the molecule. The larger the molecule, the larger the frictional coefficient (i.e. more resistance to motion of the molecule). The frictional coefficient for a spherical particle is given by

$$f = 6\pi\eta R_s \qquad (4)$$

where η is the viscosity, and R_s (Stokes radius) is the radius of the hydrated sphere.

From (1), (2), and (3), $F_e = F_f$, or

$$QE = fv \qquad (5)$$

Hence $v/E = Q/f = U$ = electrophoretic mobility, or

$$U = \frac{v}{E} = \frac{Q}{2\pi\eta R_S} \qquad (6)$$

Counter ions in the solution (from salts) form a cloud around the charged macromolecule, and partially shield the charged particle from the electric field *E*.

When the field is delivered across a "porous" membrane, friction is critical. "Porous" means that transient structural defects are present. The smaller molecules can pass through the membrane defects more readily than larger molecules, so there is an additional sieving mechanism that contributes to the effective transport

Note: Electrode nomenclature might be confusing to some of you. As mentioned above, cations move towards the cathode (where reduction occurs), so the cathode must be negative. Likewise, anion move towards the anode (where oxidation occurs), so the anode must be positive

Facilitated diffusion

Facilitated diffusion, also called carrier-mediated osmosis, is the movement of molecules across the cell membrane via special transport proteins that are embedded within the cellular membrane. Large, insoluble molecules, such as glucose, vesicles and proteins require a carrier molecule to move through the plasma membrane. Facilitated diffusion is a passive process: the solutes move down their concentration gradient and do not require the expenditure of cellular energy

Active and co-transport

In active transport a solute is moved against a concentration or electrochemical gradient by transport proteins that consume metabolic energy, usually ATP. In primary active transport the hydrolysis of the energy provider (e.g. ATP) takes place directly in order to transport the solute in question (ATPase enzymes). In secondary active transport, the energy provider acts indirectly; the energy is stored in an electrochemical

gradient to transport a target compound against its gradient,

Primary active transport is mediated by the formation of a substrate-transporter complex; Therefore, each transport protein has an affinity constant for a solute. This is equivalent to the case of an enzyme to the Michaelis-Menten constant.

$$J = \frac{J_{max}S}{K_m+S} \qquad (7)$$

Some important features of active transport in addition to its ability to intervene even against a gradient, its kinetics and the use of ATP, are its high selectivity.

Pumps

A pump is a protein that hydrolyses ATP to transport a particular solute through a membrane, and in doing so, generating an electrochemical gradient membrane potential. This gradient is of interest as an indicator of the state of the cell through parameters such as the Nernst potential

$$E = \frac{RT}{zF}\ln\frac{[ion\ outside\ cell]}{[ion\ inside\ cell]} \qquad (8)$$

With active pumping, the Goldman equation gives the resting potential

$$E_m = \frac{RT}{F}\ln\left(\frac{\sum_i^N P_{M_i^+}[M_i^+]_{out}+\sum_j^M P_{A_j^-}[A_j^-]_{in}}{\sum_i^N P_{M_i^+}[M_i^+]_{in}+\sum_j^M P_{A_j^-}[A_j^-]_{out}}\right) \qquad (9)$$

E_m is the transmembrane potential Pion is the permeability for that ion, $[ion]_{out}$ is the extracellular concentration of that ion, $[ion]_{in}$ is the intracellular concentration of that ion.

Transport by vesicles

Specialized vesicles mediate the transport by complex interactions with the membrane. Their intra-vesicular cargo is delivered to the other side of the membrane. This is called transcytosis (endo and exocytosis). The process is active meaning it requires energy and the action of the cell machinery.

Suggested reading and watching

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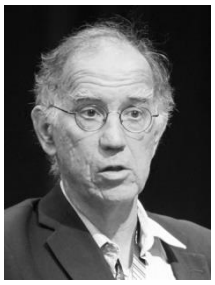

Teissié Justin was born 24 March 1947 in Poitiers, France. Got a degree in Physics at the Ecole superieure de Physique et de Chimie Industrielles de Paris (ESPCI) in 1970. Got a PhD in Macromolecular Chemistry on a project on fluorescence detection of action potential under the supervision of Prof. Monnerie (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 250 papers.

NOTES

NOTES

Nucleic acids electrotransfer *in vitro*

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Abstract: Cell membranes can be transiently permeabilized by application of electric pulses. This process, called electropermeabilization or electroporation, allows hydrophilic molecules, such as anticancer drugs and nucleic acids, to enter into targeted cells and tissues. The knowledge of the processes involved in membrane permeabilization and in gene transfer is mandatory for this promising method to be efficiently and safely used. The behavior of the membranes and the cells both while the electric field is on and after its application has therefore to be addressed. The description of the full mechanisms takes benefit from studies performed on different biological models (lipid vesicles, cells in 2D and 3D culture) and from different microscopy tools that allow to visualize the processes. Single cell imaging experiments revealed that the uptake of molecules (antitumor drugs, nucleic acids) takes place in well-defined membrane regions and depends on their chemical and physical properties (size, charge). 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 engender a complex mechanism of transfer. Gene electrotransfer indeed 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 towards the nuclei and finally gene expression.

INTRODUCTION

Gene therapy is a treatment option for a number of diseases as inherited disorders and cancer. Despite the fact that a lot of methods of vectorization have been developed during the last decades, the technique has still to be improved to be both efficient and safe (1). Among the different approaches, electroporation appears as the most promising one. This physical method can be efficiently used for the targeted deliver of molecules in a wide range of cells and tissues (2). Electroporation is nowadays a well-known technique of cell transfection used in the laboratories. Vaccination and oncology gene therapy are major fields of application of DNA electrotransfer in clinics (3, 4). Translation of preclinical studies into clinical trials started 10 years ago. The first clinical trial of plasmid electroporation carried out in patients with metastatic melanoma has shown hopeful results (5). The method has also been successfully used for the treatment of companion animals. However, despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated more than 30 years ago (6), many of the mechanisms underlying membrane electropermeabilization and DNA electrotransfer remain to be elucidated. Even if *in vitro* electrotransfer is efficient in almost all cell lines, *in vivo* gene delivery and expression in tumors can be not as efficient as in viral vectorization. It is therefore mandatory, for increasing gene transfer and expression while preserving safety, to increase knowledge about the mechanisms. This chapter aims to describe the basics aspects of membrane electropermeabilization and gene delivery in cells and by doing so to give some tips to perform experiments and optimize protocols.

MEMBRANE ELECTROPERMEABIZATION

The basics

Cells have a resting transmembrane potential which is uniform all along their plasma membrane. Exposure of living cells to short and intense electric pulses induces position-dependent changes of this transmembrane potential. Being dependent on the angle between the electric field direction and the normal to the membrane, the electric field effects are not uniform along the membrane. Maximum effects are present at the poles of the cells facing the electrodes when the resulting transmembrane potential reaches a threshold value. Above this threshold, permeabilization of the cell membrane occurs. Electropermeabilization of the plasma membrane is a prerequisite for gene electrotransfer since nucleic acids are highly charged and large molecules that cannot enter cells.

The way to conduct an experiment

Electropermeabilization can be performed in different manners depending on the way cells are grown. For cells grown on Petri dish, culture medium can be removed and replaced by a low ionic, iso-osmotic buffer. This pulsation buffer allows to limit the Joule effect and therefore help to preserve the cell viability. The composition of this medium is generally a 10 mM phosphate buffer, 250 mM sucrose and 1 mM $MgCl_2$. On a practical point of view, the bottom of the Petri dish can serve as an electropulsation chamber. For cells in suspension, cells resuspended in the pulsing buffer are placed in purchased cuvettes or in "home-made" chambers that can be easily obtained by placing the electrodes on the bottom of the Petri dish (see Figure 1). The electric pulses are delivered through a set of electrodes connected to the pulse generator. In

most experiments, square-wave electric pulses generators are used. Contrary to exponential decay generators, they allow the independent control of the amplitude of the electric field pulses E and their duration T. This is important for mammalian cells which have no cell wall and therefore are more affected by electric pulses than bacteria and yeast. The electric pulse parameters have to be selected considering the characteristics of the cells in particular their size. One key step to further ensure DNA electrotransfer and expression is to determine the best electric conditions allowing both the permeabilization of the plasma membrane and the preservation of the cell viability.

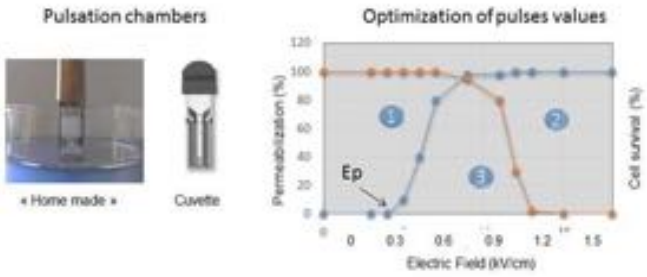

Fig. 1 Tips for your experiments. Cells are pulsed on Petri dish or on cuvettes. Permeabilized and viable cells are plotted to define the optimum conditions ((1) E < Ep or just above, poor permeabilization; (2) E>> Ep, viability loss; (3) best values).

The use of video microscopy allows visualization of the permeabilization phenomenon at the single cell level. Fluorescent indicators of membrane permeabilization, such as Propidium Iodide (PI), are very convenient to detect the electrotransfer of molecules into the cytoplasm. They can simply be added to the cells before application of the electric pulses. The uptake of the fluorescent dye into the cells is the signature of membrane electropermeabilization. Whatever the value of the pulses duration T, permeabilization only appears above a threshold value of pulse intensity E, called Ep. Therefore, the first experiment to perform consists to submit the cells to increasing values of E and determine the permeabilization efficiency (i.e. the percentage of cells that have been electropermeabilized, cells which nuclei become fluorescent). For E<Ep, which in the example of Figure 1 is equal to 0.3 kV/cm, no permeabilization occurs. Above E, increasing E leads to the progressive permeabilization of the whole cell population, that is obtained at 0.8 kV/cm. Then, the next step is the determination of the cell viability. For field values higher than 0.9 kV/cm, viability is affected. Once obtained, such kind of results easily allows to define the best conditions for membrane permeabilization and also for gene electrotransfer. In the example shown in Figure 1, the electric field values that can be used range from 0.6 to 1.0 kV/cm.

Kinetics of membrane permeabilization

Electropermeabilization of cells is a fast process that can be detected immediately after the application of electric pulses. Usually, transport across the 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 2), i.e. in the hyperpolarized area than in the depolarized area, which is in agreement with both theoretical and experimental considerations as explained in other chapters. 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, when pulses parameters have been correctly defined, E> Ep, the formation of transient permeable structures facing the electrodes allows the exchange of molecules; Propidium iodide is observed to rapidly access the cell interior in the region of the cells facing the electrodes, mainly at the anode facing site; (iii) after electropulsation, membrane can stay permeable before resealing occurs (7). Life-time of permeabilization can be assayed by adding the fluorescent dyes at various times following the pulses. If the cell membrane is still permeable, then the cell will be fluorescent. Resealing varies from a few seconds (when cells are put at 37°C just after pulsation) to several hours (when cells are maintained on ice) according to the experimental conditions (temperature and pulse parameters). However, one has to take into account that viability can be affected since ATP release will occur. It is therefore better to avoid to maintain the cells at low temperature after pulse delivery.

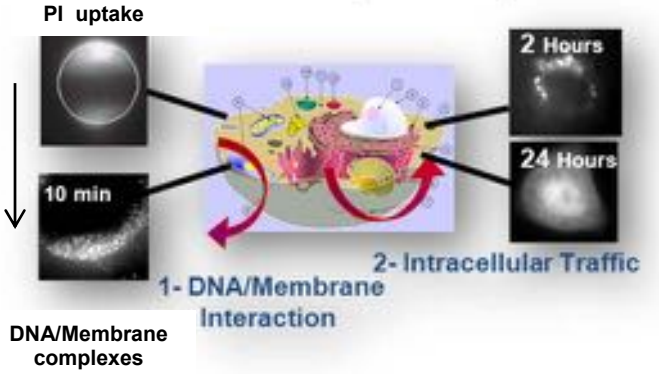

Figure 2: Molecule electrotransfer mechanisms. Left: During electric pulses application: Plasma membrane is electropermeabilized facing the 2 electrodes (PI uptake). DNA aggregates are formed. This interaction takes place only on the membrane facing the cathode. Right: About 2 h after electric pulses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

Whatever the molecules used to detect permeabilization (if they are small enough and charged), a direct transfer into the cell cytoplasm is observed. When added after electropulsation, molecules can still penetrate into the cells but less efficiently because 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 electrotransfer mechanism involved is indeed specific for the physico-chemical properties of the molecule (8).

Progress in the knowledge of the involved mechanisms, in particular in the elucidation of membranes structures that are responsible for molecules transfer, is still a biophysical challenge. Hydrophilic pores have been proposed to be created and their formation confirmed by molecular dynamics modelling. But their existence in permeabilized cells has still to be proven. Phospholipid scrambling and changes on lateral mobility of proteins have been observed suggesting that part of the membrane surface is occupied by defects or pores and that these structures propagate rapidly over the cell surface (9). One can also took advantage of atomic force microscopy to directly visualize the consequences of electropermeabilization and to locally measure the membrane elasticity. 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 (10, 11).

The fact that the entire cell surface is affected is not so obvious since permeabilization is only induced in specific regions of the cells. So, even if the entire mechanisms of membrane electropermeabilization (or electroporation) is not fully understood, and the existence of the exact structures responsible for molecules uptake still a debate, this physical method of vectorization has become one of the most efficient for gene delivery.

MECHANISMS OF ELECTROTRANSFER OF DNA MOLECULES INTO CELLS

What is known about the process

The first electroporation-mediated gene transfer experiment was published more than 30 years ago (6). Translation to the clinic benefited from increased knowledge of the mechanisms involved in the electrotransfer of nucleic acids during the last 3 decades. As for electropermeabilization, single-cell studies aided in describing the process of DNA electrotransfer.

In addition to membrane permeabilization, DNA electrotransfer is dependent on DNA electrophoresis. The oligonucleotide must indeed be present during the pulse to be later on transferred in the cytoplasm. The electrophoretic mobility of pDNA is not dependent on its number of base pairs. Short pulses with high field strength can be used but are less effective than long pulses with lower field strength. Therefore, pulses parameters have to be determined to lead the membrane to be permeable (E > Ep) while preserving as much as possible cell viability (above 30-50 %). Reporter genes are useful to optimize the protocol. As for electropermeabilization, single-cell microscopy and fluorescent plasmids can be used to visualize and determine the different steps of electrotransfection. Plasmids can be labeled with fluorescent dyes to allow visualization of its electrotransfer. 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 (E<Ep), the DNA simply flows around the membrane in the direction of the anode. Beyond the critical field value, above which cell permeabilization occurs (E > Ep), the DNA interacts with the plasma membrane.

DNA/membrane interaction

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, the formation of "microdomains" whose dimensions lie between 0.1 and 0.5 µm is observed (Figure 2). 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.

Dynamic of the process

DNA/membrane interaction and as a direct consequence gene expression depend 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. These observations 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 (12). Dynamics of membrane/complexes formation has been poorly understood because direct observations have been limited to time scales that exceed several seconds. However, experimental measurement of the transport of plasmid DNA and propidium iodide with a temporal resolution of 2 ms has been performed thanks to high speed and sensitive camera and allowed the visualization of the DNA/membrane interaction process during pulse application (13). Plasmid complexes, or aggregates, start to form at distinct sites on the cell membrane during the first pulse. Increasing the number of pulses do not lead to the creation of new sites, but to the increase in the amount of DNA. The formation of plasmid complexes at fixed sites suggested that membrane domains may be responsible for DNA uptake and their lack of mobility (as directly observed under the microscope or quantify by Fluorescence Return After Photobleaching (FRAP) measurements) could be due to their interaction with the actin cytoskeleton. As will be described later in this chapter, several publications reported evidences for the involvement of cytoskeleton (14, 15). The dynamics of the entire process is reported in Table 1. If pulse delivery occurs in a relative short time scale (µs to ms), the subsequent traffic of plasmid DNA occurs during the minutes and hours following pulse delivery.

Time Scale	Steps involved in DNA electro-mediated delivery	References
µs	Plasma membrane facing the electrodes is permeabilized	(7)
ms	Electrophoretic migration of DNA towards the membrane DNA/membrane complex formation	(7, 13)
s	Conversion of the metastable form of the DNA/membrane complex to a stable one	(12)
min	DNA translocation/diffusion across the membrane	(13)
hour	DNA transport towards the nucleus along the cytoskeleton	(14, 15)
day	Gene expression	(16)

Table 1. Kinetics of the different steps involved in gene delivery.

DNA transfer through the cytoplasm

The process of plasmid transfer through the cellular cytoplasm to the nuclear envelope is a complex process (17). In principle micro sized aggregates of DNA or vesicles filled with DNA could be too large to pass through the pores formed by electroporation. However individual DNA molecules, while they can pass through electropores, have a limited mobility within the cell and may well be totally degraded before reaching the nucleus. It is possible and worth investigating the possibility that the actin cytoskeleton reacts to the presence of DNA aggregates and plays an important role in the subsequent intracellular transport. It seems reasonable that only aggregates beyond a certain size (a few hundred nanometers) can induce a biological cellular response and can be transported by the cell. In addition, the fact that the DNA is in aggregate form means that the DNA in the center of the aggregate is relatively protected from degradation. Therefore, for gene therapy purposes, it is optimal for DNA to enter the cell as single molecules, but the subsequent transport toward the nucleus is, for biological (possibly by inducing a response of the actin cytoskeleton) and physical (diminishing enzymatic degradation) reasons, optimized if the DNA is in a micro-sized aggregate form.

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, guidelines to improve gene electrotransfer can not only result from the way pulses parameters have been selected. Expression of the pDNA is controlled by the viability of the pulsed population and 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 cell limiting factors that must be taken into account. 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 (hours compared to minutes). 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 will represent the last, but by no means the least important, obstacle for the expression of the plasmid DNA.

Passage through the nuclear envelope and gene expression

A high transport does not always result in a high level in expression. The relatively large size of plasmid DNA makes it unlikely that the nuclear entry occurs by passive diffusion. Single particle tracking experiments of DNA aggregates in living cells showed how electrotransferred DNA is transported in the cytoplasm towards the nucleus. The modes of DNA aggregates motion in CHO cells have been analyzed. Fast active transport of the DNA aggregates occurs over long distances. Tracking experiments in cells treated with different drugs affecting both the actin and the tubulin network clearly demonstrate that transport is related to the cellular microtubule network (Figure 3, (16)).

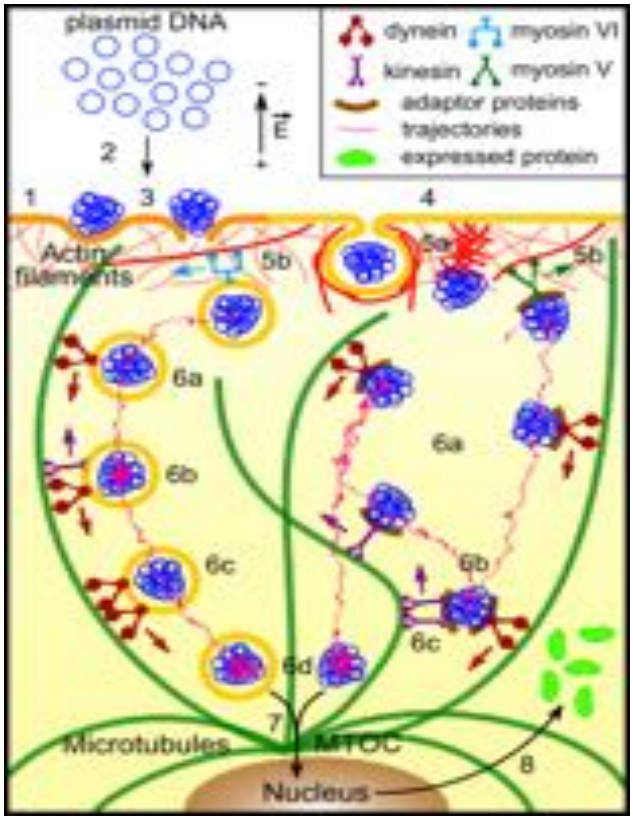

Figure 3: Schematic representation of the mechanism of DNA electrotransfer. During the electric pulses, (1) the plasma membrane is permeabilized, (2) DNA is electrophoretically pushed onto the cell membrane, which results in (3) DNA-membrane interactions. After resealing of the membrane, (4) DNA is internalized by endocytosis and other means where actin may take shape of bursts of polymerization. (5) While being actively transported in the cytoplasm by actin and tubulin networks, DNA aggregates pass through the endosomal compartments. Free DNA interacts with adapter protein in order to be transported by motor proteins. For gene expression to occur, (6) DNA has to escape from endosomal compartments. Once in the perinuclear region, (7) DNA crosses the nuclear envelope to be expressed and (8) yield proteins released.

Active transport of DNA aggregates

Several studies point towards the contribution of endocytosis in the electrotransfer of DNA, but more investigations have to be performed in order to understand what type(s) of endocytosis would be involved. It is necessary to understand as well how electric fields could stimulate such processes. Also notably, any endocytosis model would only explain the internalization of large molecules as it does not support the free membrane crossing of small molecules. It has therefore to be considered to occur in parallel to another model valid for small molecule transmembrane exchange. One model that could reconcile all the DNA internalization models would be that DNA accumulates where pores are formed and that its electrophoretically driven insertion in the membrane pulls the pore and the plasma membrane around. This would generate membrane curvature that could be recognized as an emerging endocytic vesicle and induce a similar response from the cell as for an endocytic process, with the recruitment of actin, clathrin, caveolin, dynamin and other endocytic regulators (18, 19).

Electrotransferred DNA trajectories possess portions of active transport interrupted by phases of nearly immobility (15). During the phases of active transport, DNA aggregates featured a motion on average having a velocity of 250 nm/s, persisting for 6 s and leading to a displacement of 1.3 µm. However, the distributions were rather broad with velocities from 50 nm/s to 3400 nm/s, displacements from 0.1 µm to 12 µm and active transport durations from 2 s to 30 s. These ranges are in agreements with other types of intracellular particle dynamics as observed for viruses, polyplexes, lipoplexes, receptors, endosomes and mitochondria. Lower velocities were shown to correspond to actin-associated transport. Indeed, after disruption of the microtubules using the nocodazole drug, active transport of the DNA still occurred and the measured velocities were in the range expected for myosin motors operating on actin – between 50 nm/s and 300 nm/s for myosin VI and between 250 nm/s and 500 nm/s for myosin V. In addition to motor driven transport, actin-related movement could be also due to bursts of actin polymerization which was reported to drive viruses, bacteria or endosomes from the plasma membrane to the cytosol with mean velocities ranging from 50 to 600 nm/s.

New challenges to increase gene expression

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 (20). Clear limits of efficient gene expression using electric pulses

are therefore due to, in addition to the passage of DNA molecules through the plasma membrane, to the cytoplasmic crowding and transfer through the nuclear envelope. One of the key challenge for electromediated 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. An idea, to improve transfection success, is thus to perform classical membrane permeabilization allowing plasmid DNA electrotransfer to the cell cytoplasm, and then after, when DNA has reached the nuclear envelope, to specifically permeabilize the nuclei using these short strong nanopulses. Thus, when used in conjunction with classical electropermeabilisation, nanopulses gave hope to increase gene expression (21). However this work was not yet replicated. Another idea is to combine electric pulses and ultrasound assisted with gas microbubbles. Although electroporation induced the formation of DNA aggregates into the cell membrane, sonoporation induced its direct propulsion into the cytoplasm. Twenty-four hours later, cells that received electrosonoporation demonstrated a four-fold increase in transfection level and a six-fold increase in transfection efficiency compared with cells having undergone electroporation alone (22). Sonoporation can therefore improve the transfer of electro-induced DNA aggregates by allowing its free and rapid entrance into the cells (23).

Lipid vesicles and spheroids as other models to study gene electrotransfer

Coming back to a mechanistic point of view and due to the complexity of the composition of the plasma membrane, other experimental tools can be useful to characterize the membranes domains observed during gene electrotransfer. For that purpose, giant unilamellar vesicles (GUV) represent a convenient way to study membrane properties such as lipid bilayer composition and membrane tension (24). 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. They can be obtained by simple methods such as electroformation and their composition can be very simple (one type of phospholipids) or more complex (several lipids including cholesterol). Experiments showed a decrease in vesicle radius which was observed as being due to lipid loss during the permeabilization process. Three mechanisms responsible for lipid loss were directly observed: pore formation, vesicle formation and tubule formation, which may be involved in molecules uptake. However, no interaction between plasmid DNA and the GUV membrane could be observed; a direct transfer of DNA into the GUVs took place during application of the electric pulses (25). That gives clear evidence that "lipid bubble" is not always relevant as a cell and a tissue is not a simple assembly of single cells. Therefore, it is necessary to develop and use different models, from simple lipid vesicles to tumor multicellular tumor spheroids more closed to the *in vivo* situation, for the understanding of the membrane permeabilization and DNA electrotransfer process in tissues. Each of this model has advantage and limits. Together combined they can help in the study of the full processes (table 2).

Table 2. What models can address about electropermeabilization and gene delivery processes.

Model	Membrane permeabilization	DNA electrotransfer
GUV	Direct visualization of membrane permeabilization and its consequences (deformation, lipid loss)	Failed to address DNA/membrane interaction (DNA is directly transferred inside the vesicle)
2D Cell culture	Kinetics of permeabilization and its consequences (lateral and transverse mobility of lipids and proteins)	Visualization of DNA/membrane complex formation and access to DNA traffic into the cells
3D Cell culture	Molecules diffusion and transfer that mimic *in vivo* complex situation (contacts between cells, junctions, extracellular matrix)	Allow to address DNA delivery in 3D and mimic what happens *in vivo* (decrease in gene expression from the periphery to the core)

Even if the high majority of studies underlying molecule transfer by electric fields have been performed on 2D cell culture in Petri dish or in cells cultured in suspension, 3D multicellular spheroids represent a nice, relevant, cheap, easy-to-handle *in vitro* model. 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 micro regions of tumors (26). Confocal microscopy allowed to visualize the repartition of permeabilized cells in spheroids submitted to electric pulses. Results revealed that cells were efficiently permeabilized, whatever their localization in the spheroid, even those in the core, mimicking previously observed *in vivo* situations. Propidium iodide uptake was observed to be present

but spatially heterogeneous within the 3D multicellular spheroid after electroporation, with a progressive decrease from peripheral to interior cells. In the case of large molecules as plasmid DNA, spheroids allowed showing that electrophoresis, and not tissue deformation or electroosmosis, is the driving force for interstitial transport. In addition, and at the opposite of cells in 2D cultures, only cells on one side of the outer leaflet expressed the reporter gene (27). This low expression is in fair agreement with *in vivo* experiments on tumors. Close contacts between cells and extracellular matrix may act as physical barrier that limit/prevent (uniform) DNA distribution and explain the absence of gene expression in the inner region of spheroid. The limited access of plasmid-DNA to central region of spheroid remains a significant barrier to efficient gene delivery in tissues. Taken together, these results, in agreement with the ones obtained by the group of R. Heller (28), indicate that the spheroid model is more relevant to an *in vivo* situation than cells cultured as monolayers and therefore can be useful to address the mechanisms of DNA electrotransfer. In order to assess the effects of the extracellular matrix composition and organization, as well as intercellular junctions and communication, other 3D reconstructed human connective tissue model can be used. Cell sheets, reconstructed *in vitro* by a tissue engineering approach, presents multiple layers of primary dermal fibroblasts embedded in a native, collagen-rich Extra Cellular Matrix (ECM) and can be a useful tool to study skin DNA electrotransfer mechanisms. Cells within this standardized 3D tissue can be efficiently electropermeabilized by milliseconds electric pulses (29, 30). Moreover such a tissue-engineered dermal model recapitulates the mechanical properties of human native dermal tissue unlike the classically used monolayer and spheroid models (31). A better comprehension of gene electrotransfer in such a model tissue would help improve electrogene therapy approaches such as the systemic delivery of therapeutic proteins and DNA vaccination.

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 Models of Lipid Bilayers and Electropore Formation are giving interesting new insight into the process. Electroinduced destabilization 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, that may vary according to the micro environment, 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, cells and tissues remain a priority given the importance of these phenomena for processes in cell biology and in medical applications. *In vivo* gene electrotransfer will face other challenges such as the necessity to control electric field distribution and gene expression both in space (targeted DNA delivery to the cells) and in time. Guidelines for successful DNA delivery are still required but we can be optimistic that further working to improve gene electrotransfer mechanisms will yield effective treatments.

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NOTES

Molecular Dynamics Simulations of Lipid Membranes Electroporation

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Abstract: Currently, computational approaches enable to follow, at the atomic scale, the local perturbation lipid membranes undergo when they are subject to external electric field. 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. The simulations are further used to characterize the transport of charged species through these pores. The results obtained 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.

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: Long and intense electrical pulses induce 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 [13–18]. Several MD simulations have recently been conducted in order to model the effect of electric field on membranes [19–23], providing perhaps the most complete molecular model of the electroporation process of lipid bilayers.

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 [24,25]. 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 [26] CHARMM [27] and AMBER [28], 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 [29–31] 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 [32,33].

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 [34–37]. More recent studies have considered a variety of alternative lipids, featuring different, possibly charged, head groups [38][39–42], and more recently mixed bilayer compositions [43–49]. 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 [14,17,50,51].

MODELING MEMBRANES 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 using equivalent network RC circuits in the 100 ns time scale [19,52–55]. A charging time constant in the range of hundreds of nanoseconds was also obtained from derivations based on the Laplace equation (see e.g. [56] for the first-order analysis on a spherical vesicle; [57] for the second-order analysis; and [58] 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 response of the system is mainly dielectric and is linked to the polarization of the interfacial water (see below).

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.

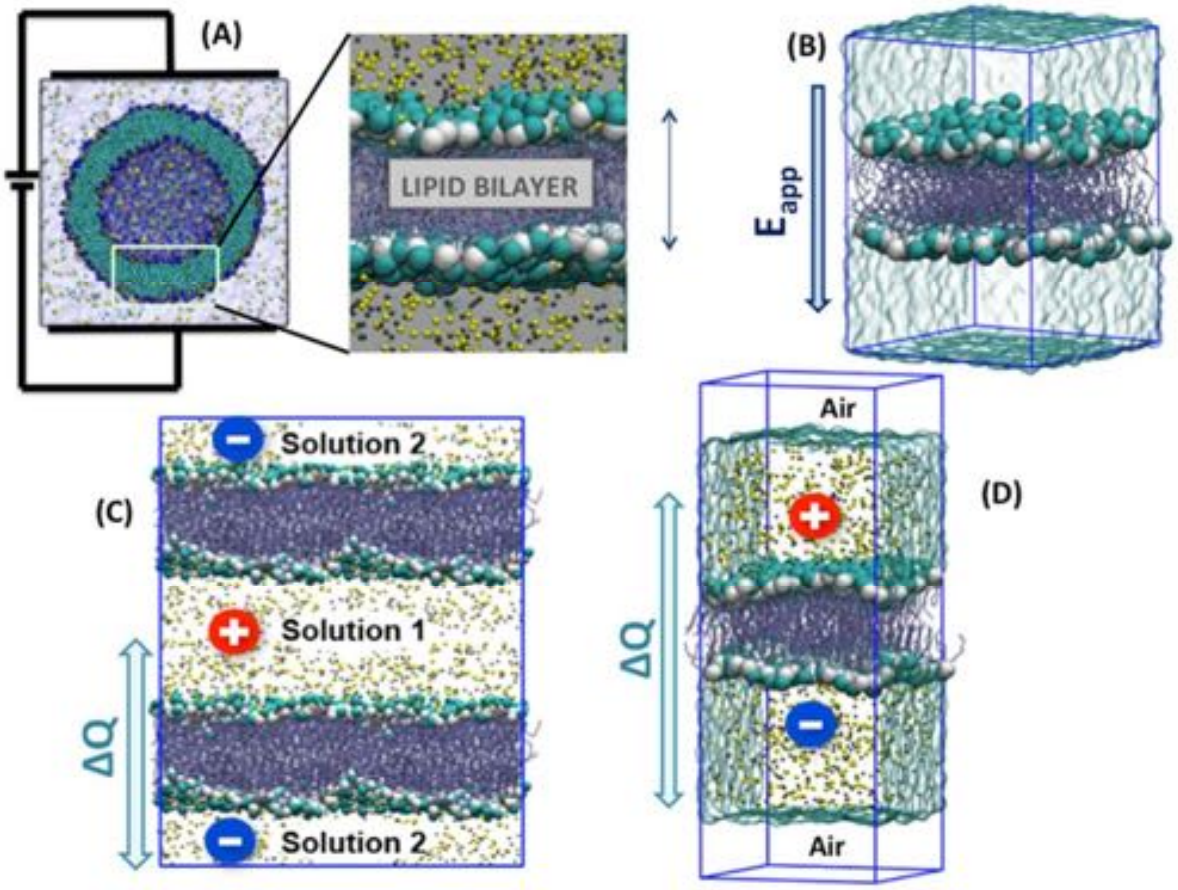

Figure. 1 Protocols for atomistic modelling of cell membranes or liposomes lipid bilayers (A) electroporation; (B) nsPEFs protocol: the system is modeled in absence of salt, and subject to an electric field E_{app} perpendicular to the bilayer (z axis). Note that in some studies ions were also considered; (C) µs-msPEFs protocol introduced in the double bilayer setup: a charge imbalance ΔQ is set across each bilayer and the scheme is implemented using classical PBCs. To prevent ions from migrating through the periodic boundary conditions, the simulation box (in blue) is extended in the direction perpendicular to the bilayer (z axis) to create a vacuum slab in the air/water interface protocol (D).

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 [59–63]. MD simulations adopting such an approach have been used to study membrane electroporation [19–23], lipid externalization [64], to activate voltage-gated K^+ channels [65] and to determine transport properties of ion channels [66–69].

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 [70]. 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 [71]. 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:

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

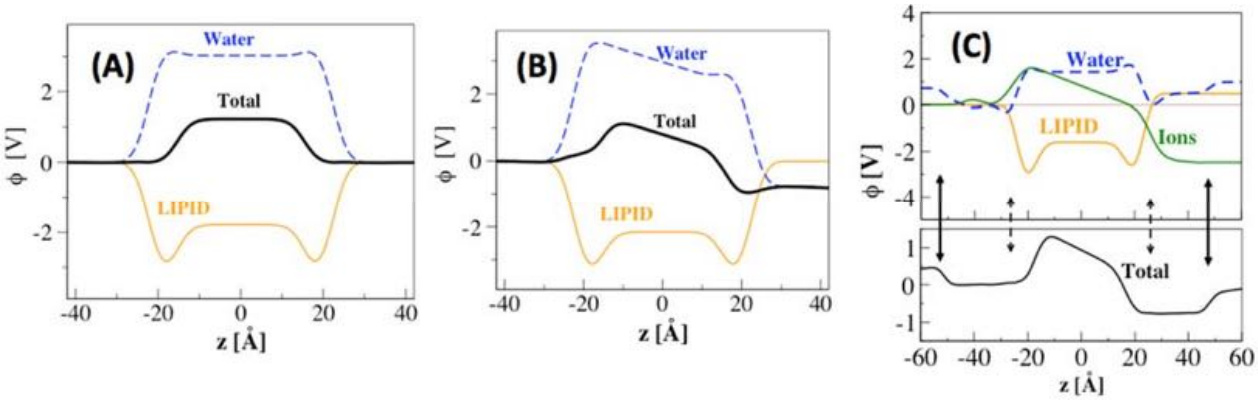

Figure. 2 Electrostatic potential profiles ϕ(z) along the membrane normal (z axis) of a POPC lipid bilayer. Bilayer (A) at rest, (B) subject to a transverse electric field (nsPEF protocol), and (C) bilayer set with a charge imbalance (μs-msPEF protocol). z=0 represents the center of the lipid bilayer. The contributions to the electrostatic profile from water (blue), lipid (yellow), ions (green) are reported next to the total one (black). The dashed arrows in panel C indicate the positions of the lipid/water interfaces and the solid arrows the position of the water/air interfaces. Note that the TM voltage U_m (potential difference between the upper and lower water baths) in the nsPEF protocol is mainly due to water dipoles reorientation, while in the μs-msPEF protocol it is mainly due to the charge (ions) distribution.

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 [21,72], and not exceeding few degrees toward the field direction at longer time scale [22]. Hence, within a very short time scale - typically few picoseconds [21] –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 hundred picoseconds) formation of water defects and water wires deep into the hydrophobic core [20]. 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 [21] 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 [22,73]. Quite interestingly, addition of salt has been shown to modulate these characteristic time scales [74].

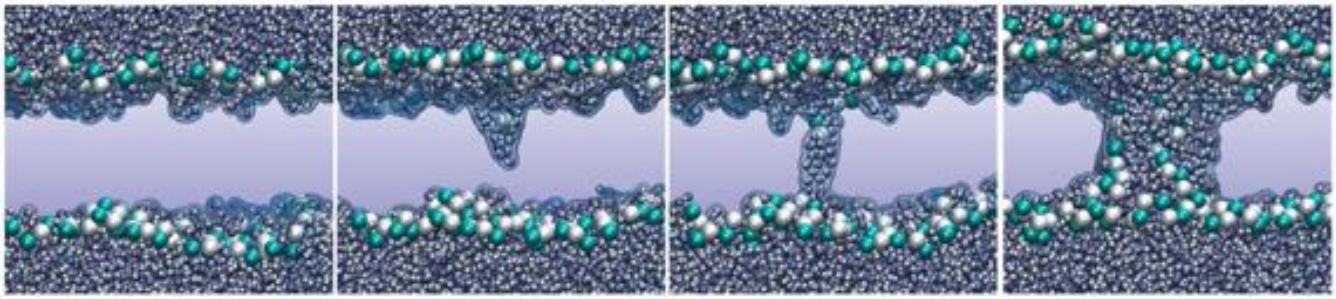

Figure. 3 Pore evolution in a POPC bilayer: The POPC headgroups are shown as cyan and white beads, the lipids tails are not show for clarity. The pore creation, in MD simulations, takes places in the range of nanoseconds.

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 [20,21]. 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 [21]. 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. [75] 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 [76]. More recently, (see sections below) it was shown that even a single 10 ns electric pulses of high enough magnitude can enhance small siRNA transport through lipid membranes [77].

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 [22][72]. Ziegler and Vernier [23] 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.

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. 1):

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 [78] (cf. Fig. 1.C). 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. [79] 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 [43]. The system is set-up as indicated in Fig. 1.D. 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 modelled finite solutions.

Fig. 2 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 electrostatic potential difference between the two electrolytes indicative of a TM potential $\boldsymbol{\Delta V}$. Quite interestingly, the profiles show clearly that, in contrast to the electric field case where the TM voltage is mainly due to the water dipole reorientation, 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 $\boldsymbol{\Delta V}$.

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 [79,80]. Simulations at various charge imbalances Q_s show a linear variation of $\boldsymbol{\Delta V}$ from which the capacitance can be estimated as $C = Q_s.\boldsymbol{\Delta V}^{-1}$. The capacitance values extracted from simulations are

expected to depend on the lipid composition (charged or not) and on the force field parameters used and as such constitute 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, 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} [78,81] 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.3). 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.

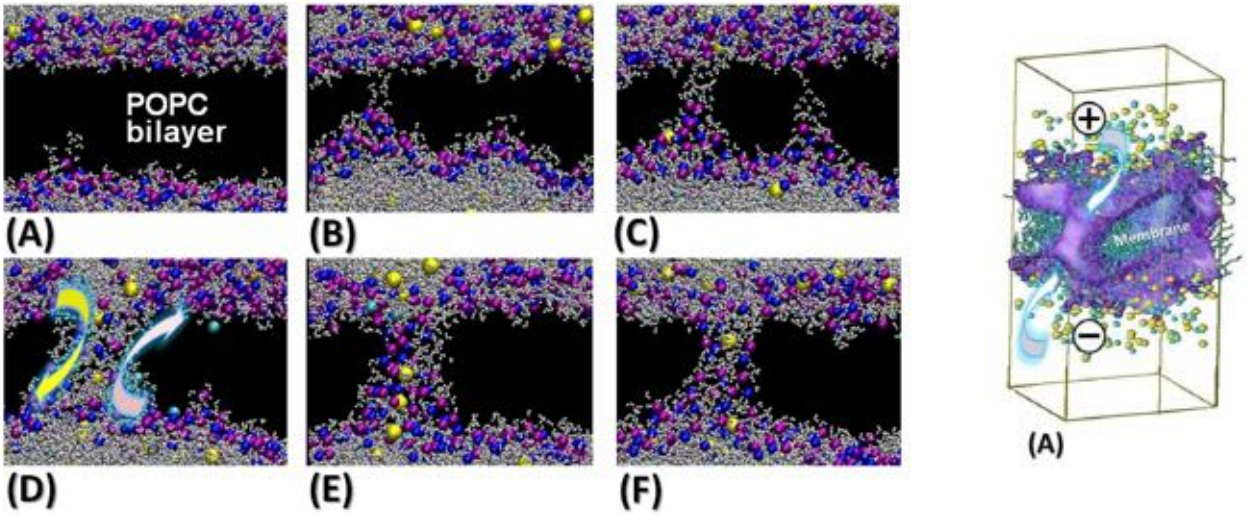

Figure. 4 *Left* Sequence of events following the application of a TM voltage to a POPC lipid bilayer using the charge imbalance method (panels A to F). Note the migration of Na+ (yellow) and Cl- (cyan) ions through the formed hydrophilic pores that are lined with lipid phosphate (magenta) and nitrogen (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 Q_s and therefore ΔV to values ≈ 200 mV. *Right* Topology of the nanometer wide hydrophilic pores formed under high transmembrane ΔV imposed by the charge imbalance method in the planar bilayer (A). The arrows highlight the subsequent ionic flow through the pores.

The MD simulations of the double bilayer system [82,83], and the results presented here for the single bilayer set-up 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 4.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 [21] 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 [84].

C- *INTERNAL ELECTRIC FIELD DISTRIBUTION AND ORIGIN OF MEMBRANES ELECTROPORAITON*

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 5.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 [85], 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. 5.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. Figs 5.B and C reveal the incidence of such reorganization on the local electric field both at the interfacial 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, where again, the TM voltages created give rise to large electric fields within the membrane core, oriented perpendicular to the bilayer.

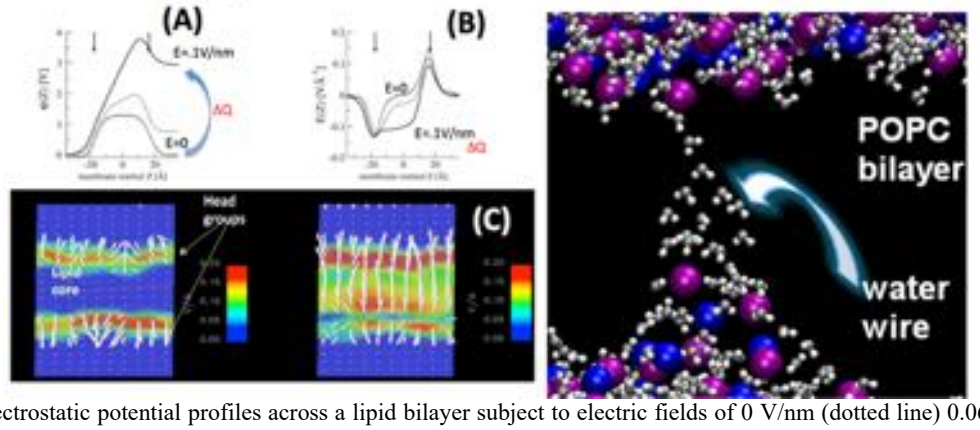

Figure. 5 (A) Electrostatic potential profiles across a lipid bilayer subject to electric fields of 0 V/nm (dotted line) 0.06 V/nm (thin line) and 0.30 V/nm (bold line), or to a charge imbalances ΔQ. (B) Corresponding electric field profiles. (C) 2d (out of plane) maps of the electric field distribution. The local electric field direction and strength are displayed as white arrows. Note that at 0mV, due to the bilayer dipole potential at rest, the larger electric fields are located at the lipid water interfaces and are oriented toward the solvent, and no electric field is present in the lipid core. When the bilayer is subject to a TM potential, a net electric field appears in the hydrocarbon region. The latter promotes dipolar orientation and penetration of water molecules (Right panel) inside the bilayer.

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 then 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. 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 [86]. 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.

Ziegler et al. [23] have shown clearly that the orientation of the lipid headgroups (dipoles) is not a determinant factor in the EP process. The general assumption that the lipid headgroups have a marginal role in the formation of the electropores, is consistent with studies on octane [20] as well as vacuum slabs [87] electroporation: These works have shown that, as in lipid bilayers, water columns can form in any water/low-dielectric/water system subject to high electric fields.

Experimental evidence shows that pores do close when the PEF is turned off. The kinetics of this process determines how long leakage from or delivery to targeted cells can last. MD simulations indicate that this process initiates with a collapse of the pore (closure) due to a rapid leakage of water outwards to the bulk, followed by a much slower reorganization that leads to lipid headgroups re-partitioning toward the external hydrophilic leaflets. Resealing kinetics is independent of the magnitude of the pore initiation

electric fields. In general, complete recovery of the original bilayer structure requires a much longer time scale [21,87,88], spanning from nanoseconds to hundreds of nanoseconds, and depends critically on the structure of the bilayer [89]. Note that addition of salt to systems undergoing the nsPEF protocol has been shown to modulate the characteristic time scales of the whole pore life cycle [88,90].

COMPLEX BILYAER MODELS: EP THRESHOLDS AND PORE FEATURES

A- *ELECTROPORATION THRESHOLDS*

Since the pioneering simulations [21,91], which considered simple lipid bilayers of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and dimyristoyl-phosphatidylcholine (DMPC), a variety of lipid bilayers have been modeled in order to understand the key elements that might modulate their electroporation thresholds. The increase of the EP threshold upon addition of cholesterol [92–94] was studied using the E field [95] and charge imbalance protocols [93]. For the former, a steady increase of the EP threshold coincides with an increase in cholesterol concentration: a two folds higher electric field was necessary for the electroporation of bilayers with the addition of 50 mol% cholesterol. Under μs-msPEFs conditions, the EP threshold was showed to level-off above 30 mol % cholesterol. Generally, the increase of the EP threshold has been linked to the increase of the stiffness of the bilayer [92,94] .

In a series of papers [96,97] Tarek's group investigated the effect on the EP threshold of ester and ether linkages, of branched (phytanoyl) tails, and of bulky (glucosyl-myo and myo inositol) lipid head groups. The authors have found that the EP threshold of a lipid bilayer depends not only on the ''electrical'' properties of the membrane, i.e. its dipole potential or membrane capacitance, but also on the nature of lipids hydrophobic tails. The authors report that there is a correlation between the lateral pressure in the water/lipid interface and the EP threshold. They suggest that an increase of the lateral pressure (in the branched lipid membrane compared with the simple lipid bilayers) hinders the local diffusion of water molecules toward the interior of the hydrophobic core, which lowers the probability of pore formation, increasing therefore the electroporation threshold.

Comparing specifically the Archeal lipids (glucosyl-myo and myo inositol head groups) to normal PC lipid, the higher electroporation thresholds for the former was attributed [96,97] to the strong hydrogen-bonding network stabilizing the head-group head group interactions. Likewise, Gurtovenko et al. [98] reported higher EP threshold for phosphatidylethanolamine (PE) lipid bilayers compared to phosphatidylcholine (PC) lipid bilayers. This effect was linked to inter-lipid hydrogen bonding taking place in the PE bilayer, which leads to a denser packed water/lipid interface and more ordered hydrocarbon lipid chains. Considering an asymmetric bilayer, composed by PC and PE lipid leaflets, the authors observed that the initial electroporation feature, i.e. the water column formation is also asymmetric, with initial steps taking place primarily at the PC leaflet. Studying more complex composition membranes, Piggot et al. [99] reported that the Gram-positive bacterial S. aureus cell membrane is less resistant to poration than the Gram-negative bacterial E.coli outer membrane (EcOM). The higher EP threshold of the EcOM was linked to the reduced mobility of the Lipopolysacharide molecules that are located in the outer leaflet. Additional factors, such as cholesterol, the presence of impurities, and other compounds, can modify the permeation properties of membrane models by acting on their stability.

B- *PORE FEATURES*

The MD results support the hypothesis that following the application of a high transmembrane voltage, the cell membrane is permeabilized by the formation of conducting hydrophilic pores stabilized by the lipid headgroups. The properties of the lipids play a determinant role in the electropores life-time and in its structural characteristics (e.g. size, shape, morphology) [87]. Other studies, considering various lipid bilayers, challenged the standard pore morphology. Tarek and coauthors pointed out that a peculiar EP process may be possible in which large long living ion-conducting water columns are not stabilized by lipid headgroups [93,97,100]. These "hydrophobic" conducting pores originate from constraints of a different nature in the lipid bilayer. The first report [100] focused on a palmitoyl-oleyl-phosphatidylserine (POPS) bilayer characterized by negatively charged headgroups. When this system was subject to a charge imbalance high enough to electroporate the bilayer, the migration of lipids along the water column turn out to be largely hindered (Fig. 5, second panel [100]). Similar conclusions were drawn for PC lipid bilayers containing more than 30 mol % cholesterol [93] or for Archaeal lipids [97] (Fig. 5). This peculiar morphology was ascribed to the repulsion of negatively charged headgroups in the first case [100], to the condensing effect of cholesterol in the second [93], and to the steric hindrance of the bulky headgroups coupled with the branched tails in the latter [97].

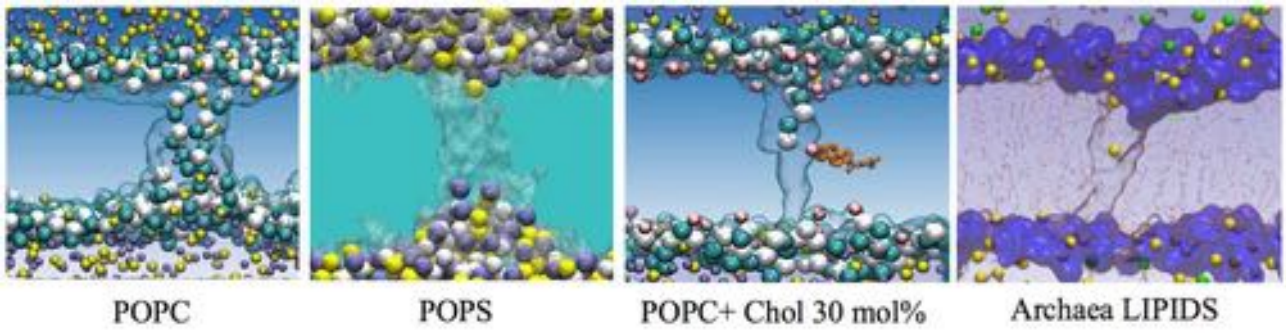

Figure 6. Various morphologies of conducting pores revealed by MD simulations. Note that beside the POPC zwitterionic lipids, pores formed in POPS, a negatively charged lipid, with addition of cholesterol, or in the complex Archaea lipids (sugar like head groups), the electropores are not stabilized by the lipid head groups.

C- *PORES STABILIZATION*

When dealing with the characteristics of electropores (e.g. size, conductance, transport of molecules) one would expect the pore to be in an energetically favorable state, i.e. one that corresponds to a stable configuration. In order to understand if the pore can be considered in a steady state for a given TM voltage and characterize its size and conductance, the two MD procedures, (introduced in previous sections) need to be improved. Indeed, the main drawback of these two protocols, as usually used, resides in the impossibility of maintaining a stable pore. In the electric field method, the pore tends to expand, leading to the breakdown of the bilayer, when it reaches the dimensions of the simulation cell box. The charge imbalance protocol, on the other hand, suffers from an important shortcoming: The imbalance is not re-set during the simulation. Thus, in the studies carried out both with the double and single bilayer schemes, the charge imbalance imposed at the beginning decreases significantly within several tens/hundreds ps (depending on the system size) of EP due to an exchange of ions through the pore. The decrease of the charge imbalance results in a TM voltage drop, which ultimately leads to pore collapse and resealing.

When using the nsPEF protocol, the lowering of the electric field intensity after pore creation was shown to result in its stabilization [22]. Using the same strategy, Fernández et al. [95] could modulate the size of the pore and showed that it depends only on the strength of the stabilizing electric field. More recently our group [101] used a scheme to maintain a constant charge imbalance, refining thereby the µs-msPEFs approach to obtain size-controlled steady pores. The protocol used is identical to the procedure proposed by Kutzner et al. [84] to study the transport in ion channels using the double layer scheme. In this procedure, named "swapping", the number of ions in the two solution baths is frequently estimated and, if the latter differs from the initial setup, a "swapping" event takes place: An ion of one solution is exchanged by a water molecule of the other solution bath (see the supplementary material for more information). Note that to overcome the limitation of simulating the bilayer in the NVT ensemble (constant volume), the swapping procedure can be coupled with the NPγT ensemble (constant surface tension) to maintain the bilayer surface tension constant (null) and mimic, therefore, experimental conditions [101].

D- *PORE CHARACTERIZATION*

A first attempt to link experimental evidence of pore conductance and radius estimation was carried out by Kramar et al. using a linear rising current technique combined with MD simulations performed under similar conditions [102]. Their findings suggest that the opening and closing of a single pore under conductance in the 100-nS scale would be possible for a pore diameter of ~5 nm.

More systematic investigations, using the nsPEF [95,103] and µs-msPEF [101] modified protocols allowed to better characterize the conductance of electropores. For simulations carried out under the two protocols and when applying TM voltages below the EP threshold, the pore formed could be stabilized to different radii for tens of ns. Quite interestingly, the pore radii, and the pore conductance were found to vary almost linearly with the applied voltage. Moreover, the pores were found to be more selective to cations than to anions [101,103,104]. This selectivity arises from the nature of the lipid molecules constituting the pore: The negatively charged phosphate groups that form the walls of the pore attract sodium ions, which hinders their passage across the bilayer, but also makes the pore interior electrostatically unfavorable for other sodium ions [105]. This, already, suggests that the transport through electropores is sensitive to the type of solutes, showing a different affinity for different charged species.

TRANSPORT OF MOLECULES

Although numerous molecules are implicated in EP and/or concerned by its applications (e.g. drugs, genetic material, dyes, …), very few have been investigated with MD simulations. Apart from few studies in which electropore-mediated flip-flop of zwitterionic PC lipids [106–108] was reported, most simulations concerned charged species for which transport involved electrophoresis [21,77,109]. In the following, we discuss the results obtained using the two simulations protocols.

A- nsPEFs

nsPEFs can induce externalization of phosphatidylserine (PS), a phospholipid usually confined to the inner leaflet of the plasma membrane that and can trigger several recognition, binding and signaling functions. MD studies of PS bilayers [19,110] showed how PS externalization is a pore-mediated event occurring exclusively with an electrophoretic drift.

A decade ago, Tarek [21] reported the first MD simulation on the transport of a short DNA double strand using high intense electric fields. It was shown that the uptake occurred only in presence of the pore by electrophoretic drift. Since then, to our knowledge, only two MD studies have been reported on the transport of molecules under nsPEFs. In 2012 Breton et al. [77] showed that a single 10 ns high-voltage electric pulse can permeabilize giant unilamellar vesicles (GUVs) and allows the delivery of a double-stranded siRNA (-42e charge, 13.89 kDa) through the formed pore, by electrophoresis (Fig. 7 [77]). Comparing experimental evidence with MD simulations they could show in particular that: (i) following the application of an electric field, the siRNA is pushed toward the lipid headgroups forming an siRNA- phospholipids headgroups complex that remains stable even when the pulse is switched off; (ii) no transport is detected for electric fields applied below the EP threshold; (iii) when the Eapp is above the EP threshold (Eth) the siRNA is electrophoretically pulled through the electropore and translocated within a 10 ns time scale; (iv) if the Eth is turned off before the complete transition, the pore collapses around the molecule which is, hence, trapped.

Recently, Salomone et al. [109] used a combination of nsPEFs and the chimeric peptides (CM18-Tat11) as efficient delivery vectors for plasmid DNA using endocytotic vesicles. To provide molecular details about the processes taking place, the authors modeled the peptide and its fragments. They reported from MD simulations that, when subject to high electric fields, Tat11, a small cationic peptide (residues 47-57 of HIV-1 Tat protein; +8e charge, 1.50 kDa) can translocate through an electroporated bilayer within few nanoseconds without interacting with the phospholipid headgroups. In contrast, the amphipathic peptide CM18, even when located near a preformed pore, remains anchored to the lipid headgroups and does not translocate during a 12 ns high electric field pulse.

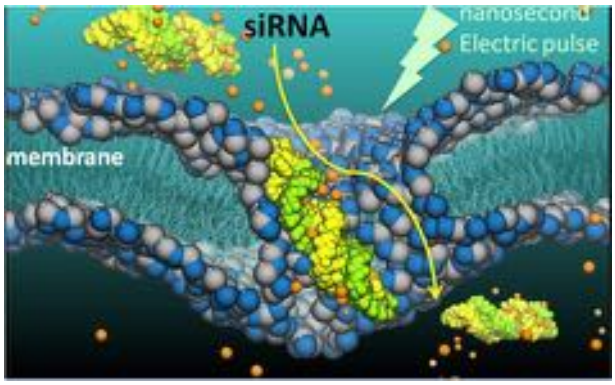

Figure 7: A single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance for the design of protocols aimed at using nanosecond-pulse siRNA electro-delivery in medical and biotechnological applications [77].

B- µs-msPEFs

We present below the latest results from MD simulations of the uptake of molecules through lipids bilayers subject to µs-msPEFs. We focus our attention on Tat11 and the siRNA double strand to compare their mechanism of transport to the one reported using the nsPEFs [77,109]. These data have been reported in [111].

Transport of siRNA

In 2011 Paganin-Gioanni et al. [76] investigated siRNA uptake by murine melanoma cells, when subject to electric pulses (1 Hz of repetition frequency) using time lapse fluorescence confocal microscopy. A direct transfer into the cell cytoplasm of the negatively charged siRNA was observed across the plasma membrane exclusively on the side facing the cathode. Noting that when added after electropulsation, the siRNA was inefficient for gene silencing because it did not penetrate the cell, the authors concluded that the siRNA transport takes place during the electric pulse and is due to electrophoresis through electropores. The same group reported also that 0.17 kV/cm - 5 ms pulses, named EGT, are more effective in terms of silencing than the more intense less lasting HV pulses (1.3 kV/cm - 0.1 ms). They showed on the other hand that a double pulse procedure, consisting of one HV followed by a long below-EP-threshold pulse does not increase the efficiency of the delivery. All together,

their evidence suggests that, for msPEFs, the key factors for an efficient delivery are the voltage above the EP threshold and the duration of the pulse.

In order to investigate the siRNA transfer into cells under conditions similar to the µs-msPEFs experiments, we have performed a set of simulations where the system was subject to several voltages (see Table 1). We first electroporate a bilayer patch by submitting it to a high charge imbalance. Once the pore was large enough (arbitrary value of ~2 nm radius) we lowered ΔQs to stabilize it to different radii as in [101]. These configurations were then used to start the simulations with siRNA placed near the pore mouth and were continued at the desired voltage.

Table 1 Pore radius R and crossing time tc estimated at specific TM voltages (Um) for the two molecules considered. The pore radius (diameter) is estimated as the minimum lipid to lipid distance along the pore lumen

System	t_s (ns)	U_m (V)	R (nm)	t_c (ns)
POPC_1024+siRNA	100	0.16 ± 0.16	2.0 ± 0.6	> 100
	35	0.55 ± 0.19	3.3 ± 0.2	32.5
POPC_1024+Tat$_{11}$	40	0.43 ± 0.16	1.6 ± 0.2	32.8
	14	0.70 ± 0.24	2.0 ± 0.1	11.3

ts – simulation time; Um – transmembrane voltage create by the charge imbalance; R – minimum pore radius maintained by a given Um (see SM); tc – crossing time of the molecule through the electropore.

For the lowest transmembrane voltages Um run, the siRNA approached the large pore (~4 nm diameter) mouth then started sliding through it while interacting with the lipid headgroups lining it. The complete translocation of the siRNA did not occur however within the first 100 ns of the run. In a completely independent run, we repeated the simulation by maintaining a higher voltage, namely 0.55 V. The siRNA approach, pore entry and sliding under these conditions (Fig. 7) were similar to the lower voltage run. However, at 0.55 V despite its anchoring to the lipid headgroups, a complete translocation from the upper to the lower water bath occurred in ~30 ns. Two factors contributed probably to this speed up. Compared to the previous conditions, not only the electrophoretic force pulling the siRNA is indeed higher, but the pore size increases too under this higher voltage.

All together the simulations mimicking µs-msPEFs experiments, demonstrate that the translocation of siRNA through the pore driven by the application of TM voltages above 0.5 V takes place in the nanosecond time scale, as reported for the nsPEFs. Noticeably, in both simulations carried out under electric field or under the charge imbalance, the siRNA remains anchored to the lower leaflet of the membrane after translocation without diffusing in the bulk solution even if the voltage is maintained.

Experiments performed on mouse melanoma cells applying ms-long pulses evidenced that tuning the duration of the pulse is essential for an efficient siRNA uptake. In fact the authors found more effective the EGT (0.17 kV/cm, 5 ms) class of pulses than the HV (1.3 kV/cm, 0.1 ms) one. No direct measurement of the TM voltage was carried out during these experiments and the authors assume that it is around 0.25 V, since it was observed that the EP threshold value is always about 0.20 mV for many different cell systems [112]. Corroborated by our findings, one can speculate that the transport of siRNA when subject to longer pulses could be facilitated by the formation of a pore population having larger diameters. This population of larger pores would allow siRNAs to flow through the pore and to access directly the cytoplasm increasing the transport efficiency.

Transport of Tat11

The translocation for Tat11 differs from the highly charged siRNA because no specific interactions between this peptide and the lipid headgroups take place during the process, resulting in a faster uptake. Under a TM voltage Um ~0.70 V, the molecule, initially parallel to the membrane and located near the pore opening, first rotates to align its dipole along the local electric field (Fig. 10, t = 0 ns), then drifts though the center of the pore with a radius of 2 nm (Fig. 10, t = 8 ns), over the same time scale reported by the nsPEFs procedure [109]. The Tat11 reaches the lower bath where it freely diffuses (Fig. 8, t = 12 ns). At lower Um (~0.43 V) Tat11 translocates in 32.8 ns (see Table 1), presumably as a consequence of a higher hindrance of the pore (the pore radius decreases to 0.4 nm) and of a reduction of the electrophoretic drift.

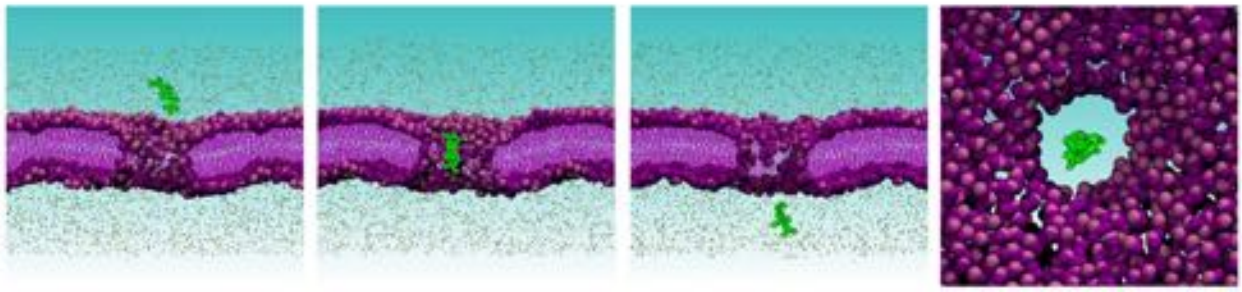

Figure. 8 The process of Tat_{11} transport in three frames corresponding to 0, 8, and 12 ns. In the right panel the top view clearly shows no interactions between the molecule and the pore walls. The POPC headgroups are shown as mauve and violet beads, the tails as purple lines; sodium and chloride ions are colored in yellow and gray; Tat_{11} is green (adapted from [111]).

Considering a patch of 256 lipids, and applying an electric field that generates a 1.6 V across the bilayer, Salomone et al. [109] reported that Tat11 translocates through an electropore within 10 ns. This seems inconsistent with our results since one should expect that under our conditions, i.e. subject to a voltage Um of ~0.43 V, the time needed for Tat11 transport would be much longer. Indeed, if one considers only the ratio of electrophoresis, translocation of Tat11 should be three times slower at the lower voltage. In addition, a second inconsistency concerns the sizes of the pores created. Indeed in [109] the pore created has a radius of ~1.7 nm, much smaller than one expected from our results: we generated a pore of radius ~1.6 nm under Um ~0.43 V (Table 1). We have recently reported size effects in simulations of lipid bilayers electroporation, and shown specifically that patches of 256 lipids are too small to study electroporation: Pores generated in MD simulations using such patches are much smaller than those generated using larger patches (1024 lipid).

Despite these discrepancies, it is very interesting to note that both when applying both an electric field and charge imbalance, the translocation of a small charged molecule such as Tat11 occurs on the tens of nanosecond time scale.

DISCUSSION AND PERSPECTIVES

A current goal in improving our understanding of 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 simulations have become extremely important to study EP in atomic detail. In the last decade, a large number of 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.

Our investigation of the electrotransfer of small charged molecules, siRNA (-42e) and Tat11 (+8e) through a cell membrane model subject to microsecond pulse electric fields (µs-msPEFs) provided a novel insight. For transmembrane voltages of few hundred millivolts we report for siRNA a complete crossing translocation from one side of the bilayer to the other within several tens of nanoseconds despite its strong anchoring with the zwitterionic phospholipids headgroups. Tat11 on the other hand, is transported (within ~10 ns) without any interaction with the pore. Interestingly, for both molecules, we found that the transport process takes place at the same time scale (nanosecond) as much shorter pulses (nsPEFs) that we previously reported. Importantly, we recall that experiments are performed on cells, while our investigation concerns lipid bilayers. In cells, one should also consider the cytoskeleton and possible interactions with molecules e.g. siRNA in its way to the cytosol, slowing down the process of translocation.

In summary, we have designed MD protocols suitable for the characterization of the transport of uncharged and charged species driven by µs-msPEFs that can help to shed light on the uptake mechanism of drugs by cell membranes. Systematic studies carried out with this protocol in presence of other relevant drugs (e.g. bleomycin) or dyes (e.g. propidium iodide, YO-PRO,…) are expected to drastically broaden our understanding of the uptake mechanism, thus providing further insights may lead to improvements in related experimental techniques and therapeutic effectiveness.

It is worth mentioning another aspect that needs to be considered as well when studying electric field effect on cells. It has been suggested over a decade ago, that membranes can be oxidized upon electroporation. Experimental evidence reports, indeed, that pulsed electric fields can increase the extent at which lipid acyl chain peroxidation occurs. In particular, it has been demonstrated that the application of external electric fields alters the phospholipid composition and properties of liposomes, vesicles and cells [113–119]. The presence of oxidized lipids within biomembranes is known to modify their physical properties and, in particular, their permeability [120–123]. We cannot

therefore exclude that uptake under PEFs experiments may be, at least partially, taking place through diffusion across oxidized/permeabilized lipid bilayers and not uniquely across electropores. Simulations along these lines should improve our characterization of the electro-transport of molecules across membranes driven by electric fields.

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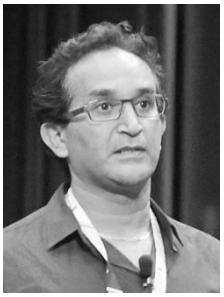

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NOTES

NOTES

Nanoscale and Multiscale Electropulsation

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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 for some applications.

Of course, some important *effects* of electropulsation 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 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.

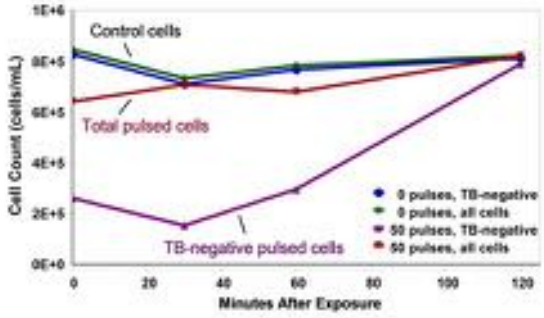

Figure 1. Nanoelectropulsed Jurkat T lymphoblasts recover over 2 hrs from initial Trypan blue permeabilization after exposure to 50, 20 ns, 4 MV/m pulses at 20 Hz.

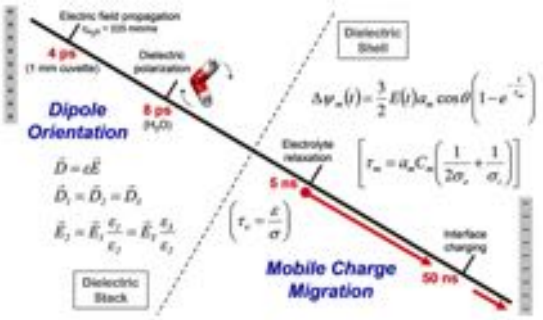

Figure 2. Timeline representing the sequence of events following electrical polarization of a biological tissue or aqueous suspension of cells. The dielectric properties of the system are important in the sub-nanosecond regime. For longer times the distribution of fields and potentials is dominated by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

NANOSECOND BIOELECTRICS

From longstanding theory that models the cell as a dielectric shell [1–4] came the notion that sub-microsecond 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, and apparently confirmed [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 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 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 (< 100 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] are negligible for the electropermeabilizing conditions that are most commonly studied (μs, kV/m pulses), 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 propidium-negative, the cell volume of Jurkat T lymphoblasts exposed to a series of 50, 20 ns, 4 MV/m pulses increases, and they become 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 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 indicates 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], an *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 (YP1) [21], a more sensitive indicator of membrane permeabilization than propidium (PPD) [22]. Additional direct evidence comes from patch clamp

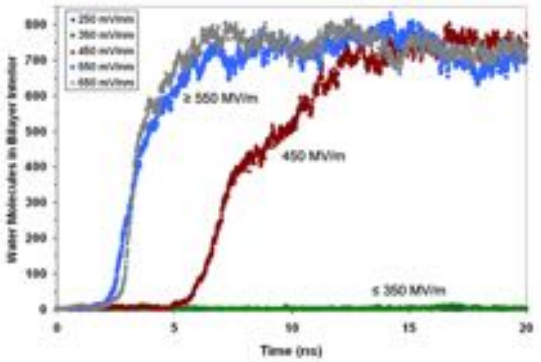

Figure 3. Electric field-driven intrusion of water into a simulated lipid bilayer.

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.

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

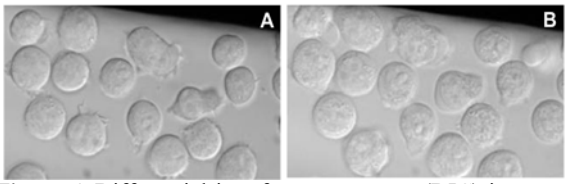

Figure 4. Differential interference contrast (DIC) images of Jurkat T lymphoblasts before (A) and 30 s after (B) exposure to 5 ns, 10 MV/m electric pulses (30 pulses, 1 kHz). Note swelling, blebbing, and intracellular granulation and vesicle expansion, results of the osmotic imbalance caused by electropermeabilization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

Experiments and molecular models of membrane permeabilization. Figure 4 shows a simple and direct response of cells to pulse 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, which also create 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

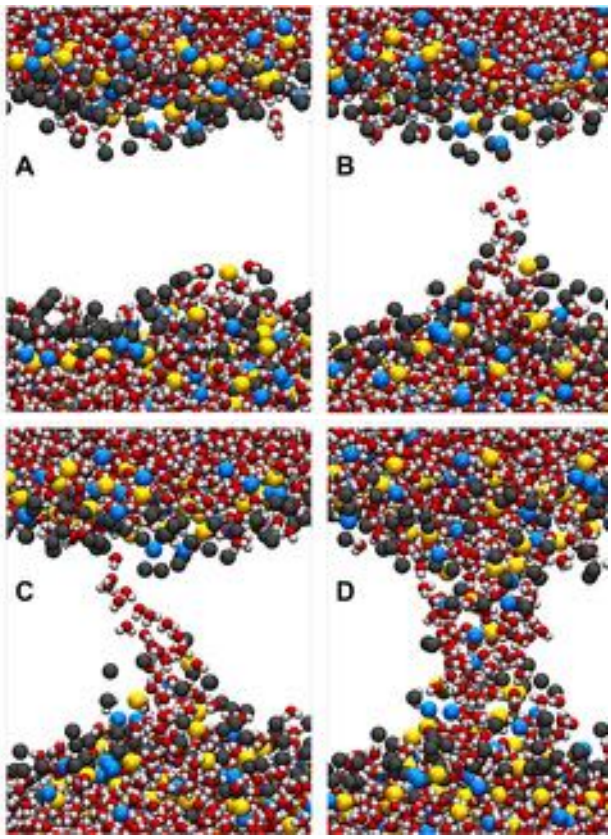

Figure 5. Electropore creation sequence. (A) Molecular dynamics representation of a POPC lipid bilayer. Small red and white spheres at the top and bottom of the panel are water oxygen and hydrogen atoms. Gold and blue spheres are head group phosphorus and nitrogen, respectively, and grey spheres are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

as a lowering of the activation energy for the

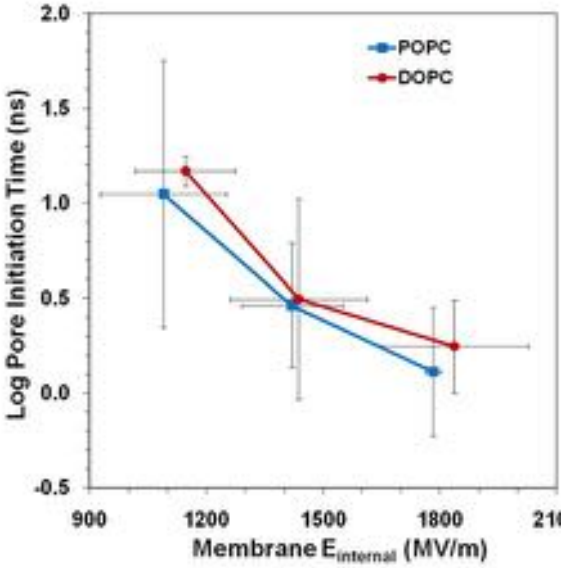

Figure 6. Electropore initiation time is a nonlinear function of the magnitude of the porating electric field. Pore initiation time (time required to form the water bridge shown in Fig. 1C) is exponentially dependent on the applied electric field, expressed here as the electric field observed in the 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].

formation of the pore-initiating structures described above. We can use simulation results like those in Fig. 6 to reconcile molecular dynamics representations with continuum models, and ultimately both of these to experiment. For example, the relation between electric field and pore creation rate is described in the Krassowska-Weaver stochastic pore model in the following expression,

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

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. We are in the process also of validating 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.

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.)

Because of the complexity of all of the structures, systems, and processes which comprise the permeabilized membrane of a living cell (the electropermeome), a comprehensive analytical understanding of permeabilization (pore?) lifetime remains a major challenge for both models and experimental approaches.

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

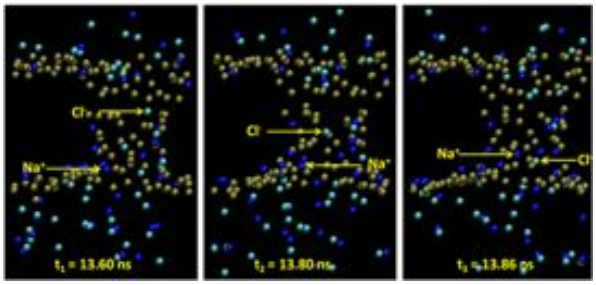

Figure 7. Sodium and chloride ions migrating through a lipid nanopore in the presence of an external electric field.

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

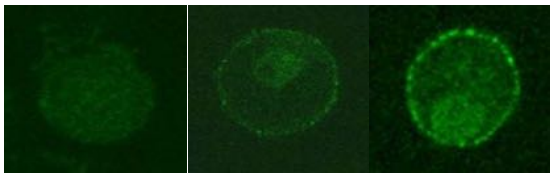

Figure 8. Immunocytochemical labeling of dopamine-β-hydroxylase (DβH) using an anti-DβH antibody coupled with a fluorescently-tagged 2° antibody. DβH is externalized by exocytotic fusion of vesicles with plasma membrane. Left panel, control. Center panel, 2 min after treatment with the pharmacological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

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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Electrochemotherapy from bench to bedside: principles, mechanisms and applications

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Abstract: Electrochemotherapy consists of administration of the chemotherapeutic drug followed by application of electric pulses to the tumour, 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 tumours. Preclinical studies elaborated on the treatment parameters, route of drug administration and proved its effectiveness on several experimental tumour models. Based on the known mechanisms of action, electrochemotherapy has been successfully tested in the clinics and is now in standard treatment of cutaneous tumours and metastases. Electrochemotherapy as a platform technology, is now being translated also into the treatment of bigger and deep seated tumours. With new electrodes and new electric pulse generators, clinical trials are on-going for treatment of liver metastases and primary tumours, of pancreas, bone metastases and soft tissue sarcomas, as well as brain metastases, tumours in in oesophagus 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, tumour drug entrapment (vascular lock), vascular-disrupting effect and involvement of the immune response. Based on all these 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].

PRECLINICAL STUDIES

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 cisplatin [3]. 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 [4-7]. 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 [4-7,8,9].

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

- The drugs can be given by different *routes of administration*, they can be injected either intravenously or intratumourally. The prerequisite is that, at the time of application of electric pulses to the tumour, a sufficient amount of drug is present in the tumour. 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 tumours. After intratumoural administration, this interval is even shorter and the application of electric pulses has to follow the administration of the drug as soon as possible (within a minute) [4-7].

- 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 [10-12].

- 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 tumours showed that amplitude over distance ratio above 1000 V/cm is needed for tumour electroporation, and that above 1500 V/cm, irreversible changes in the normal tissues adjacent to the tumour occur. 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 tumour is high enough. The minimal number of pulses used is 4; most studies use 8 electric pulses of 100 μs [4,7,11,13-15].

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 tumours, 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 tumours, whereas treatment with bleomycin or cisplatin alone or application of electric pulses alone has no or minimal antitumour effect.

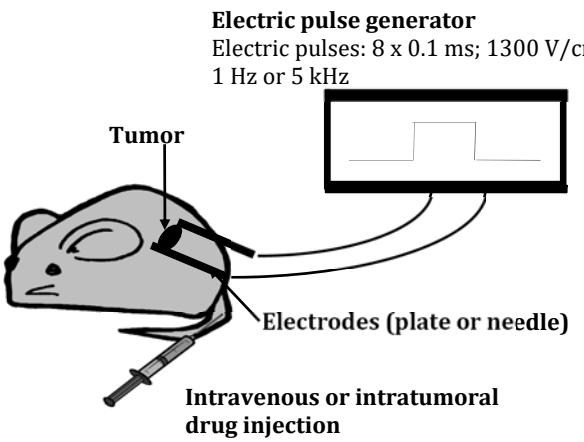

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumourally at doses which do not usually exert an antitumor effect. After an interval which allows sufficient drug accumulation in the tumors, electric pulses are 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.

Mechanisms of action

The principal mechanism of electrochemotherapy is *electroporation* of cells in the tumours, which increases the drug effectiveness by enabling the drug to reach the intracellular target. This was demonstrated in studies which measured the intratumoural drug accumulation and the amount of drug bound to DNA. Basically, the amounts of bleomycin and cisplatin in the electroporated tumours were up to 2-4 fold higher than in those without application of electric pulses [4-7]. 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* [16,17]. Restoration of the blood flow in normal tissue is much faster than that in tumours [18,19]. The vascular lock in the tumour induces *drug entrapment* in the tissue, providing more time for the drug to act.

The cytotoxic effect of electrochemotherapy is not limited only to tumour cells in the tumours. Electrochemotherapy also acts on stromal cells, including endothelial cells in the lining of tumour blood vessels, which undergo cell death [19]. Consequently, by vascular-disrupting action of electrochemotherapy, a cascade of tumour 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 [20-22]. This vascular-disrupting action of electrochemotherapy is important in clinical situations where haemorrhagic tumour nodules need to be treated [23].

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 [24]. Due to massive tumour 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, IL-12, GM-CSF and TNF-α [24-28].

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 tumours, 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.

CLINICAL STUDIES

The first clinical study was published in 1991 on head and neck tumour nodules [29], which was thereafter followed by several others [2]. These clinical studies demonstrated the antitumor effectiveness of electrochemotherapy using either bleomycin or cisplatin, given intravenously or intratumourally. 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 tumour type, drug used, route of administration and type of electrodes, was tested [30]. 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 tumour nodules, regardless of tumour histology and drug or route of administration used.
- At 150 days after treatment, the local tumour control rate for electrochemotherapy was 88% with bleomycin given intravenously, 73% with bleomycin given intratumourally and 75% with cisplatin given intratumourally, demonstrating that all three approaches were equally effective in local tumour control.
- Side effects of electrochemotherapy were minor and tolerable (muscle contractions and pain sensation).

The results of the ESOPE study confirmed previously reported results on the effectiveness of electrochemotherapy and Standard Operating Procedures (SOP) for electrochemotherapy were prepared [31].

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 tumours bigger than 3 cm in diameter can be successfully treated by electrochemotherapy in successive electro-chemotherapy sessions [32,33]. In general, electrochemotherapy provides a benefit to patients especially in quality of life [33], because electrochemotherapy is nowadays used predominantly in palliative intent [32,33].

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 [34].

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

- Effective in treatment of tumours of different histology in the cutaneous or subcutaneous tissue.
- Palliative treatment with improvement of patient's quality of life.
- Treatment of choice for tumours 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, tumour nodules can be treated by electrochemotherapy with injection of bleomycin intravenously or intratumourally and by electrochemotherapy with cisplatin given intratumourally. The choice of the chemotherapeutic drug in not based on tumour histology, but depends on the number and size of the nodules. After drug injection, the tumour nodules are exposed to electric pulses. The interval between intravenous drug injection and application of electric pulses is 8-28 min, and after intratumoural injection, as soon as possible. Different sets of electrodes are available for application; plate electrodes for smaller tumour nodules and needle electrodes for the treatment of larger (3 cm) and thicker tumour 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 [30-33].

The treatment after a single electrochemotherapy session in most cases results in complete tumour 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 [35]. 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 [36].

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 reports have already been published in treatment of colorectal liver metastases *(Figure 2)*, pancreatic tumors, and bone metastases. However, the technology can be implemented also in treatment of other localizations, such as head and neck tumors.

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.

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.

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NOTES

Electrochemotherapy in clinical practice; Lessons from development and implementation - and future perspectives

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Abstract: In just two decades electrochemotherapy has developed from an experimental treatment to standard therapy. This paper describes this development and also goes into the details of how a new technology can become implemented, to benefit patients. Electrochemotherapy is a technology that involves the use of electric pulses and chemotherapy. Thus the development of this technology has required specialists in biology, engineering and medicine to pull together, in order to achieve this accomplishment. This paper describes the development of equipment, as well as standard operating procedures, for treatment with electrochemotherapy. This chapter also deals with sharing knowledge about the use of the technology, and ensuring access for patients.

DEVELOPMENT OF ELECTROCHEMOTHERAPY

Initial studies on the organization of the cell membrane, and on deformation of this membrane by electric forces, were performed through the particularly the 1960s and 70s. In 1977 rupture of erythrocytes was described in a Nature paper [1], and another highly influential paper was Neumanns study from 1982 [2], demonstrating DNA electrotransfer which is now one of the most frequently used laboratory methods in molecular biology.

A very active field in cancer therapy in the 1970s and 80s was resistance to drug therapy, and there was great optimism that understanding resistance to therapy could ultimately lead to a cure for cancer. Different important cellular resistance systems were discovered, e.g. the multidrug transporter p-glycoprotein, that enables cancer cells to export chemotherapy [3]. In this landscape electroporation was a new technology that allowed circumvention of membrane based resistance by simply plowing a channel through cell membrane, allowing non-permeant drugs inside.

A number of studies were published about enhancement of cytotoxicity by electroporation [4,5] in vitro, and also in vivo [6], principally from Lluis Mir's group at Institut Gustave-Roussy. It was also here that, in a remarkable short time-frame, the first clinical study was reported, preliminary results in French in 1991, and the final publication in 1993 [7]. A few years later [8], the first studies from the US came out, as well as studies from Slovenia [9], and Denmark [10].

Out of a wish to create electroporation equipment for clinical use, which would be able to perform both gene therapy and electrochemotherapy, which could be adapted by the user to accommodate developments, and which was a useful instrument for the treating physician, i.e. by showing precise recordings of voltage and current along with the treatment, the Cliniporator consortium was formed. This European consortium developed and tested the Cliniporator [11,12].

A subsequent European consortium, named ESOPE (European Standard Operating Procedures for Electrochemotherapy) set out to get the Cliniporator approved for clinical use, to produce electrodes for it, to test the system in a clinical protocol, as well as to make concluding standard operating procedures.

Four groups went into the clinical study of which three had previous experience with electrochemotherapy. And the methods used differed between those three centers.

In France, a hexagonal electrode was used, with 7.9 mm between electrodes and a firing sequence allowing each of seven electrodes to be pairwise activated 8 times, a total of 96 pulses delivered at high frequency, with a voltage of 1.3 kV/cm (voltage to electrode distance ratio). Patients were sedated, bleomycin was given iv, and the procedure took place in an operating theatre [7].

In the Slovenian studies, patients were treated with cisplatin intratumorally, and with plate electrodes using 1.3 kV/cm, anesthesia not described. Pulses were administered as two trains of each four pulses [9].

In Denmark we used intratumoral bleomycin, a linear array electrode of two opposing rows of needles activated against each other using 1.2 kV/cm, 8 pulses at 1 Hz. Local anesthesia with lidocaine was used [10].

In other words, there was agreement about the overall purpose, but three different approaches. The ESOPE study [13] brought these three approaches together, and on the technical side, the three different electrodes were manufactured, and the final conclusion of the different methods and electrodes were defined in collaboration.

The standard operating procedures [14] are very detailed, allowing a newcomer to the field to immediately implement the procedure. Thus it is described how to administer the drug and pulses, how to make treatment decisions, and how to evaluate response and perform follow-up.

The standard operating procedures, together with the availability of certified equipment, marked a dramatic change in the use of electrochemotherapy. Thus when the standard operating procedures were published in 2006 only few European centers were active, and after the publication of the procedures the number of centers quickly rose and is today over 140. It would be estimated that this number will continue to grow, and also that the generators now being placed in various institutions will be increasingly used also for new indications.

IMPLEMENTATION

In an ideal world, new developments in cancer therapy become immediately available to patients. But experience shows that from the development of the technology, and the emergence of the first results, there is still quite a road to be traveled in order for the individual patient to be able to be referred, if the treatment is relevant to the particular case. First of all, equipment must be present at the individual institution, along with knowledgeable surgeons and oncologists trained to provide the treatment. The logistical set up must be in place, and this includes availability of time in the operating rooms and competent nursing support. Patients need to know that the treatment is an option. As electrochemotherapy is an option for patients suffering from different types of cancer, it requires continuous work to address specialists in the different fields. Information available on the internet can be an important resource for patients, as well as professionals.

Various countries have different approval mechanisms for new treatments, and endorsement can be a time-consuming affair. The most renowned national agency is the National Institute of Health and Care Excellence (NICE) in the UK, which has a rigorous scrutinization of new technologies and where central documents are freely available. NICE has guidances for electrochemotherapy for cutaneous metastases, and primary skin cancers respectively [15,16]. These national recommendations, as well as the integration of electrochemotherapy into specific guidelines (see e.g. [17]) are very important for the improving accessibility to treatment.

RESEARCH

A very important point is that the standard operating procedures were a very important foundation – but must be followed up with more detailed experience and further developments. Several groups have published further studies on electrochemotherapy, broadening the knowledge base and answering specific questions of clinical importance [18-26].

Furthermore, electrochemotherapy is now being developed for a number of new indications, including mucosal head and neck cancer, gastro-intestinal cancers, lung cancer (primary and secondary), gynecological cancers, sarcoma, bone metastases, as well as brain metastases. For each of these indications standard operating procedures will need to be developed, in order to allow dissemination of the treatment.

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NOTES

NOTES

Gene electrotransfer *in vivo*

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Abstract: Gene electrotransfer consists of administration of nucleic acids (DNA, RNA oligonucleotides…) followed by application of electric pulses to the specific tissue in order to enable delivery of nucleic acids into cells and consequently the therapeutic action of delivered genetic material. Due to the size of nucleic acids the electrical parameters of gene electrotransfer vary greatly depending on the tissue to be transfected and also on the desired level and duration of expression as well as accompanied tissue damage. Besides optimization of electrical parameters for specific application, design of therapeutic plasmid DNA or RNA molecules can also influence the therapeutic outcome. Initial studies on gene electrotransfer were mainly focused on the evaluation of electrical parameters for efficient gene delivery to different tissues, such as skin, muscle, liver and tumors using various reporter genes encoding fluorescent proteins, luciferase and β-galactosidase. Therapeutic field of gene electrotransfer is mainly divided into two fields: DNA vaccination and cancer gene therapy. DNA vaccination against infectious diseases and cancer on one hand and antiangiogenic and immunomodulating gene therapies against cancer on the other hand are the prevalent areas of research. Furthermore, increasing number of clinical trials, especially in USA, are registered using electroporation for delivery of therapeutic plasmid DNA. The perspectives of therapeutic gene electrotransfer for cancer therapy lie mainly in different combination with standard local therapies, such as radiation therapy or electrochemotherapy, with the aim to turn local treatments into systemic ones. In addition, a lot of preclinical work is dedicated to optimization of therapeutic plasmid DNAs, development of new electrodes and evaluation of electrical parameters, which will lead to better planning and design of clinical trials.

INTRODUCTION

The *in vitro* application of electroporation for the introduction of DNA into the cells was evaluated and tested in 1982 by Neumann et al [1], 6 years before the use of electroporation for delivery of antitumor chemotherapeutic drugs (electrochemotherapy) into the tumor cells [2]. However, *in vivo* studies only slowly followed and the first *in vivo* study was performed in 1991 by Titomirov et al [3], evaluating the usefulness of exponentially decaying pulses for delivery of genes to the mouse skin. Later on, the transfection of brain, liver, tumor and muscle using different reporter genes were successfully demonstrated using different types of electric pulses [3–7]. Due to the physicochemical properties and the size of nucleic acids compared to small chemotherapeutic drugs, the mechanism of entry of nucleic acids is different than that of small molecules. In tissues, other, tissue and cell related parameters also influence the transfection efficiency, such as cell size, shape and organization in the tissues, presence of the extracellular matrix and tissue heterogeneity (presence of different types of cells in the particular tissue). In addition, the construction of plasmid and its administration can also influence the level of transfection as well as its duration. Therefore, a vast amount of studies in the field of *in vivo* gene electrotransfer were dedicated to evaluation of different parameters of electric pulses for different tissue type as well as for different application (Figure 1). Currently, therapeutic use of gene electrotransfer is focused in mainly two fields: DNA vaccination and cancer gene therapy [8,9].

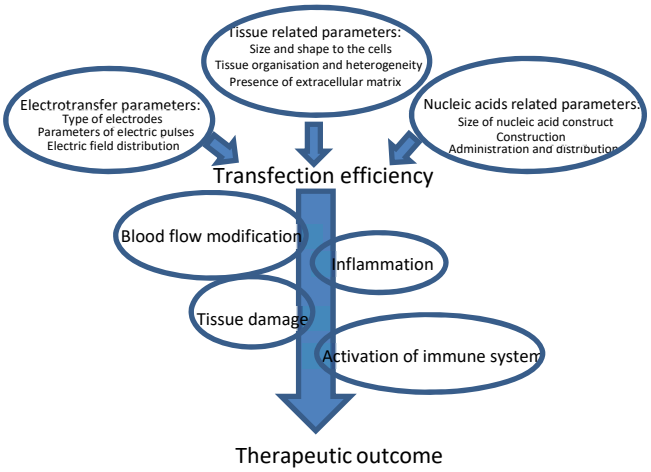

Figure 1: Different parameters can influence the transfection efficiency and therapeutic outcome of gene electrotransfer.

PRECLINICAL GENE ELECTROTRANSFER OF REPORTER GENES

Reporter genes used in preclinical studies on gene electrotransfer were mainly encoding either different fluorescent proteins or luciferase. Both enable to visualize the transfection of tissues (gene expression in cells in tissues) *in vivo* using different types of *in vivo* imaging, either whole body imaging or at the cellular level [10,11]. Most of the studies were performed in muscle and skin, as these tissues are easily accessible

and therefore represent an obvious target tissue for DNA vaccination. Besides easy accessibility for gene electrotransfer, muscle cells are long lived and they can produce relatively high quantities of therapeutic proteins that are also released into the blood stream, thus acting systemically. On the other hand, skin also represent a great target tissue, not only due to the easy accessibility, but mainly because of the numerous immune cells present in the skin that can elicit effective immune response of the organisms needed for DNA vaccination [12,13](Figure 2).

As mentioned in the introduction, numerous different parameters of electric pulses were used, either short (~100μs) high voltage (in the range of ~1000 V) electric pulses or long (up to 100 ms) low voltage (up to few 100 V) pulses were used. Moreover, even a combination of high voltage and low voltage pulses were tested and showed improved transfection in skin and muscle compared to single type of pulses used for transfection [14–16]. In tumors, the combination of pulses did not result in improved transfection [18]. In addition, the influence of orientation and polarity of the applied electric pulses were also evaluated in tumors, demonstrating that increased transfection efficiency is obtained only by changing the electrode orientation, but not pulse polarity[19].

The main type of electrodes used in the studies was either plate or needle and more recently also non-invasive multielectrode arrays [15,19,20]. Other types of electrodes that were tested for gene electrotransfer were spatula electrodes for gene delivery to muscle [22] and other types of noninvasive electrodes, such as needle free, meander and contact electrodes for skin delivery [21–24]. Selection of electrode is very important for appropriate electric field distribution in the tissue which is a prerequisite for effective gene electrotransfer[24, 25].

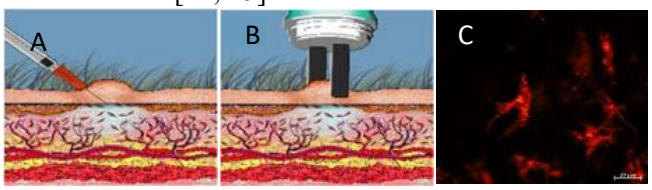

Figure 2: Gene electrotransfer to skin. **A** injection of plasmid DNA subcutaneously. A bubble on the skin will be formed. **B** If using plate electrodes, they are positioned in a way that the bubble is encompassed between the two plates. **C** Intravital confocal microscopy of cells in mouse skin expressing DsRED fluorescent protein at the depth of 30 μm.

Besides electrical parameters, the type of the nucleic acid used for electrotransfer can also affect the transfection efficiency. Namely, it was shown that smaller siRNA can more easily crossed the plasma membrane compared to larger plasmid DNA molecules, however the duration of the expression (or effect) is shorter [26–28]. Therefore, the plasmid DNA are still the most often used in gene electrotransfer studies. To improve the safety and targeting of the plasmid DNA delivery as well as to minimize the undesired tissue damage, the plasmids with tissue specific promoters, devoid of antibiotic resistance gene and with minimal or no bacterial backbone were constructed and evaluated in combination with electroporation [29–32].

Due to the size of plasmid DNA and the presence of nucleases in the blood and also tissues, the most suitable was of plasmid DNA administration is local injection. The distribution of the plasmid DNA in different tissues has different time frame, therefore it is also very important the timing between the injection of plasmid DNA and application of electric pulses. For muscle is was shown that it should be as soon as possible, while for the tumors, depending on the histological type, it can be up to 30 min after the injection of the plasmid [33–35]. Improved distribution and consequently better transfection efficiency can be achieved also by pretreatment of muscles and tumors with extracellular matrix degrading enzymes, such as hyaluronidase and collagenase [36,37].

In vitro, it was shown that size, orientation and shape of the cells influence the permeabilisation of the cell membranes and thus also transfection efficiency. The same is also valid *in vivo*. Tissues with more organized structure, such as muscle are more easy to transfect than highly heterogenic tissue, such as tumors [16]. In addition, in tumors with large cells higher transfection efficiency was obtained compared to tumors with smaller cells [38,39].

The importance of careful selection of plasmid DNA and electrical parameters for specific application, was recently reinforced by experiments showing that gene electrotransfer of plasmids devoid of therapeutic gene can induced complete regression of tumors and that cytosolic DNA sensors activating innate immune response were upregulated following gene electrotransfer [42]. The inflammation and induction of immune response was demonstrated also for muscle and skin transfection [41,42].

PRECLINICAL AND CLINICAL GENE ELECTROTRANSFER OF THERAPEUTIC GENES

The preclinical studies using therapeutic genes were mainly dedicated to evaluation of gene electrotransfer for DNA vaccination or treatment of various diseases, such as cancer, where therapies are targeted either directly to tumor cells or aim to increase the immune response of the organism against cancer cells.

In general, gene therapy can be performed using two different approaches. The first one is *ex vivo* gene

therapy, where cells, including stem cells, are removed from patient, transfected *in vitro* with the plasmid or viral vector, selected, amplified, and then reinjected back into the patient. The other approach is *in vivo* gene therapy, where exogenous DNA is delivered directly into host's target tissue e.g. locally to tumor or peritumorally and for systemic release of the therapeutic molecule into skeletal muscle depending on the type of therapeutic molecules and intent of treatment [45].

Gene electrotransfer was first used for DNA vaccination in 1996 [46]. Currently, numerous studies, using gene electrotransfer mainly to muscle and skin for DNA vaccination against infectious diseases, arthritis, multiple sclerosis, inflammation are undergoing. In addition, several clinical trials, against infectious diseases, such as HIV, hepatitis are going on. Gene electrotransfer of plasmid DNA resulted in stimulation of both arms of adaptive immune system, humoral and cellular [8,9] .

In cancer gene therapy, gene electrotransfer of therapeutic genes directly into tumors facilitates local intratumoral production of therapeutic proteins, enabling sufficient therapeutic concentration and thus therapeutic outcome. This is especially important in case of cytokines, where high systemic concentrations are associated with severe toxicity.

The first evaluation of intratumoral electrogene therapy for cancer treatment was performed 3 years after the first DNA vaccination study in 1999 in murine melanoma tumor model [47]. Since then, a variety of therapeutic genes, mostly encoding cytokines, but also tumor suppressor proteins, siRNA molecules against various targets, such as oncogenes, have been tested in a numerous animal tumor models. Overall, results of preclinical studies indicate, that intratumoral therapeutic gene electrotransfer enables efficient transgene expression with sufficient production of therapeutic proteins, which can lead to even complete tumor regression and in some cases to induction of long-term antitumor immunity in treated animals.

Some of the most significant antitumor effect to date in cancer gene therapy have been achieved with employment of active nonspecific immunotherapy, i.e. use of cytokines. Gene electrotransfer of genes, encoding different cytokines, has already shown promising results in preclinical trials on different animal tumor models. Cytokine genes, which showed the most potential for cancer therapy, are interleukin (IL)-2, IL-12, IL-18, interferon (IFN) α, and GM-CSF[47–52]. Currently, the most advanced therapy is using IL-12, which plays important role in the induction of cellular immune response through stimulation of T-lymphocyte differentiation and production of IFN-γ and activation of natural killer cells[54]. Antitumor effect of IL-12 gene electrotransfer, has already been established in various tumor models, e.g. melanoma, lymphoma, squamous cell carcinoma, urinary bladder carcinoma, mammary adenocarcinoma and hepatocellular carcinoma[53]. Results of preclinical studies show that beside regression of tumor at primary and distant sites, electrogene therapy with IL-12 also promotes induction of long-term antitumor memory and therapeutic immunity, suppresses metastatic spread and increases survival time of experimental animals[53]. On preclinical level, gene electrotransfer to tumors was also employed in suicide gene therapy of cancer, replacement of oncogenes therapies, introduction of wild type tumor suppressor genes etc [47,54–56]. Another approach in cancer gene therapy, which is currently being widely investigated, is based on inhibition of angiogenesis of tumors. The basic concept of antiangiogenic gene therapy is transfection of cells with genes, encoding inhibitors of tumor angiogenesis. Electrotransfer of plasmids encoding antiangiogenic factors (angiostatin and endostanin) was demonstrated to be effective in inhibition of tumor growth and metastatic spread of different tumors[57–59]. Recently, RNA interference approach was evaluated, using siRNA molecule against endoglin, which is a co-receptor of transforming growth factor β and is overproduced in activated endothelial and also certain tumor cells. Gene electrotransfer of either siRNA or shRNA molecules against endoglin resulted in vascular targeted effect in mammary tumors as well as antitumor and antivascular effect in melanoma tumors that are expressing high level of endoglin [60,61].

Muscle tissue is, besides in DNA vaccination, used also as a target tissues due to the possibility of high production and secretion of therapeutic proteins. Gene electrotransfer to muscle was evaluated with the aim to treat various muscle diseases, for local secretion of angiogenic or neurotrophic factors or for systemic secretion of different therapeutic proteins, such as erythropoietin, coagulation factors, cytokines, monoclonal antibodies, etc. [62–64]. In cancer gene therapy, gene electrotransfer of plasmid DNA encoding cytokines IL-12, IL-24, and antiangiogenic factors was evaluated with encouraging results.

Clinical studies on gene electrotransfer with plasmid DNA encoding cytokine IL-12 in patients with melanoma, as well as in veterinary patients show great promise for further development of this therapy[65,66]. In human clinical study, 24 patients with malignant melanoma subcutaneous metastases were treated 3 times. The response to therapy was observed in treated as well as in distant non-treated tumor nodules. In 53% of patients a systemic response was observed resulting in either stable disease or an objective response. The

major adverse side-effect was transient pain after application of electric pulses. In post-treatment biopsies, tumor necrosis and immune cell infiltration was observed. This first human clinical trial with IL-12 electrogene therapy in metastatic melanoma proved that this therapy is safe and effective[66]. In veterinary oncology, 8 dogs with mastocytoma were treated with IL-12 gene electrotransfer. A good local antitumor effect with significant reduction of treated tumors' size, ranging from 15% to 83% (mean 52%) of the initial tumor volume was obtained. Additionally, a change in the histological structure of treated nodules was seen as reduction in the number of malignant mast cells and inflammatory cell infiltration of treated tumors. Furthermore, systemic release of IL-12 and IFN-γ in treated dogs was detected, without any noticeable local or systemic side-effects[67]. Again, the data suggest that intratumoral IL-12 electrogene therapy could be used for controlling local as well as systemic disease.

For example, results of intramuscular IL-12 gene electrotransfer in canine patients indicate that it is a safe procedure, which can result in systemic shedding of hIL-12 and possibly trigger IFN-γ response in treated patients, leading to prolonged disease free period and survival of treated animals [68].

PERSPECTIVES

In oncology, local ablative treatments are very effective, however they lack a systemic component. Therefore, much effort is dedicated to development of treatments, that would act systemically or that would add a systemic component to the local treatment. With the progress of knowledge in tumor immunology, new immunomodulating therapies were developed for treatment of cancer and are currently combined with standard treatment with great success. DNA vaccination and immune gene therapies with cytokines aim to stimulated antitumor immunity and are thus good candidates to be combined with local therapies[68,69].

Several studies combining electrochemotherapy or radiotherapy with gene electrotransfer have been evaluated preclinically. The most promising immune-gene therapy that already reached clinical trials in veterinary and human oncology, is gene electrotransfer of IL-12. In the preclinical studies IL-12 gene electrotransfer was combined with electrochemotherapy and radiotherapy in different tumor models. Intramuscular gene electrotransfer of IL-12 combined with electrochemotherapy with cisplatin increased the percentage of complete regression of fibrosarcoma SA-1 tumors to 60% compared to 17% complete regression after electrochemotherapy alone [71]. When combined with radiotherapy even 100% complete response of LPB tumors was obtained [72]. Intratumoral IL-12 gene electrotransfer resulted in ~2.0 radiation dose modifying factor [73].

Clinically, only several studies were performed in client owned dogs, combining electrochemotherapy with either bleomycin or cisplatin and intratumoral or peritumoral application of IL-12 gene electrotransfer [73–76]. The results of these clinical studies are very promising and further studies, hopefully also in human oncology are foreseen.

Gene electrotransfer holds big potential for further development, which might lead to new clinical trials in both DNA vaccination and gene therapy application. Plasmid design is crucial for appropriate therapeutic protein production and effect, therefore the research is focused on codon optimization, the use of various promoters (tissue specific and inducible), the incorporation of various immunostimulatory motifs in the plasmid sequence and the use of plasmids devoid of antibiotic resistance gene, which is in compliance with Regulatory Agencies. In addition, physical factor, such as elevated temperature can also lead to improved gene electrotransfer. Furthermore, new types of electrodes, such as microneedles and non-invasive multi-electrode arrays with carefully selected parameters of electric pulses are evaluated and will lead to efficient gene electrotransfer with minimal side effects and discomfort for the patients.

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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, the dermis and the hypodermis. 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 multiple lipid bilayers. The dermis is the layer of skin beneath the epidermis that consists of connective tissue. It also contains many nerve endings hair follicles, sweat glands, lymphatic vessels and blood vessels.

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 pain 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.

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

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.

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.

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

Electroporation has been widely used to introduce DNA into various types of cells in vitro. It allows efficient delivery of DNA into cells and tissues in vivo, thereby improving the expression of therapeutic or immunogenic proteins that are encoded by plasmid DNA. Electrogene transfer 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 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.

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. 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. In particular, several groups have shown that microarray electrodes are more efficient to increase gene expression in the skin than plate electrodes.

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. Host defense peptides,

in particular LL-37, are emerging as potential therapeutics for promoting wound healing and inhibiting bacterial growth. However, effective delivery of the LL-37 peptide remains limiting. We have shown that skin-targeted electroporation of a plasmid encoding hCAP-18/LL-37 promote the healing of wounds.

Vaccination is another interesting application of electrotransfer into the skin. Intradermal electrotransfer enhanced the expression of DNA encoded antigens into the skin and both humoral and cellular immune responses have been induced. Robust antibody responses were elicited following vaccine delivery in several tested animal models. Hence, it is developed as a potential alternative for DNA vaccine delivery and is currently tested in clinical trials, inclucing for DNA cancer vaccines.

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

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Development of devices and electrodes

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Abstract: Since first reports on electroporation, numerous 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 constantly 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]–[6]. 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, 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 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 [7]. 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 [8]–[10].

THERAPEUTIC AND TECHNOLOGICAL APPLICATIONS OF ELECTROPORATION

Nowadays electroporation is widely used in various biological, medical, and biotechnological applications [11]–[16]. 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 [17]. 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.

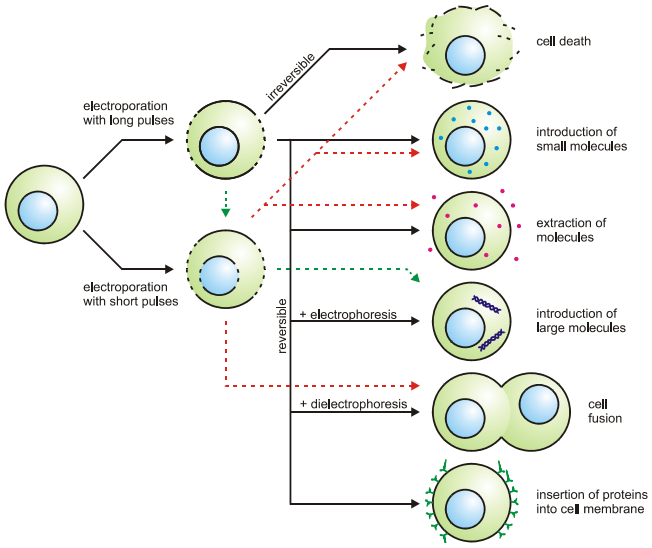

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications 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 [18]. 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 [19]. New technological developments were made available for in treating deep seated tumours, where 3000 V, 50 A and 100 µs pulses are being delivered [20]. Recent advances in treating liver metastasis, bone metastasis and soft tissue sarcoma have been reported [20]–[22].

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 [12], [23], [24]. Smilarly as in electrochemotherapy pulses of 50 or 100 µs with amplitudes up to 3000 V are used [25]. The number of pulses delivered to the target tissue is however considerably higher. If in electrochemotherapy 8 pulses are delivered, here 90 or more pulses are used. Pulse repetition frequency needs to be low 1 or 4 Hz in order to avoid excessive heating [26].

GENE ELECTROTRANSFER

Exogenous genetic material can be delivered to cells by using non-viral methods such as electropermeabilization [27]. 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 [28]. Although no consensus is reached yet, it can however be stated that longer pulses are generally used in gene transfection than in electrochemotherapy with few exceptions [29]. 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) [30]. 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 [31].

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 these 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 dielectrophoretically connecting 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 [32]. 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 [33], [34]. Electrofusion of cells of different sizes can be achieved by nanosecond pulsed electric fields [35].

ELECTROEXTRACTION

Electroporation can be used to extract substances (e.g. juice, sugar, pigments, lipid and proteins) from biological tissue or cells (e.g. fruits, sugar beets,

microalgae, wine and yeast). Electroextraction can be more energy and extraction efficient, and faster than classical extraction methods (pressure, thermal denaturation and fermentation) [36]–[40]. An economic assessment of microalgae-based bioenergy production was recently made [41]. Recommendations guidelines on the key information to be reported in biotechnological studies because of variability in results obtained in different laboratories [42].

ELECTRO- PASTEURIZATION AND STERILIZATION

Irreversible electroporation can be used in applications where destruction of microorganisms is required, i.e. food processing and water treatment [43]. 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 considered to be superior as it allows treatment of large volumes. Such mode of operation requires constant operation requiring higher output power of pulse generators [13], [44].

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 [45]. 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 [46] with models of electric field distribution [47]–[51]. 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 [52], [53].

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 the local electric field to which the cell is exposed [45]. 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 [54]–[58]. 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 represents a considerable 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 [59]–[61]. 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 [62], which further leads to increased sample conductivity [63].

ELECTRIC PULSES

For better understanding and critical reading of various reports on electroporation phenomenon and electroporation based applications, complete disclosure of pulse parameters needs to be given. Electric pulses are never "square" or "rectangular", but they are characterized by their rise time, duration/width, fall time, pulse repetition frequency. Rise time and fall time are determined as time needed to rise from 10% to 90% of the amplitude, drop from 90% to 10% of amplitude, respectively. Pulse width is most often defined as time between 50% amplitude on the rise and 50% amplitude on the fall. Pulse repetition frequency is the inverse of the sum of pulse width and pause between two consecutive pulses. These may seem trivial when discussing pulses of 1 ms, but become an issue when discussing ns or even ps pulses [64]. The cell membrane damage and uptake of ions is significantly reduced when using bipolar ns pulses instead of monopolar [65]. Shapes other than "rectangular" have been investigated with respect to electroporation efficiency [66]. 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 part of the pulse i.e. the peak 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 [30].

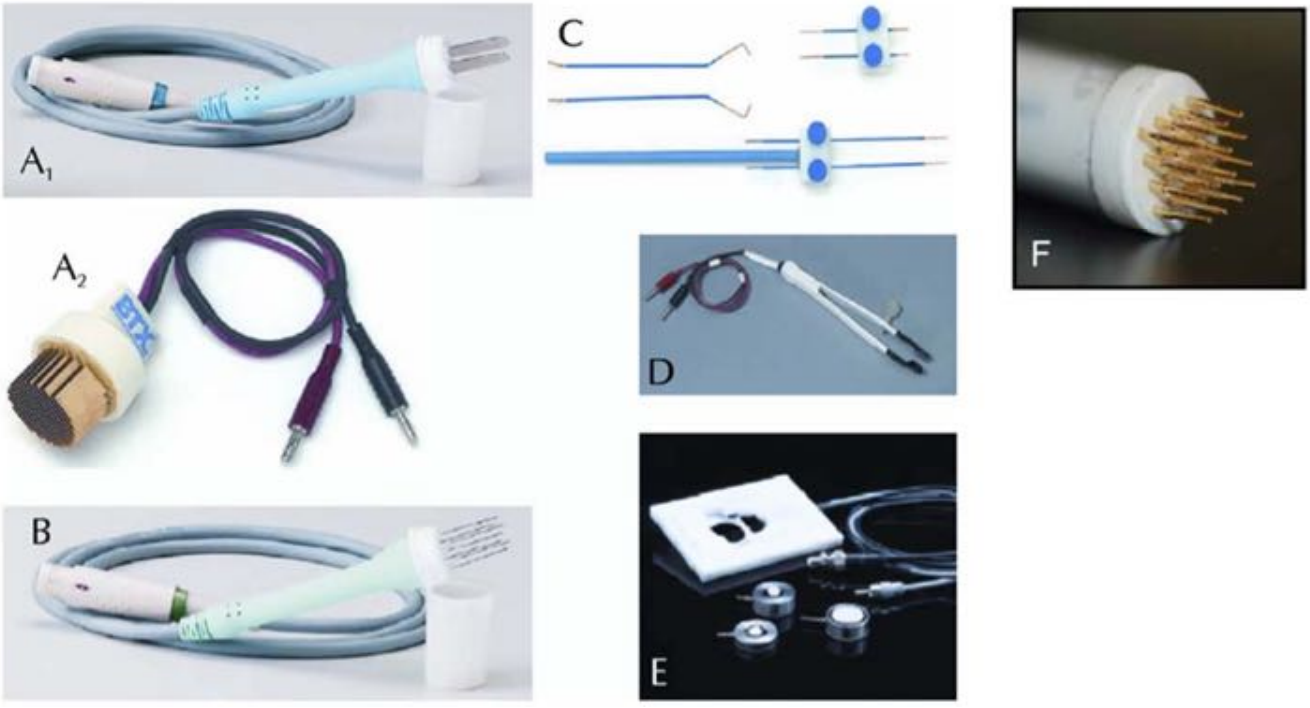

Figure 2: Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: A_1 and A_2 –parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes, E – coaxial electrodes and F – multiple electrodes array. Electrodes A_1 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 [29].

ELECTROPORATORS – THE NECESSARY PULSE GENERATORS

Electroporator is an electronic device that generates signals, usually square wave or exponentially decaying pulses, required for electroporation [1]. Parameters of the signal delivered to electrodes with the treated sample vary from application to application. Therefore, in investigating of electroporation phenomenon and development of electroporation based technologies and 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 [67].

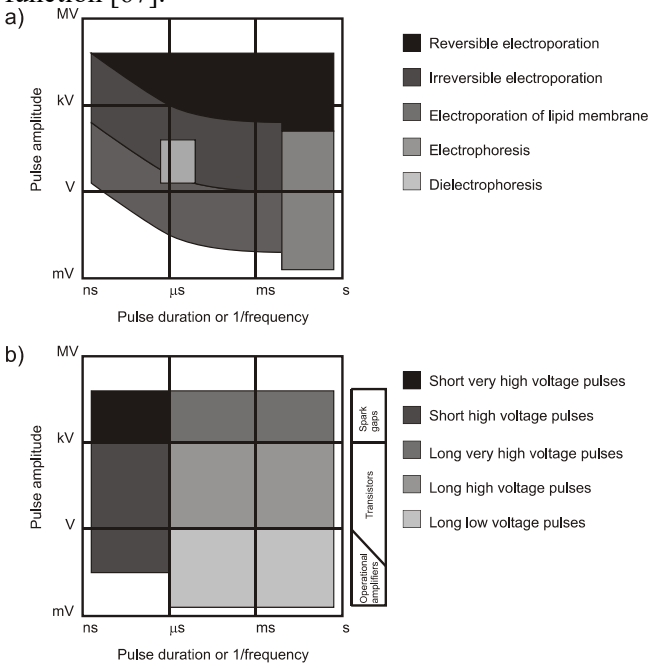

Figure 3: Areas of amplitude and duration of electrical pulses which are used in the research of electroporation and related effects (a). Five different areas of electroporation pulse generation (b). To amplify or to generate very-high-voltage electroporation pulses (over a few kV) spark gaps and similar 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].

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 [68], updated in 2010 [3] and in 2017 [6].

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

In nanosecond applications rise time of the pulse is sometimes shorter than the electrical length (the time in which an electrical signal travels through the line) between the source and the load. In this case, the impedance of the load and the transmission line has to match the impedance of the generator, so that there are no strong pulse reflections and consequently pulse prolongations.

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, reduce electrolyte wear and electrolytic contamination of the sample, 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 [54], [56], [69]. Short bipolar pulses were also investigated as they mitigate nerve/muscle stimulation [70]–[72], but these pulses may already fall into vicinity of "cancellation effect" [72].

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... [7].

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 [1]. Cliniporator (IGEA, s.r.l. Carpi, Italy) was certified in EU (CE mark) as a medical device and is offered on the market along with standard operating procedures for electrochemotherapy of cutaneous and subcutaneous tumors. NanoKnife (AngioDynamics, Queensbury, USA) was certified in EU and approved by the FDA for surgical ablation of soft tissue, including cardiac and smooth muscle. Some electroporators are now available under the license for clinical evaluation 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 and electropermeabilisation - pieces of puzzle put together

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Until now, two main generic approaches have been used to detect the cell permeabilization after the application of electric pulses to cells or tissues. They are based either on the detection of electrical changes of the tissue/cells (bioimpedance measurements, or simply conductance determinations) or on molecular exchanges across the membrane (diffusion or electrotransfer of markers, like fluorescent small molecules, radioactive compounds, plasmids coding for reporter genes, etc.). The second approach, based on the transport of a given molecular species, is very depending on the physic-chemical characteristics of the marker used (molecular weight, net charge, fluorescence yield, merker-target interactions (if any), mode of transport [1], …)

The models built to describe the phenomena occurring at the cell membrane (even at artificial membranes, whether these artificial membranes were planar membranes or membranes of vesicles of different sizes and compositions) have been mainly based on the physical principles that could explain the transport of molecules across the membrane. The input of the bioimpedance measurements, while very useful in practical terms, has brought a limited contribution to the understanding of these phenomena. However, in the transport phenomena there are parameters not related to the structural features of the membrane before, during and after the pulses. Indeed, as already mentioned, there is an impact of the size of the molecules, their charge, the gradient of concentration between the inside and the outside, the sensitivity of their detection inside the cells, etc. There are a number of examples, whatever the duration of the pulses, nanosecond pulses or microseconds pulses, that can be reported. In this context, it is important to highlight that penetration of Calcium ions can be detected at electric field amplitudes for which many other electropermeabilization markers do not yet reveal the electropermeabilization of the cells. This allows manipulating cytosolic calcium content in conditions where cell survival is fairly well protected [2,3].

Several new techniques have been recently applied to explore the changes in the membrane itself, independently of any transport phenomenon. Some of these techniques come from technologies that were not previously used to analyse the effects of the electric pulses on the lipid bilayers or the cell membranes.

On the one hand, the use of Giant Unilamellar Vesicles (composed of a defined lipid species and having the size of an animal cell) has allowed analysing chemical changes occurring in the lipid bilayers during the delivery of the pulses [4]; molecular dynamics has started to bring the explanations for these reactions to occur. It is important to note that these two approaches (experimental and in silico) restrain their analysis to the lipid part of the complex cell membranes.

On the other hand, using cells in culture, non linear optical methods are producing new elements of the puzzle. Spontaneous Raman microspectroscopy has brought new information about modifications of proteins that could occur during (or, maybe, after) the delivery of the electric pulses [5]. Confocal Raman microscopes has brought spatial as well as dynamical information on the changes in the Raman spectra that reflects these changes in the proteins [6].

Because biological objects are immersed in water-based media, Confocal Raman microscopes must be used to eliminate the non-resonant Raman contribution of the water. Coherent Raman microspectroscopy, like the Coherent AntiStockes Raman Scattering microspectroscopy, seems more attractive because of the enhancement of the signal caused by the "coherence" provided by the use of two lasers accordingly tuned. Enhancement of the signal with respect to spontaneous Raman signal can reach 10^8 times. Coherent AntiStockes Raman Scattering microspectroscopy has recently provided us with information on changes in the interfacial water (the few layers of water molecules organized at the surface of the membranes) and even of the interstitial water. After the pulses delivery, an important loss of the interfacial water signal has been recorded, which means that the alterations of the membrane structure consecutive to the pulses application also affects the water surrounding the membrane (to be submitted). We are thus acquiring information on the changes occurring in the membranes independently of any transport phenomenon. This information has now to be introduced into the models that tentatively describe the phenomena occurring at the membranes, to continue improving the knowledge of the electroporation/electropermeabilization of cells as well as of even much smaller biological objects [7].

However, there is another level of perturbations that has also to be taken into account, for which information is rapidly accumulating: the cell reactions to the stress caused by the electric pulses delivery. It corresponds to the ensemble of the biological aspects linked to the electric pulses delivery, with kinetics that can be orders of magnitude longer than the duration of the electric pulses and even of the duration of the recovery of the cells impermeability to classical electropermeabilization markers.

The construction of any new model is therefore becoming incredibly complex. This just reflects the complexity of the phenomena that have been presented in the Electroporation-Based Technologies and Treatments school. The viscous, elastic and viscoelastic models of membranes electrical breakdown are far behind us. The models describing the generation of stable pores are also insufficient nowadays. Models including several terms to explain the evolution of the permeability and the conductivity of the cell membranes are arising [8]. It is a hope that they will be able to give clues about the many questions that are still unsolved. For example, considering the "irreversible electroporation", it is still unknown what the "irreversible" event is …

All the aspects developed here above will be discussed in the frame of a new model of the phenomena occurring in the membranes of the cells exposed to the electric pulses. This model will be presented, and terminology will be delivered for a correct use of terms that have been used indistinctly until now. Therefore a distinction between "electroporation" and "electropermeabilization" will be brought in the context of the cells "electropulsation", as parts of a puzzle that collectively we want to put together.

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Lluis M. Mir was one of the pioneers of the research of electropermea-bilization (electroporation) and the applications of this technique for antitumor electrochemotherapy and DNA electrotransfer. He is the author of 193 articles in peer-reviewed journals, 21 chapters in books, and over 500 presentations at national and international meetings, invited lectures at international meetings and seminars. He received the Award for the medical applications of electricity of the Institut Electricité Santé in 1994, the Annual Award of Cancerology of the Ligue contre le Cancer (committee Val-de-Marne) in 1996, the Award of the Research of Rhône-Poulenc-Rorer in 1998, the medal of the CNFRS under the auspices of the French Sciences Academy in 2012, the Frank Reidy Award in Bioelectrics in 2015 and the Balthazar van der Pol Gold Medal of the International Union of Radio Sciences in 2017. He is an Honorary Senator of the University of Ljubljana (2004). He is also fellow of the American Institute of Biological and Medical Engineering. He has been visiting professor of the Universities of Berkeley (USA), Bielefeld (Germany) and Jerusalem (Israel). He is the director of the laboratory of Vectorology (UMR 8203 of CNRS, Universiy Paris-Sud and Institut Gustave-Roussy), and he is also the founder and co-director of the European Associated Laboratory on Electroporation in Biology and Medicine of the CNRS, the Universities of Ljubljana, Primorska, Toulouse and Limoges, the Institute of Oncology Ljubljana and the Institut Gustave-Roussy.

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INVITED LECTURERS

Non Thermal Plasmas for Biomedical Applications: Studies, Issues and Challenges

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Abstract: The non-equilibrium low temperature atmospheric pressure plasma (LTAPP) created by electric discharge have characteristics which make them particularly attractive for the development of new applications in the medical field and particularly for new therapeutic applications. This area, now called "Plasma Medicine" has seen dramatic developments in recent years, particularly since 2005-2007. It has been shown, in particular, that many applications can be approached concerning blood coagulation, prevention of the development of bacteria on operated organs, dentistry, dermatology, care on persistent wounds or ulcers, regenerative medicine or treatment of tumors, to name but a few examples. The enthusiasm aroused by research concerning the biomedical applications of cold plasmas is such that now specialized sessions are organized in all major plasma symposia or conferences. If the processes involved are far from being understood, the first clinical applications are emerging. The areas where progress have been most dramatic are certainly the treatment of chronic wounds (wounds or ulcers) for which clinical trials on a large number of patients have already been carried out, and cancer treatment with the first case study recently published. After discussing the sources and dedicated reactors, we will present some important recent developments, notably on the antitumor effect and then present the main issues and challenges.

WHY LTAPP, WHY NOW

Plasmas are natural sources of active species. Globally neutral, these ionized gases contain high electron and ion concentrations together with excited species and emit light usually in a broad wavelength domain. In specific conditions, they can also generate intense electric field in the surrounding. In air, in addition to the charged species, they can easily lead to the production of molecules or radicals, reactive oxygen species and reactive nitrogen species (RONS) whose role has already been evidenced in established therapies [1]. Whether the plasma is produced directly in the air or in a rare gas opening into the ambient humid air, it leads to transfers of energy to nitrogen and oxygen molecules, and thus produces OH or NO radicals, which can then lead to the production of heavier RONS. These radicals are known, for the first, as one of the precursors of the chain of processes reinforcing the oxidative stress within living cells and tissues [2], or favorising angiogenesis [3] or tissue repair for the latter [4]. It should be emphasized from the outset that the plasmas will lead to a locoregional and not a systemic treatment as is the case of absorption of drugs and will not generally lead to the production of products or by-products to be eliminated by the body or to be reprocessed.

All plasma types, of course, will not be suitable for use for therapeutic purposes. The two main parameters are directly fixed by the living nature of the target: operating at atmospheric pressure and at temperature below 42°C (ambient temperature if possible). Thermal plasmas, much warmer (a few hundred to a few thousand degrees) are already present in operating rooms. These are "argon knives" or electrocoagulators. These radio frequency (RF) plasma minitorches, generally using argon or argon-helium mixtures at flow rates of a few liters per minute, are used in surgery for their thermal characteristics. They allow rapid coagulation when cutting tissues or organs, thus avoiding significant bleeding. These instruments allow precise cutting and shallow necrosis, but cannot be used for therapeutic purpose where healthy tissues should be affected to a minimum.

Cold plasmas, mostly at low pressure, have been used for more than thirty years in the context of the sterilization and decontamination of fragile objects. They have long demonstrated their bactericidal and anti-microbial power of great interest to the healthcare sector. One may wonder why research into medical applications has begun relatively recently. Three factors have undoubtedly contributed to this. First of all, plasma scientists, specialists in cold plasma at atmospheric pressure, had for many years more focused on the production of radiation sources, in particular laser, and on the transformation of gaseous effluents for depollution purposes. Their concerns were therefore quite far from the world of biology, but the field of plasma medicine involves a particularly close collaboration between plasma scientists, biologists and doctors that it is not easy to initiate. Second, electronics in general and electronics for shaping and controlling high-voltage pulses in particular have undergone considerable progress simplifying the generation of atmospheric plasmas and allowing the realization of

particularly compact and transportable systems. Finally, the incentive and then the enthusiasm for multidisciplinary research, particularly those related to biology, have helped to create favorable conditions for bringing communities together [5].
There are now laboratories and institutes dealing with "Plasma Medicine" all over the world (e.g. among the mains ones: INP Greifswald, Germany; Nyheim Plasma Institute at Drexel University, Philadelphia, USA; Plasma Bioscience Research Center, Kwangwoon University, South Korea; Old Dominon University, FRRC for Bioelectrics and PEMI, Norfolk, USA; IIFS, Nagoya University, Japan; GREMI/CBM, CNRS/University of Orleans, France).

SUITABLE REACTORS

Alongside research directly related to therapeutic applications, there is a considerable effort to develop new discharge systems to achieve appropriate LTAPP [6]. The reactors making it possible to produce the appropriate plasmas (see fig. 1) are essentially of two main types:
1) Dielectric Barrier Discharges (DBD) (fig. 1a) where one of the electrodes is covered with a dielectric layer avoiding any arcing [7],[8]. These discharges at atmospheric pressure generally lead to the production of a large number of small plasma filaments randomly distributed on the surface of the electrode. The plasmagen medium (gas mixture where the plasma develops) is directly the ambient air. The reactor must be close to the surface to be treated (fraction of mm to a few mm) and the target acts as the second electrode. The term Floating Electrode DBD, or FE DBD, is generally used. They are mostly powered by high-frequency (from a few kHz to a few tens of kHz) AC power supplies at voltages of a few kV. This type of reactor is particularly suitable for surface treatments.
2) Plasma jets, themselves divided into two broad categories, RF and microwave plasmas [9], [10], [11]- see fig. 1b - and plasmas created by annular DBD [12],[13],[14]- see fig. 1c. Applied voltages range from kV to tens of kV. Initiated in rare gases, essentially helium and argon, they emerge into the air in the form of a plasma plume whose active species and photons transfer their energy to the air constituents located around. The used gas flow rates range usually in the domain from 0.1 to 10 liters per minute. The flow has a strong influence on the length and behavior of the plume and the production of the active species. The plasma itself is composed of a bright ionization front followed by a less emitting plasma column linking the latter to the discharge electrode zone. The plasma column together with the ionization front form a Pulsed Atmospheric pressure Plasma Stream (PAPS) which front is travelling at high velocity in the range from 10^6 to 10^8 cm/s, depending on discharge conditions [15]. In the case of RF devices where the plasma exit temperature (70-80° C) is considerably higher than the required processing temperature, the reactor must be kept at a sufficient distance from the target, in the order of cm. The plasma jets, which can be used over large parameter domains (gas flow, gas mixture, applied voltage, frequency, discharge cycle), are, compare to DBDs, more flexible and are suitable for precise or robotic applications.

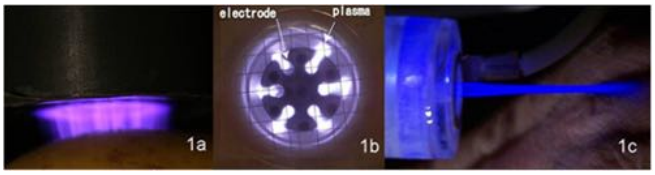

Figure 1: Plasma sources at atmospheric pressure for therapeutic applications - 1a) FE DBD (GREMI, Orléans, France) air, gap 4mm, 20kV, 1kHz; 1) MicroPlaSter (AD TEC, UK / MPI EP, Garching, Germany) argon at 2.2 liters per minute, 2.3 GHz, 100 W; 1c) annular DBD plasma jet (LPEI, Norfolk, USA) He, 3.5 l / min, 5kV, 5kHz.

It is important to note that with most types of used discharge among those mentioned above, the plasma reactor is always in the vicinity of the target (tissue, organ), which can cause problems of manipulation or access. To overcome this problem, GREMI has developed a new category of reactors, called Plasma Gun [13], which allow to generate plasma plume at a very long distance (from a few tens of cm to more than few meters depending on discharge parameters) in dielectric tubes or capillaries whose diameter varies from a few tens of micrometers to a cm particularly suitable for the treatment of targets that are difficult to access or that can be treated endoscopically (fig.2).

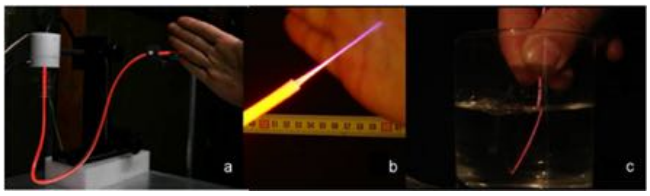

Figure 2 : a) Plasma Gun (Ne, 400 cm^3/mn, 1kHz) ; b) Plasma Gun Plume (Ne, 1.5 l/mn, 10Hz); c) Plasma Gun Plume ending in water at the output of a very thin capillary of 200 µm inner diameter (Ne, 50 cm^3/mn, 50 Hz)

CLINICAL TRIALS

Treatment of chronic wounds and ulcers is the most advanced therapeutic plasma application [1],[16],[17], for which clinical trials have been conducted in Germany on several hundred patients having infected ulcers, mainly on the lower part of the leg. The results

are particularly encouraging - see fig. 3 and 4 - and show the interest of the research carried out in this field. There are now four centers in Germany conducting clinical trials in this domain located in Munich, Rostock, Greifswald and Berlin.

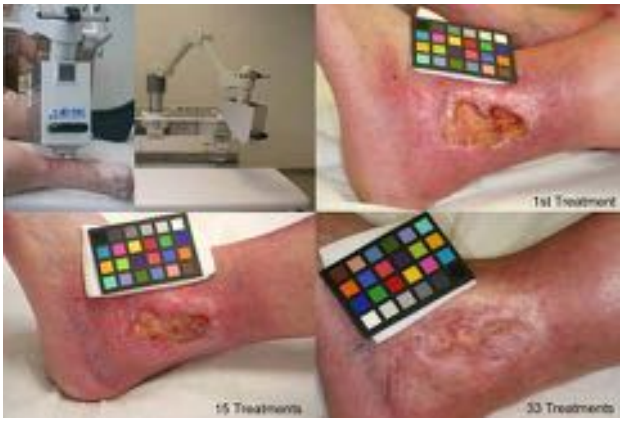

Figure 3: Ulcer Treatment with an argon plasma: treatment device (MicroPlaSter, 2.3 GHz, 100 W, 2.2 l / min, 2 min exposure daily) and evolution of the ulcer after 1, 15 and 33 treatments. Data from the Schwabing Hospital in Munich / MPI EP Garching, Germany, courtesy of G. Isbary et al.

The treatments perform at the Schwabing Hospital in Munich [4] (fig. 3) are carried out with a device, MicroPlaster, developed in partnership between the MPI EP in Garching and an Adtec company in the United Kingdom. The argon plasma of the Microplaster (argon at 2.2 liters / min, 2.3GHz, 50-100V, 100W), was applied in addition to routine care for two minutes

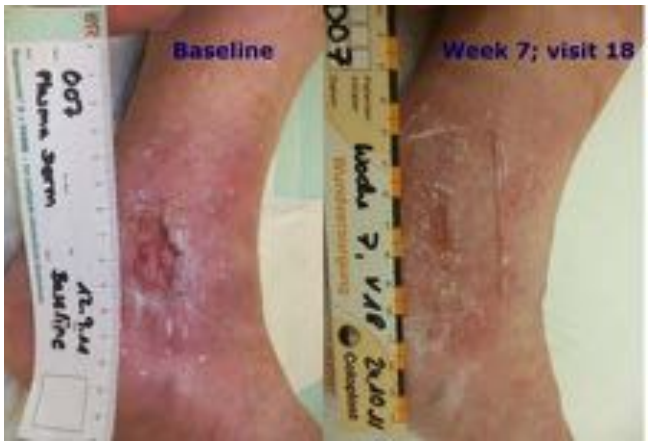

Figure 4: Ulcer Treatment with a PlasmaDerm from CINOGY. Data from University Medical Center Göttingen, Germany, courtesy of S. Emmert et al.

every day until healing or sufficient improvement, wound de-infection and tissue reconstruction. In order to work under perfectly reproducible conditions and insofar as the output temperature of the plasma is slightly too high, the distance to the treated tissue is controlled by means of an ultrasound device.It is important to note that the treatments do not cause any side effects or particular pain. Ulcer treatment is also performed using RF jet [18] or a direct DBD discharge [19]. This latter has been developed by a group in Göettingen, which recently move to Rostock, using a device called PlasmaDerm, from Cinogy. Results are also very encouraging as can been seen on fig.4.

ANTITUMOR EFFECT OF COLD PLASMAS

The use of LTAPPs in the treatment of certain types of cancer also appears to be extremely promising. Starting in 2005, plasma effect on cancer cells *in vitro* has been demonstrated on almost any tested cancer cell lines using a large variety of plasma devices based both on DBDs or plasma jets. Results can be found in reviews such as [20], [21]. The first demonstration of an anti-tumor effect in vivo has been done by the Orléans group in 2009 [8], on mice carrying human brain tumors (Glioblastoma) with short-term effects (regression of tumor mass) and long-term effects (60% increase in life expectancy of mice). It should be noted that this type of tumor is known to be particularly chemo- and radioresistant. This work was the first in a long series of *in vivo* experiments that led to particularly important results on the plasma/chemotherapy association on pancreatic cancer and recently ended on the first case study on head and neck carcinoma [22] realized by the Greifswald group (University of Greisfwald and INP) using a kINPen Med [18] working with argon (AC at 1MHz pulsed at 2.5 kHz) (fig.5).

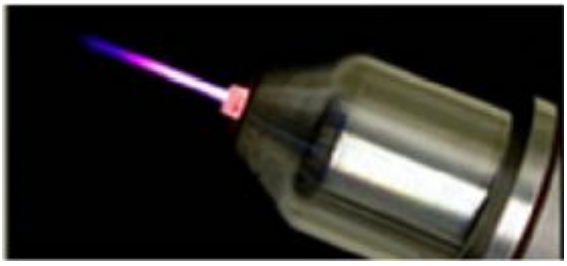

Figure 5: kINPen Med plasma jet from INP Greifswald

The first series of experiments were carried out using a FE DBDs by treating the tumors grafted on the upper thigh through the skin of the nude mice. It was first shown in preliminary experiments dedicated to toxicity studies that the application of plasma over significant period of time (depending on the DBD system used) did not cause short-term side effects, neither significant increase in skin temperature at treatment location or did not affect the cardiac or respiratory rhythm of the animals or had no systemic behavior side effect.

Experiments with the FE DBD on the colon quickly confirmed the antitumor effect of cold plasmas observed on glioblastoma, but the most interesting results were obtained with plasma jets on orthotopic colon [23] and pancreas cancer [24] and melanoma [25], [26]. Tumors are called "orthotopic" when they are specific to the organ on which they are grafted. The experiments involving orthotopic tumors are much more representative of clinical situations and *a priori* more predictive of the expected response in humans. Although tumors are treated only on their accessible surface, it is important to note that immunohistological analyzes showed that apoptosis was induced throughout the whole tumor volume.

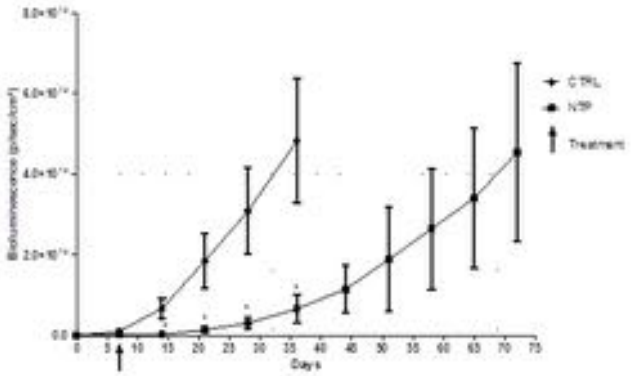

Figure 6: Carcinoma of the colon - Follow-up of tumor growth by bioluminescence imaging, tracer of tumor activity, of tumors grafted on nude mice. Comparison between a control group (CRTL) and a group treated with Plasma Gun (NTP) (treatment at day 7, arrow in the figure; He, 200 cc/mn; treament time: 10 min).

In the Plasma Gun-treated colon cancer experiments [23], performed on mice grafted with cancer cells which were previously stably transfected with firefly luciferase gene (thus allowing bioluminescence imaging (BLI)), comparative monitoring of the control mouse group and the group of mice treated with plasma (a single application of Plasma Gun for 10 min 7 days after tumor grafting) shows a very significant reduction in tumor growth in the treated mice (see fig.6) In this particular case, the plasma treatment resulted in a 115% increase in their life expectancy (the survival time is set by a specific bioluminescence value measured, in this case 4 10^{10} photons / s / cm2, associated with a tumor volume). Beyond the tumor reduction, these experiments also revealed a very marked reduction, or even an absence in most cases, of metastase proliferation. This observation is not yet explained but is of the greatest interest and will be the subject of future studies.

Pancreatic cancer is the gastrointestinal cancer with the poorest prognosis, with a 5-year survival rate of 5%. To evaluate the effectiveness of LTAPP treatment, an orthotopic model of pancreatic carcinoma MIA PaCa-luc representative of the pathophysiology of human pancreatic cancer was developed (CERB / CIPA-TAAM collaboration) [24]. The experiments were carried out on four groups of nude mice orthotopically grafted followed by BLI *in vivo*: an untreated control group but whose individuals underwent the same surgery as other in the other groups; a group treated only with gemcitabine at 200 mg / kg, a reference chemotherapy for pancreatic cancer; a group treated only by Plasma Gun (He, 340 cm3 / min, 13kV, 2 kHz); and a group with combined gemcitabine and Plasma Gun as shown in the inset of fig. 7. The obtained results (fig.7) are extremely encouraging. Not only did they allow for the first time to show an antitumor effect of a plasma jet on the pancreas, but also for the first time to demonstrate a beneficial effect of the combination Chemotherapy / Cold atmospheric plasma. It can be seen that 36 days after the injection, the reduction in tumor volume is greater in the Plasma Gun group (68%) than in the gemcitabine group (30%).

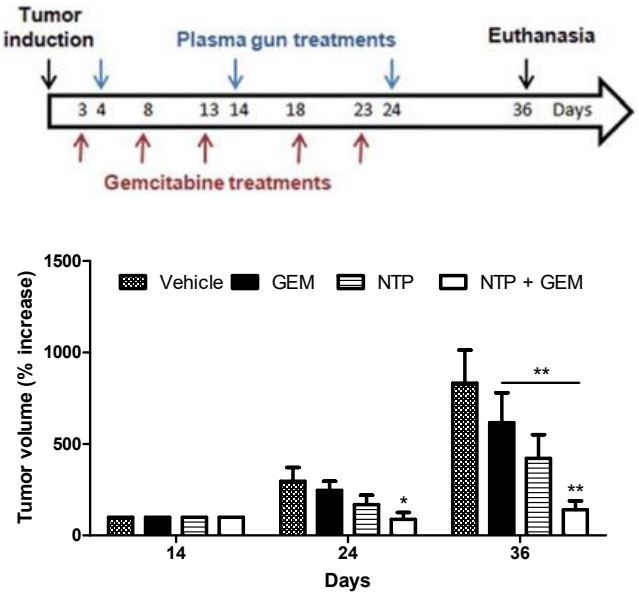

Figure 7: Adenocarcinoma of the pancreas - Comparison of the evolution of tumor volume on days 14, 24 and 36 after injection between a control group (Ctrl), a gemcitabine (GEM) treated group (200mg / kg) By Plasma Gun (Plasma) and a combined treatment group (Plasma + GEM). The sequencing of the treatments (alone or in combination) is indicated in the bottom diagram.

The most striking result is of course that the treatment involving chemotherapy and LTAPP leads to an even greater reduction in the order of 90%. These experiments open the way to combined treatments, particularly at the end of a surgical procedure in the immediate vicinity of the ablated part, which, even if they do not lead to a complete cure, can either reduce the used doses of chemotherapy for patient comfort or

improve the life expectancy in the medium term. This work will of course be continued in order to try to determine an optimum in the sequencing of the two treatments. Indeed, one can reasonably hope that the combined action Chemotherapy / Plasma Jet can still be amplified, or even be synergistic, by applying an ideal protocol that remains to be determined. As with colon carcinoma, plasma treatment on the adenocarcinoma of the pancreas has led to a significant reduction in the proliferation of metastases in many cases.

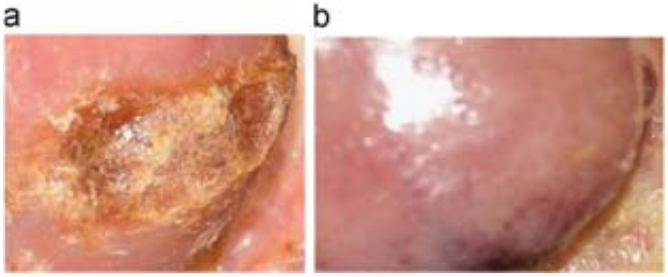

Figure 8: (a) Infected cancer ulcer of the tongue in an area with pathohistologically confirmed cancer cells and (b) wound healing under kINPenMed treatment [22]

As mentioned before, the first case study on cancer plasma treatment has been carried out on head and neck cancer. This study has been performed by the group of HR Metelmann in Greifswald using a kINPen Med [22] on 12 patients, aged between 53 and 77, afflicted with advanced squamous cell carcinoma of the head and neck to both decontaminate infected cancer ulcerations and evaluated anti-cancer effects. Treatment cycle was determined as: application of plasma jet 1 mn/cm^2, 3 times per week. Patients underwent between 1 and 9 cycles for a clinical follow-up between three weeks and 12 months. The treatment appear of benefit for select patients and the data suggested a decreased request for pain medication, a reduction of typical fetid odor related to reduction of microbial load. In some cases superficial partial remission of tumor and even wound healing of infected ulcerations has been observed following kINPenMed exposure (fig.8).

The precise mechanisms behind all the observed phenomena are far from being clearly identified and understood, but the experiments have shown the important role played by the reactive oxygen species (ROS). Biological characterizations have shown that plasmas induce double strand breaks in DNA, leading to massive apoptosis (programmed cell death) of the cells demonstrated, in particular, by bioluminescence studies. Moreover, it was possible to observe *in vitro* and *in vivo* a cell cycle arrest of the tumor cells leading to a halt in their proliferation. The mechanisms involved are close to those induced by radiotherapy but without the side effects associated with ionizing radiation. The healthy cells, less sensitive to oxidative stress, appear much less affected under treatment conditions which in the same time leads to apoptosis and necrosis of the tumor cells. This last point is still under discussion and needs much more experiments to be clearly solved.

ELECTRIC FIELD

While LTAPP are usually sources of intense pulsed electric field, up to recently, quite little attention has been paid on its potential role in the plasma treatment observed results. Most of them have been considered only has the effect of the direct interaction of the plasma itself with the cells or tissues. Some previous studies have emphasized on the potential benefit of combined plasma and PEF treatments (e.g. [27]), but in fact, direct effect of plasma produced electric field (EF) have been rarely considered. This may have partly resulted from the difficulty to measure it in real conditions of applications. The diagnostics of EF delivered in DBD or plasma jets has so far been quite limited. Besides modeling studies, experimental measurements based on the intensity ratio of excited nitrogen ions and molecules have been introduced and applied for surface DBD plasmas [28] and tentatively to He jets [29]. In the latter case, peak EF amplitudes as high as 100 kV/cm were reported [29] suggesting same order of magnitude as those measured in air for volume DBD . Using stark polarization spectroscopy, a nonintrusive time resolved measurement of EF generated by helium plasma jet reveals that peak EF amplitude between 10 and 20 kV/cm exists in the plasma ionization front [30]. Correlation between the front propagation velocity and EF was demonstrated by the same group and generalized to argon and air plasmas. Recently, a new device using an electro-optic sensor based on the Pockels effect (Kapteos probe) allowed precise characterization of plasma jet, especially Plasma Gun [31] showing pulsed EF in the range of few kV/cm to few tens of kV/cm, of few tens of nanoseconds to few microseconds in duration, depending on discharge parameters. It also allowed to show that plasma produced EF was penetrating quite deeply in the tissue [32], probably directly inducing effects that, to a certain level, can be compared to the ones obtained by PEF treatments. Recent results on cell permeabilization tend to support this hypothesis [33], [34] , [35]. Many studies are currently carried out on that particular topic and should bring new interesting route for plasma treatment alone or in combination with other physical or chemical therapies.

NEXT

All the results obtained up to now concerning therapeutic applications of plasmas let hope that many new applications will be develop in a near future. However, they will require major research efforts for a better understanding of the processes involved in the plasma/cell or plasma/tissue interactions, the evaluation of long-term effects and the difficult but, of course, indispensable passage from animal models to large-scale clinical trials. It will be also very important to understand the role played by the tissue oxygenation observed when applying plasma on tissues [36] [37]. In the forthcoming period, among the main challenges the community will face, we can point out: the development of devices suitable for endoscopic applications for treatments in body parts or organs difficult to access, or which are not compatible with surgery, or of complex structures such as lungs; the development of wide area plasmas or plasma jet array devices allowing the treatment of large tissue or organ surfaces. There are already great progresses in those domains [38] [31] [39], but still an enormous work to be performed to make the recently existing devices more reliable and well adapted to the targeted diseases to be treated. Beside challenges linked to improvement of the comprehension of the involved processes and plasma/living matter interactions, and to developpment of new plasma source generation, it can be added that the recently open field of plasma immunotherapy/plasma vaccination [40] will raised new and very exciting problems to solve. The latter is probably one of the very promising ways together with direct or combined plasma with physical (PEF, Ionizing radiations, phototherapy) or chemio treatments to develop in the very next future new plasmas based therapies.

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Eric Robert, 47 year old, is a CNRS senior research scientist since 1999 at GREMI, Orléans University. In 1997, he obtained his Ph.D on the topic of high-pressure rare gas plasmas excited by energetic flash X-ray source. Since then, his main interest was focused on energetic radiation sources ranging from UV to X-ray photons. He developed plasma-based sources dedicated to specific technological needs: mercury free lightning, new generation of lithography, high-pressure spray diagnostics, and more recently for biomedical applications. He is co-author of 72 peer reviewed papers, together with 3 patents. He supervised 7 PhD students and 10 post-doctoral fellows. He is currently involved in 4 national scientific programs, two H2020 programs, 2 technology transfer grants, and 2 research networks: European COST action on Plasma in interaction with liquids and french GdR ABioPlas (Biomedical application of non-thermal plasmas). He is member of the board of directors of the International Society for Plasma Medicine and of the new branch of the French Society of Physics (SFP Physique et vivant) and recently joined Cosmétosciences steering committee.

NOTES

NOTES

Preserving freshness in frozen and dried vegetables using reversible electroporation

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Abstract: Preserving freshness in fruits and vegetables that have been subjected to "harsh" processing operations such as freezing/thawing and dehydration is a technological challenge. This challenge can be addressed by the application of pulsed electric fields (PEF), in the reversible form of the technology. This paper describes the application of reversible PEF for the quality improvement of frozen/thaw leafy vegetables and dried basil leaves. For improving the freezing tolerance of leaf tissues, a cryoprotectant (for example trehalose) is first infused in the extracellular space of the tissue using vacuum impregnation (VI). The impregnated tissue is subjected to PEF which could (i) distribute the cryoprotectant in the extra and intracellular spaces of the tissue and/or (ii) cause stress responses that would lead to an increase resistance to abiotic stress such as freezing. The combination of VI and PEF drastically increase the freezing tolerance of the treated leaves. Another application that is discussed in this paper is the preservation of quality on aromatic herbs such as basil during dehydration. The method is based on the observation that application of pulsed electric field can lead to stomatal opening, suggesting a differential influence of the electrical treatment on the stomatal complex relative to its influence on the surrounding epidermal cells. Forced stomatal opening facilitates water loss from the tissue, enhancing the rate of dehydration and the quality of the final product.

FRESHNESS OF VEGETABLES

Throughout decades, food scientists have developed technologies that would allow the preservation of fruit and vegetables with the freshness characteristics that nature provides. The challenge is to prolong freshness as long as possible so that the products can be transported and commercialized without losing their innate quality characteristics such as flavour, texture and nutritional value. These technologies either target the growth of microorganisms that would potentially cause deterioration of the product and/or target the metabolic activity of the product in order to influence their respiration rate (for a review see [1]) and, in consequence, their deterioration process.

Excluding external factors of deterioration (e.g. microbial contamination, mechanical damage), keeping freshness is closely linked with keeping cell vitality and tissue structure. These main conditions for keeping freshness have been met with success by continuous developments in postharvest and minimal processing (washing, cutting and packing) technologies. However, "harsh" processing technologies such as cooking, drying and freezing/thawing provoke a total loss of cell vitality in the processed plant tissue as well as a profound influence on tissue structure and composition.

Is it possible to have vegetables that after freezing and thawing keep their freshness? Is it possible for herbs to keep their structure and aroma compounds intact after drying? This paper aims at answering these questions by describing advances in the area of vegetables "harsh" processing using reversible electroporation as an aid to keep freshness after processing.

REVERSIBLE ELECTROPORATION

Cells may survive the application of the pulsed electric field (PEF), recovering the permeability properties of their plasma membrane as well as their normal metabolic functions. If this is the case, the applied electroporation has been reversible. However, there are still consequences regarding metabolic responses to the stress caused by the electroporation.

Even if the cells survive the electric field, they are subjected to stress due to the opening of pores and the struggle of the cells to recover their normal functionality (for a review see [2]). PEF involves the production of reactive oxygen species (ROS), adenosin triphosphate (ATP) hydrolysis to rebuild gradients of charges across cell membranes, and/or other physiological events taking place during increased membrane permeability as well as long after pore resealing. An increased cytosolic calcium concentration has also been reported after the application of PEF. Transient elevations of cytosolic Ca^{2+} concentration may evoke downstream stimulus-specific responses including the signal transduction leading to the activation of mitogen-activated protein kinases (MAPK) which is a common stress reaction of plant cells in defence-related signal transduction pathways [3]. PEF-induced stress responses may be a key factor influencing the increased freezing tolerance of vegetables.

Reversible electroporation and survival

Using electrical resistance measurements, it has been demonstrated that at a certain applied voltage, the electrical resistance of the tissue rapidly decreases with increased number of applied pulses [4]. As illustrated in Fig. 2, the decrease of survival of rucola leaves was mostly associated with the portion of the curve where the electrical resistance does not change further (higher number of pulses).

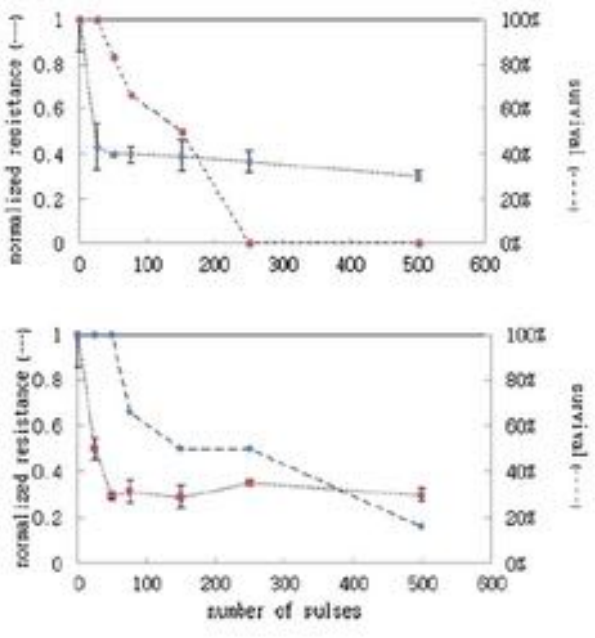

Figure. 1: Resistance and survival of rucola leaves at increasing number of pulses at different PEF protocols. Pulses were applied at 600 V/cm. In the upper figure, monopolar square pulses were applied with field width of 250 μs and 1 ms interval between pulses. In the lower figure, bipolar square pulses were applied with field width of 250 μs and 1 ms interval between pulses. Each point, joined with a dotted line, represents the average of three electrical resistance measurements. The error bar represents the standard deviationof the mean. Survival, plotted with points joined with a dashed line, is reported as a percentage of leaves that fully survived the treatments from at least 30 leaves from different packages. Tissue survival was tested with fluorescent microscopy, using fluorescein diacetate (FDA; Sigma Aldrich, USA, λex = 492 nm, λem = 517 nm), as described in [5].

IMPROVING THE FREEZING TOLERANCE OF LEAVES

The main disadvantage of freezing is that it can lead to tissue damage due to the formation of ice crystals; the cell membranes are ruptured, and the product becomes soggy after thawing. The freezing and thawing techniques used today are successful for products with low moisture content and products with relatively strong cell walls, for example, green peas, carrots, maize, and peppers. Products with weak cell walls, e.g., leafy vegetables such as spinach or soft fruits such as strawberries, become soggy upon freezing and thawing, they exude liquid, and lose much of their consumer appeal. Efforts are being made to improve methods of freezing in order to widen the application to products that presently cannot be frozen and where material losses in the food chain are high. An innovative method for improving the freezing tolerance of plant tissues has been developed. The method consists of two kinds of pre-treatment applied to the plant tissue prior to freezing: vacuum impregnation (VI) and a pulsed electric field (PEF). VI allows the introduction of a cryoprotectant molecule (e.g. trehalose) into the extracellular space of the tissue, while the subsequent application of PEF may facilitate the distribution of the cryoprotectant in the intracellular space [5,6] (Figure 2). However, specific measurements of intracellular trehalose upon PEF have not been done so far.

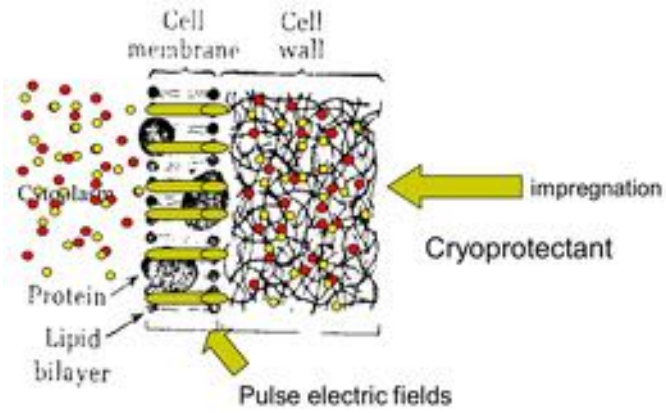

Figure 2: Schematic of the process developed for increasing freezing tolerance on plant tissues. A solution containing a cryoprotectant is vacuum impregnated in the extracellular space of the tissue. Reversible electroporation is applied to the impregnated tissue to facilitate the distribution of the cryoprotectant in the intracellular space.

Remarkably, with the combination of these unit operations, cell viability is preserved after a freezing-thawing cycle allowing cells to keep their turgor and, in consequence, avoid tissue collapse after thawing [6] (Fig. 3).

Mechanisms of survival

Survival mechanisms are not well understood. Demir et al [7] proposed that several stress-related metabolic responses could interplay. Vacuum impregnation with trehalose (a well-known cryoprotectant), might trigger metabolic responses in the leaves. Panarese et al. [8] showed that impregnated spinach leaves with trehalose doubled their gross metabolic activity, which may be provoked by the accumulation of trehalose-6-phosphate (T6P) [9]. T6P acts as signalling molecule for metabolic response to abiotic stress [10]. The application of PEF to the impregnated leaf increases further their gross metabolic

activity but does not increase further the T6P concentration (Figure 4)

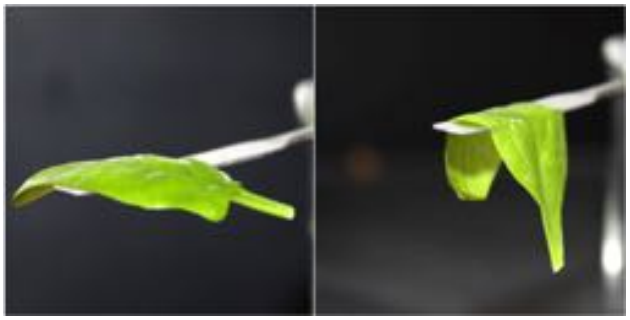

Figure 3: Spinach leaves treated with a freeze-thaw cycle. (left) The leaf cells were infused with the cryoprotectant trehalose before the freezing-thawing cycle. PEF treatment was used for the delivery of the cryoprotectant into the cytoplasm of the cells. (right) Control sample that did not undergo PEF-assisted infusion of the cryoprotectant before the freezing-thawing cycle (Pictures were kindly provided by Optifreeze AB, Lund, Sweden)

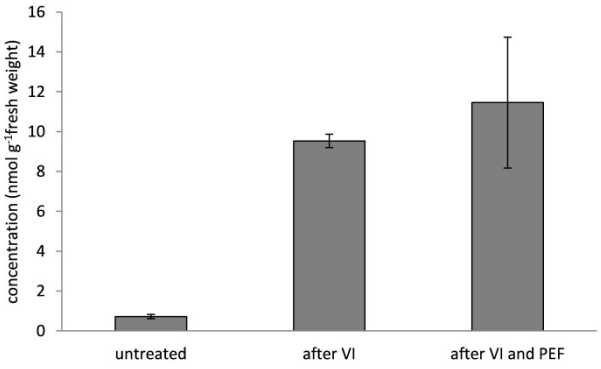

Figure 4 Concentration of trehalose-6-phosphate determined in untreated spinach leaves, leaves subjected to VI only, and a combination of VI and PEF. The error bars represent the standard deviation of four replicates

As stated above, PEF-induced stress responses may play a crucial role on freezing survival. Electroporation as such could provoke the increase of the concentration of trehalose inside the cells, promoting the acquisition of freezing tolerance, although, as pointed out by Dymek [11] there is, so far, no hard evidence that electroporation leads to enhanced transport of trehalose to the cytoplasm.

The application of PEF was, however, needed for survival as only VI with trehalose, and the concomitant accumulation of T6P in the cells, was not enough for survival [5].

IMPROVING THE QUALITY OF DRIED AROMATIC HERBS

The application of reversible permeabilization for the dehydration of basil aims at improving the retention of aroma compounds and rehydration properties. The method is based on the observation that application of pulsed electric field can lead to stomatal opening, suggesting a differential influence of the electrical treatment on the stomatal complex relative to its influence on the surrounding epidermal cells [12,13]. Forced stomatal opening could potentially facilitate water loss from the tissue to enhance the rate of dehydration.

Figure 5 shows an open stoma where electroporated guard cells are shown with propidium iodide accumulation

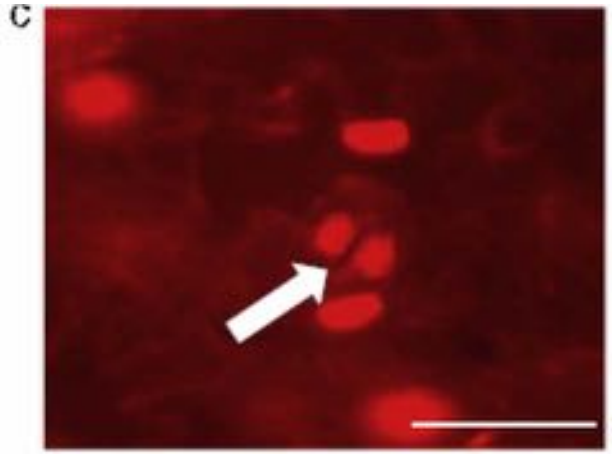

Figure 5 Typical microscopic picture of opened stoma showing electroprated guard cells with the accumulation of propidium iodide. The stoma is indicated with an arrow. The scale bar represents 50 µm.

Figure 6 shows how opening stomata with PEF, or rather, avoiding their closure during drying, accelerates the drying time for basil dried with air at 40 °C and 2 m/s of air velocity.

At 40 °C of drying temperature, the required time for dehydrating the leaves to 10 % of humidity is almost half of that of the untreated leaves.

Regarding structure, the SEM pictures shown in Figure 7, show a more intact cell structure in the dried leaves where reversible PEF was used as pre-treatment [14].

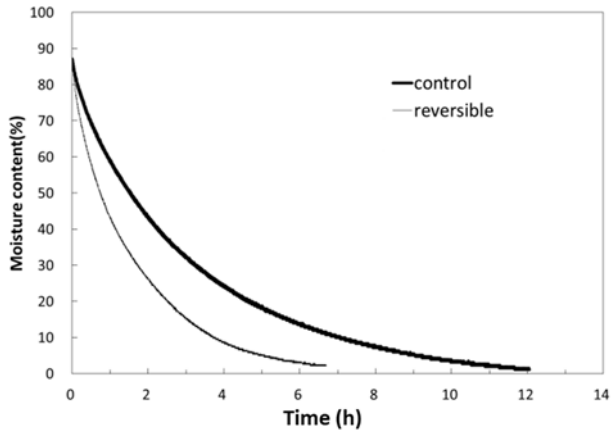

Figure 6: Drying curves for basil leaves dried at 40 °C and 2 m/s of air velocity. The thicker line represents the drying curve of the control, the thinner line represents the drying curve of the PEF-treated leaves where stomata were electroporated.

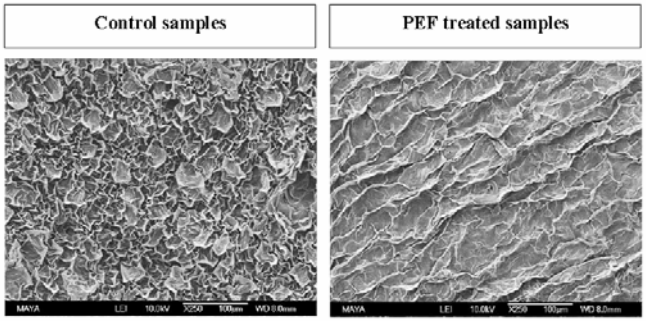

Figure 7: Scanning electron microscope pictures of the top surface of untreated and PEF treated basil leaves dried at 40 °C and 2 m/s of air velocity

However, there is still room for optimization of the PEF parameters prior drying. When aroma compounds were analysed by GC-MS in basil leaves before and after electroporation (in conditions designed to elecroporate stomata) the results showed that some aroma compounds were lost solely by the application of PEF Further differentiation in the aroma composition of the leaves is noticed when irreversible permeabilization was applied (Figure 8). The explanation of this loss relies on the effect of the electric field on the vesicles containing the aroma compounds. These essential oil glands may completely collapse due to PEF and during the drying process, however, some of them remain intact [14] (Figure 9).

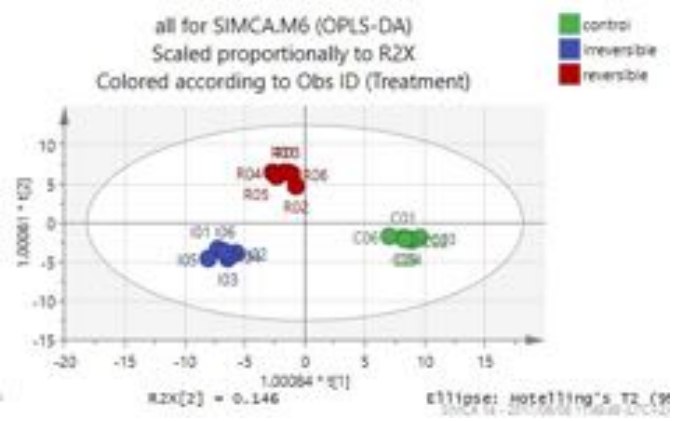

Figure 8: Ortogonal projections to latent squares with discriminatory analysis (OPLS-DA) of aroma compounds of basil leaves before and after PEF treatment. The cluster identified with "C" represents the aroma compounds of the control. The cluster identified with "R" represents aroma compounds in leaves where PEF treatment was applied in such way that the leaf's stomata were permanently opened during the drying process and cell viability was preserved (reversible permeabilization). The cluster identified with "I" represents aroma compounds in leaves where PEF was applied in such a way that the viability of the cells was not kept (irreversible permeabilization)

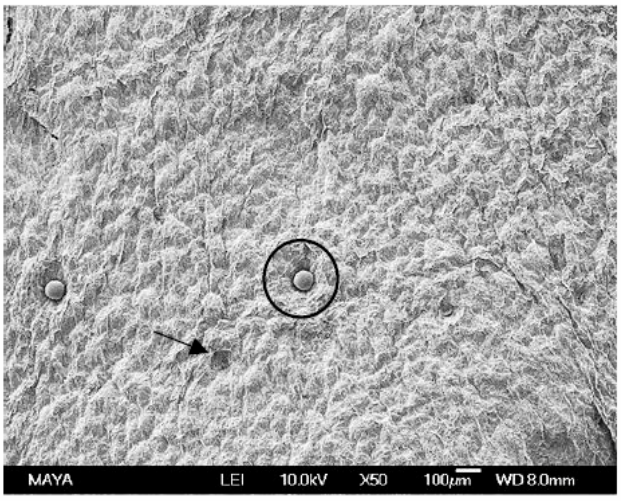

Figure 9: Scanning electrone microscope pictures of the bottom surface of basil leaves dried at 40 °C. Intact oil glands are indicated with a circle and collapsed oil glands with an arrow.

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Federico Gómez Galindo is the author of 40 articles in SCI-ranked journals cited over 600 times excluding self-citations. His h-index is 16. In 2015 he received the distinction of “Excellent Teaching Practitioner” from the Pedagogical Academy of the Faculty of Engineering at Lund University

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Image guided and computer assisted local tumor ablation in the liver with special focus on Irreversible Electroporation

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INTRODUCTION

Local percutaneous image guided ablations are feasible techniques for minimally invasive tumor treatment. The primary aim of these treatments is to preserve organ function without compromising the oncological outcome.

MAIN

In clinical routine thermal ablations as radiofrequency ablation (RFA) or microwave ablation (MWA) are frequently used. In the liver RFA of small tumors became part of the treatment guidelines of hepatocellular carcinoma [1]. However, proximity to critical main biliary and vascular structures represent a relative contraindication for thermal ablations of tumors due to the risk of 1) unwanted loss of heat energy ("heat sink effect") due to vessel induced cooling resulting in incomplete tumor ablation and 2) coagulation necrosis to "critical" adjacent vessels of bile ducts [2-5].

In case of such contraindications for thermal ablations there are alternative local treatments. As one example, irreversible electroporation (IRE) is an innovative local ablation technique that bases on high-voltage pulses to induce cell apoptosis without causing thermal damage to the target tissue [6]. Two to six electrodes have to be placed in parallel and in an exact geometry around the tumor. Image guidance is performed by CT or ultrasound. Nevertheless, a comfortable software based 3D ablation planning tool is missing and the majority patients were negatively preselected due to multiple pre-treatments and contraindications for standard first line treatments as surgery or thermal ablations. The intervention time in IRE varies from two to three hours and is therefore longer than in most other ablations. Under strict ECG synchronization and general anaesthesia with full relaxation the ablation protocol is technically safe and complication rate was low [3,5,7]. Beside directly at the needle tip itself there is no relevant heat development [8].

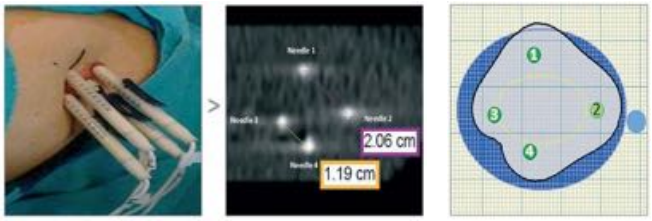

Figure 1: Planning of ct-guided IRE: (left) multi-needle placement, (middle) ct-based measurement of needle distances, (right) 2D planning on manufacture`s software

In general, the selection in between different ablation modalities is still a challenging process. This decision is highly dependent on the special expertise of the applying interventional radiologist, local availabilities and economic considerations. Clinical experience shows that the size and shape of an ablation lesion is often hard to predict resulting in a risk of under-treatment with local tumor recurrence or over-treatment with causing damage to adjacent structures. A partially clinical validated web based tool (Fig. 2) enables planning and simulation of different treatment modalities (RFA, MWA, IRE and Cryotherapy) in multiple organs (liver, lung and kidney). Such developments may furthermore facilitate the compilation of guidelines for the choice in between different ablation modalities. Prospectively software based ablation planning and simulation could overcome this and assist the interventional radiologist in finding the optimal patient specific treatment. For RFA treatment such a software tool has been developed and validated in the scope of a prospective clinical multicentre trial (EU project ClinicIMPPACT - www.clinicimppact.eu) [9,10].

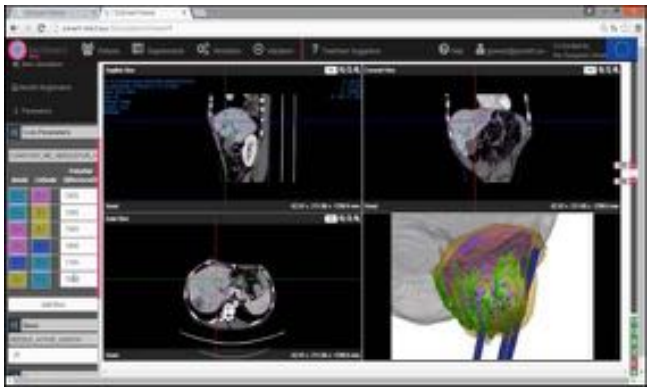

Figure 2: Planning and Simulation of IRE of a liver tumor with a web-based application (EU-project GoSmart: *www.gosmart-project.eu* or *http://smart-mict.eu*).

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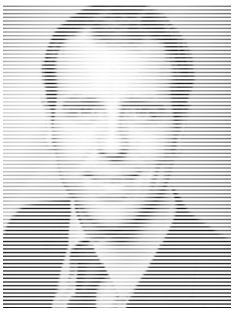

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Electromanipulation of Mesenchymal Stem Cells

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Abstract: Human mesenchymal stem cells are promising tools for regenerative medicine due to their ability to differentiate into many cellular types such as osteocytes, chondrocytes and adipocytes amongst many other cell types. These cells present spontaneous calcium oscillations implicating calcium channels and pumps of the plasma membrane and the endoplasmic reticulum. These oscillations regulate many basic functions in the cell such as proliferation and differentiation. Therefore, the possibility to mimic or regulate these oscillations might be useful to regulate mesenchymal stem cells biological functions. Interestingly, pulsed electric fields can permeabilise plasma and/or organelles membranes and therefore generate cytosolic calcium peaks by recruiting calcium from the external medium or from internal stores. According to the pulse(s) electric field amplitude, it is possible to generate a supplementary calcium spike with properties close to those of calcium spontaneous oscillations, or, on the contrary, to inhibit the spontaneous calcium oscillations for a very long time compared to the pulse duration. Through that inhibition of the oscillations, Ca2+ oscillations of desired amplitude and frequency could then be imposed on the cells using subsequent electric pulses without causing a loss of cell viability. This new tool can be useful to further investigate the role of Ca2+ in human mesenchymal stem cells and/or to manipulate their fate.

MESENCHYMAL STEM CELLS AND CALCIUM OSCILLATIONS

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells [1] originated from the embryonic mesoderm (mesenchyme) and present in many adult tissues such as bone marrow (bMSCs), adipose tissue (aMSCs), muscle, dermis, umbilical cord… [2,3]. These cells gained a lot of momentum in the last decade due to their ability to differentiate into a wide variety of cells including osteoblasts, myoblasts, fibroblasts and chondrocytes. They also express key markers of cardiomyocytes, neuronal and endothelial cells [4]. This ability makes them a very promising candidate for cell therapy and regenerative medicine in order to heal damaged tissues and organs.

Calcium oscillations

Mesenchymal stem cells present spontaneous asynchronous Ca^{2+} oscillations (Fig. 1) implicating Ca^{2+} channels and pumps of the plasma membrane (PM) and the endoplasmic reticulum (ER) [5]. These oscillations seem to start by an ATP autocrine/paracrine signaling [6] followed by Inositol triphosphate (IP3)-induced Ca^{2+} release from the ER [7] which stimulates the store-operated calcium entry (SOCE) via the plasma membrane store-operated Ca^{2+} channels (SOCCs) [8]. Afterward, the excess of Ca^{2+} is removed from the cytosol by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), the plasma

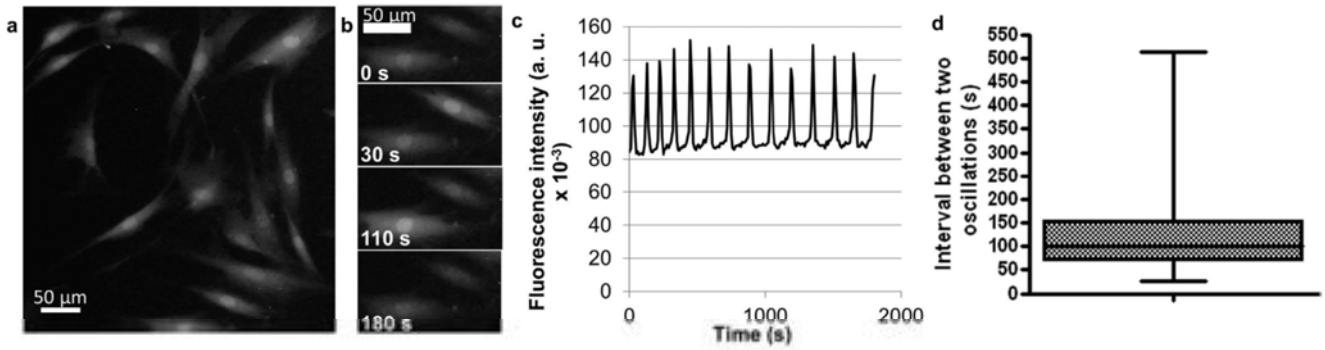

Figure 1. Attached haMSCs, preloaded with Fluo-4 (5 µM), presenting spontaneous Ca^{2+} oscillations in complete DMEM (with Ca^{2+}). (**a**) Snapshot in epifluorescence of Fluo-4 labelled haMSCs. (**b**) Focus on two Fluo-4 labelled cells at different times of observation. The cells presented asynchronous spontaneous oscillations. (**c**) Ca^{2+} oscillations of one haMSC extracted from a movie of 30 min (one image every 10 s). The Ca^{2+} oscillations displayed regular amplitude and rhythm. (**d**) Distribution of the oscillation periods of oscillating haMSCs. 197 cells were followed from four experiments. 175 out of 197 cells (89%) displayed oscillations and for 160 of them (81%), all along the observation period. These 160 cells were used to prepare the plot. There was a large intercellular variability. The average time between two oscillations was 82 s ± 96 s, mean ± SD and the median time was 100 s. The minimum was 26 s and the maximum was 514 s.

membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/ Ca^{2+} exchanger (NCX) [9]. Ca^{2+} is the most important second messenger in the cell, and it regulates many important cellular processes such as ATP synthesis, apoptosis, cellular motility, growth, proliferation and gene expression. Hence, Ca^{2+} oscillations contain embedded information that have to be decoded by the cell, and Ca^{2+} signaling pathways play a key role in controlling cell behavior and differentiation processes of MSCs.

It was shown that the Ca^{2+} oscillations frequency is different between undifferentiated MSCs and MSCs on route of differentiation and it differs between the various outcomes of the differentiation process (the level of differentiation and the differentiated cell type). While the MSCs exhibit regularly repeated Ca^{2+} oscillations, MSCs undergoing osteodifferentiation display a decrease in the frequency of their spontaneous Ca^{2+} oscillations while primary myoblasts present still another pattern of oscillations [10]. This shows that each cell type possesses its own pattern of Ca^{2+} oscillations frequency and shape.

Sun *et al.* reported that differentiated MSCs present less Ca^{2+} oscillations than undifferentiated hMSCs and that blocking these oscillations by using a 10 V/m continuous electric field (EF) facilitates differentiation into osteogenic lineage[11]. Several other studies have pointed out the key role of the intracellular Ca^{2+} for stem cells and differentiation [12]. Moreover, various reports have shown that electromagnetic fields are able to influence the differentiation of stem cells by modulating the intracellular Ca^{2+} [13]. However, the exact correlation between the intracellular calcium oscillations and the differentiation process is still unclear.

CALCIUM PEAKS INDUCED BY ELECTRIC PULSES

Presently, pulsed electric fields (PEF) are widely used in research as a non-invasive physical means to permeabilise cellular membranes. The plasma membrane permeabilization can be temporary, allowing the internalization of non-permeant molecules of interest like drugs or nucleic acids. Using one or several pulses of ultrashort duration causes changes in the cell membrane structure that permits the access to the cell cytosol to molecules that cannot cross the plasma membrane under normal conditions [14]. Microsecond pulsed electric field (μsPEF) of about 100 kV/m are commonly used to induce PM permeabilisation to different types of molecules (small ions [15,16], drugs [17], DNA [18]). The higher the EF amplitude, the greater the permeabilisation [19]. Since a decade, a new type of PEFs has been used: the

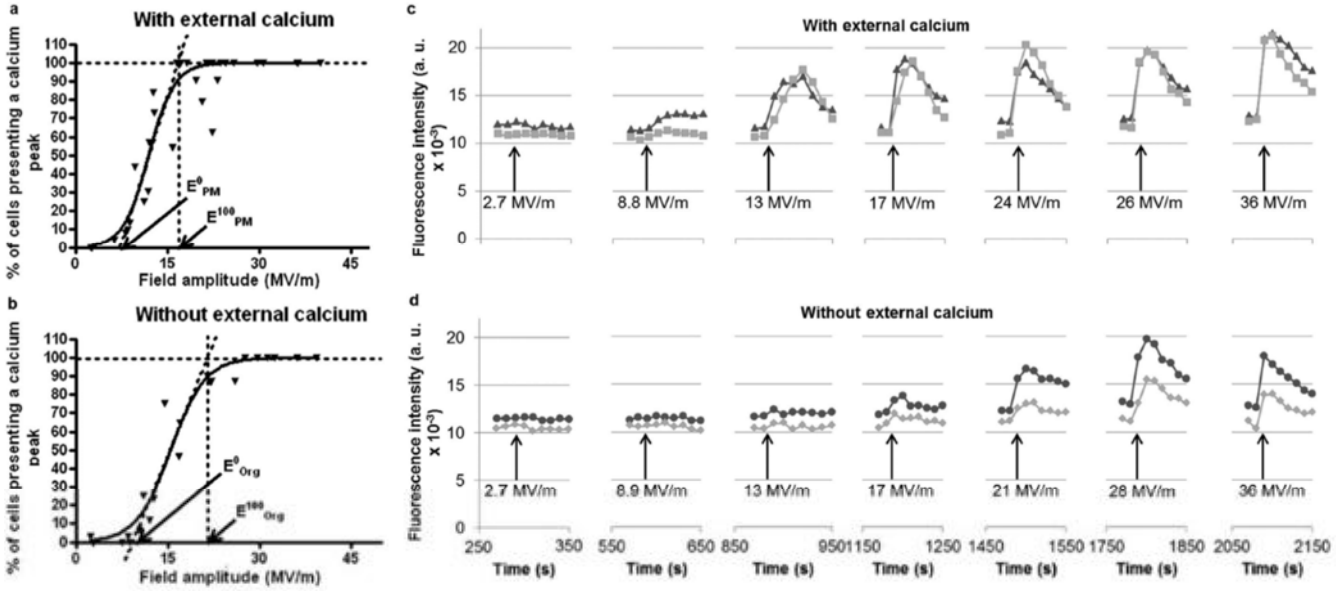

Figure 2. Effect of the PEF amplitude on the induced Ca^{2+} peaks with or without external Ca^{2+}. (**a**,**b**) Field amplitudes needed to start to permeabilise the attached haMSCs (E^0) and to permeabilise all the attached haMSCs (E^{100}) in the presence (**a**) or in the absence of external Ca^{2+} (**b**). E^0_{PM} and E^{100}_{PM} are referring to the plasma membrane whereas E^0_{Org} and E^{100}_{Org} are referring to the organelles membranes. These experiments have been repeated at least 4 times in the presence and in the absence of Ca^{2+}. 17 to 30 cells per experiment were observed for each tested electric field amplitude. Lower electric field was needed to induce Ca^{2+} peaks in the presence of external Ca^{2+} than in the absence of Ca^{2+}. (**c**,**d**) Cytosolic Ca^{2+} peaks recorded in two different experiments with adherent haMSCs responding to seven consecutive nsPEFs amplitudes ranging from 2.7 MV.m^{-1} to 36 MV/m^{-1}. The plotted cells were representative of the general cell behaviour. (**c**) 2 cells Ca^{2+} profiles from an experiment performed in the presence of external Ca^{2+} (complete DMEM). (**d**) 2 cells Ca^{2+} profiles from an experiment performed in the absence of external Ca^{2+} (S-MEM). The shape of the beginning of the Ca^{2+} peaks was depending on the electric field amplitude.

nanosecond PEFs (nsPEFs) that are about 1 000 to 10 000 fold shorter in duration and 30 to 300 fold higher in amplitude.

Normally, Ca^{2+} is an ion that only crosses the plasma and ER membranes through channel proteins. However, in a medium containing Ca^{2+}, applying a PEF (µsPEF or nsPEF), with an amplitude high enough to reach the plasma membrane permeabilization threshold, allows the Ca^{2+} entry from the cell outside [20,21]. This influx of extracellular calcium induced by the electric pulse is the result of the electropermeabilization of the cell membrane and not due to the activation of Voltage-Operated Calcium Channels (VOCCs). Moreover, if the combination of the electric field amplitude and the pulse duration is high enough, the Ca^{2+} influx is massive and triggers the additional release of Ca^{2+} from the cell inner stores, resulting in a calcium burst/peak [20]. Up to certain electric field amplitude, only the PM is electropermeabilized. At higher electric field amplitude, PEFs can generate cytosolic Ca^{2+} peaks by permeabilising not only the PM but internal membranes as well such as the ER membranes. This allows the induction of Ca^{2+} spikes in the absence of external Ca^{2+} by the direct release of the Ca^{2+} stored in the ER [20,21] to the cytosol (Fig. 2).

ELECTROMANIPULATION OF CALCIUM OSCILLATIONS

Ca^{2+} oscillations play an important role in the transduction of many physical stimuli in the cells. For example, tissue strain and shear stress on mouse tibia are transduced by repetitive Ca^{2+} spikes in the osteocytes. The spikes frequency and amplitude depend on the mechanical magnitude [22]. Electromagnetic fields (EMF) can also promote MSCs differentiation to osteoblasts through Ca^{2+}-related mechanisms [23]. Those EMF were either direct current of very low electric field amplitude (0.1 V/cm) and a long time exposition (30 min) [11] or biphasic electric current (1.5 µA/cm^2 for 250 µs) [24]. These fields either activate VOCCs if their amplitude is large enough or do not alter the membrane potential if the electric field is too low. None of the electromagnetic fields used was permeabilizing the cell PM.

Recently, it has been shown that calcium oscillations can be very precisely manipulated using PEFs (µsPEFs or nsPEFs) of a high electric field amplitude that are capable to permeabilize the cells PM or both the PM and the ER without affecting cell viability [21,25].

When a PEF is applied to the cells, an electrically-induced Ca^{2+} spike is observed synchronously in a given percentage of the cells (as a function of the electric field amplitude) (Fig. 2).

At low and moderate electric field amplitudes, the Ca^{2+} spike induced by a single PEF can be very similar to a spontaneous oscillation (same amplitude and duration). This electrically induced Ca^{2+} spike does not inhibit the natural calcium oscillations, can be applied at any time of an oscillation without interfering with this latter, and after the pulse, the oscillations continue normally at their own rhythm, and with their normal shape.

At high electric field amplitudes, the electrically induced Ca^{2+} spikes inhibit the spontaneous oscillations occurrence for some time after which oscillations reappear (Fig. 3). The percentage of cells that are unable to display further spontaneous Ca^{2+} oscillations increases proportionally with the electric field amplitude of the inhibitory electric pulse. The spontaneous Ca^{2+} oscillations inhibition can last some minutes or be longer, to reach tens of minutes, also depending on the electric field amplitude of the inhibitory electric pulse.

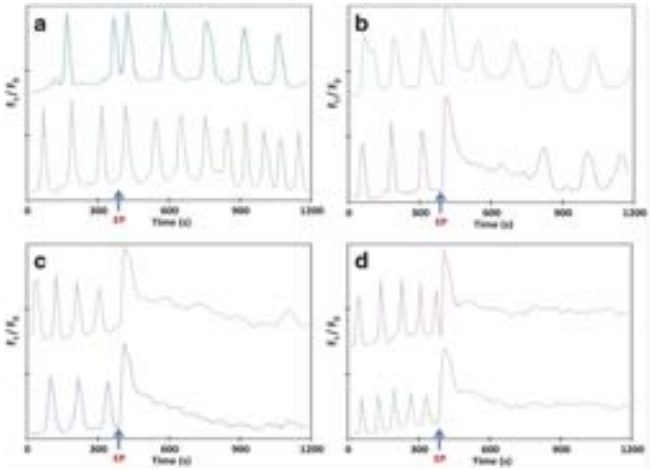

Figure 3. Examples of changes in the pattern of Ca^{2+} oscillations caused by the delivery of one 100-µs electric pulse of different field amplitudes, **a** 300 V/cm, **b** and **c** 600 V/cm, **d** 900 V/cm

During this inhibition period the cells are still reacting to PEFs and present electrically induced Ca^{2+} spikes with different amplitudes depending on the applied electric field amplitude (Fig. 4).

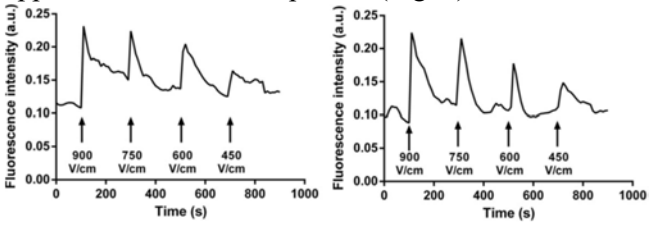

Figure 4. Two examples of calcium spike manipulation in haMSCs. After the delivery of one 100-µs electric pulse at 900 V/cm (that abolishes the spontaneous Ca^{2+} oscillations), pulses of various field amplitudes were delivered to mimic Ca^{2+} oscillations at experimentally controlled times.

The transient inhibition of the Ca^{2+} oscillations allows to impose to the cells experimentally induced Ca^{2+} spikes at a desired frequency. Hence, it is possible to precisely control MSCs spontaneous Ca^{2+} oscillations by pulsed electric fields (PEFs) with a complete preservation of the cell viability. The delivery of PEFs constitutes an easy way to control Ca^{2+} oscillations in mesenchymal stem cells, through their cancellation or the addition of supplementary Ca^{2+} spikes. Indeed, unlike chemical factors which cause continuous effects, the direct link between the microsecond electric pulse(s) delivery and the ulterior occurrence/cancellation of the cytosolic Ca^{2+} spikes allows to mimic and regulate the Ca^{2+} oscillations in these cells. The electrical control of Ca^{2+} oscillations could be in the future a promising tool to further investigate the role of Ca^{2+} in MSCs physiology and to better understand the correlation between the Ca^{2+} oscillations in MSCs and biological processes such as proliferation and differentiation.

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NOTES

SHORT PRESENTATIONS

Numerical Modelling of the Electroporation in Stomatocyte Red Blood Cells

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INTRODUCTION

During the last years, the poration of cell membrane induced by pulsed electric fields has been widely investigated [1]. To this aim, a nonlinear, dispersive and space-time numerical model has been developed to evaluate the transmembrane voltage and pore density along the perimeter of realistic irregularly shaped cells. In particular, stomatocyte red blood cell has been considered as test case.

MATHEMATICAL MODELING

The 3-D quasi-static formulation of the Maxwell's equations is used in conjunction with the nonlinear Smoluchowski equation and the general multirelaxation Debye-based relationship. Moreover, the general shape of the cell is described by using the Gielis' formula [2,3]:

$$r = \hat{r}\sqrt{x^2 + y^2} \quad (1)$$

$$x = k_x R(\theta)\cos\theta \quad (2)$$

$$y = k_y R(\theta)\sin\theta \quad (3)$$

$$R(\theta) = \left(\left| \frac{\cos(m_1\theta/4)}{a_1} \right|^{n_1} + \left| \frac{\sin(m_2\theta/4)}{a_2} \right|^{n_2} \right)^{-\frac{1}{b_1}} \quad (4)$$

In particular, such formula has been used to model the irregular geometry of stomatocyte red blood cell illustrated in Fig. 1.

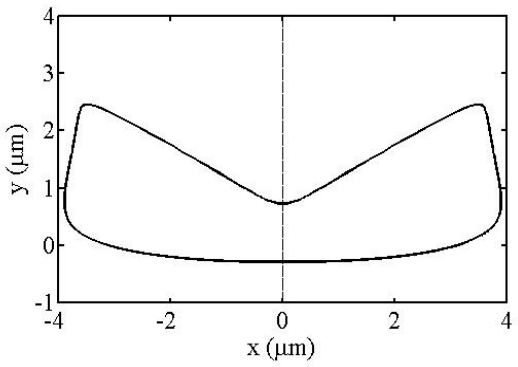

Figure 1: Geometric shape of stomatocyte red blood cell. Gielis' superformula parameters k_x = 5.31 μm, k_y = 0.73 μm, m_1 = 8.5, m_2 = 1.5, n_1 = 143, n_2 = 8, a_1 = 1, a_2 = 0.3 and b_1 = 9.

NUMERICAL RESULTS

In our simulations, the biological cell is exposed to a pulsed electric field generated by using two parallel–plate electrodes placed at the top and bottom surfaces of cylindrical lossy and dispersive medium. The pulse duration, rise and fall time are 7 ns, 2 ns and 2 ns, respectively. Moreover, a time delay of 4 ns and a time computational windows of 20 ns are considered. Fig. 2 shows the pore density along the cell perimeter at different time instants, considered with respect to t_0=0 ns. It can be observed that two nanoseconds after the pulse application a consistent electroporation emerges. A significant electroporation can be observed at the bottom and the top of the cell and the non electropored zone is mainly localized around the cell sides.

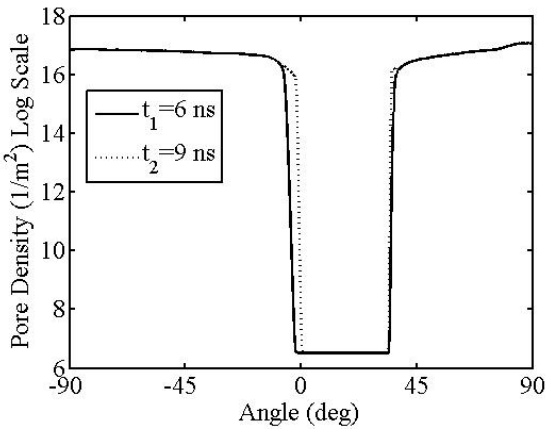

Figure 2: Pore density along the cell perimeter at different time, considered with respect to t_0=0 ns. Applied pulsed electric field having amplitude of 50 kV/cm, duration of 7 ns, rise time t_r= 2 ns and fall time t_f = 2 ns.

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Influence of electropermeabilization on the membrane stiffness

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INTRODUCTION

Living cells are permeabilized with electric pulses of high intensity enabling the transport of small and large molecules across the membrane. The influence of electric pulses on the physical properties of the cell membrane and the cytoskeleton is largely unexplored. Recently it was shown that cytoskeleton plays an active role in electropermeabilization [1,2]. Deformation of membranes has been used in the past to understand the changes in physical properties of membrane and membrane cytoskeletal adhesion.

METHODOLOGY

To elucidate the changes in membrane properties after electropermeabilization, tether extraction experiments were performed on NIH/3T3 cells. To prepare the cells for the experiment, the NIH/3T3 cells are plated on a 4 well imaging chamber and the growth media is replaced with an electropermeabilization buffer. Relevant electric pulses which induce electropermeabilization and is commonly used in the electropermeabilization community were applied to cells. No pulse is applied to the control cells.

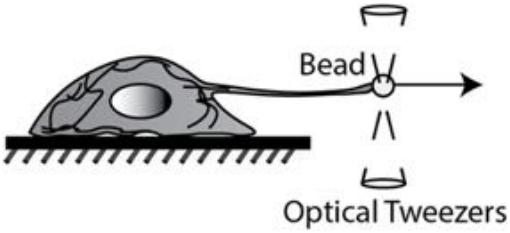

Figure 1: A schematic of membrane tether extraction experiment.

The membrane tethers are created by bringing a functionalized bead in contact with the cellular membrane and pulling the bead with the use of optical tweezers. The force-displacement curve to pull a tether from the cell membrane can then be used to extract meaningful information [3,4].

RESULTS

The membrane tether extraction experiments resulted in the characteristic force-time curve as seen in Figure 2. It was observed that the average value of force barrier shifted from 125pN in control experiments to 50pN in experiments performed after electropermeabilization. Therefore, it seems there is a change in membrane physical properties after electropermeabilization.

FUTURE PERSPECTIVE

Preliminary results show that different pulse parameters have different effects on the membrane physical properties. To fully understand what the changes in force-time curves mean, we aim to understand the mechanism of membrane force barriers in cells.

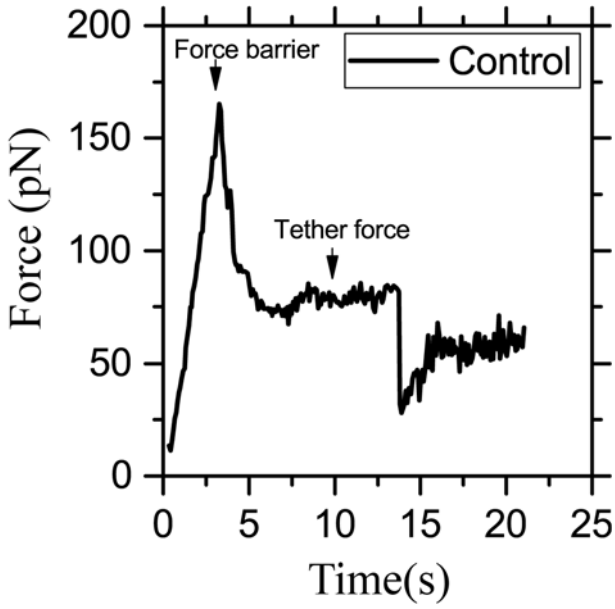

Figure 2: Force-Time curve for membrane tether extraction curve performed on control cells. The force barrier and equilibrium tether force are marked on the curve.

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Real-time nonlinear optical spectroscopy analysis corroborated by fluorescence microscopy

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INTRODUCTION

Cell membrane electropermeabilization (EPN), involving cell membrane reversible or irreversible restructuring, under extremely short and intense electric pulses, plays a key role in a number of preclinical and medical research applications.

Hence, the knowledge of EPN bio-physical nature and dynamics (i.e. pore formation and resealing processes) is crucial for the optimization of the delivered protocols, and for predicting treatments efficacy under established biological (e.g. buffer conductivity, cell dimensions) and electromagnetic conditions (e.g. pulse duration, amplitude, shape).

MATERIALS AND METHODS

To understand bio-physical mechanisms underlying such short electric pulses exposure, an innovative and advantageous approach is proposed based on real-time molecular vibrational modes imaging using fast Coherent anti-Stokes Raman Scattering (CARS) of *in vitro* samples.

My Matser internship provided support to the CARS activity which aims to study the reaction of membrane lipid and interfacial water in the EPN phenomenon through the analysis of the stretching of the water molecules.

EPN phenomenon of living cells to support CARS analysis was detected by fluorescence imaging using Yo-Pro and Fluo-4 dyes. It was performed on a coplanar waveguide (CPW) with an inter-electrode distance of 0.5 mm. Indeed, this structure designed for wide band electromagnetic exposures, was realized on a CaF2 substrate[1].

During my internship, I worked to perform these two techniques considering different electric pulsed exposures (200 pulses of 10 nanosecond (ns) and a pulse repetition frequency of 1Hz or 8 pulses of 100 microsecond (µs) at 1Hz). Different amplitudes were taken into account depending on type of cells.

Cells involved in these experiments were:

- DC3F: Chinese Hamster lung fibroblasts
- MSC: Human Mesenchymal Stem cell
- Erythrocyte: Mouse Red blood cells.

This choice has been done to take into account different levels of biological complexity in the study of the EPN phenomenon.

RESULTS

All the results obtained by CARS spectroscopy were referred to the interpretation of the water at the interface of cell membranes reported in the work of Scherman et al., (under submission). However, we were able to detect the O-H asymmetric stretching before and after electropermeabilization for DC-3Fcells (Fig. 1). Knowing that CARS signal is related to the amount of water and lipid molecules, the detection of the expected asymmetric O-H stretch under EPN clearly was more apparent using DC-3F cells. This assymetric O-H stretch (assigned to the bands lying from 3280 to 3400cm $^{-1}$) is compatible with the situation of the water located "in" the membrane, the so called "interstitial" water. Indeed, in the case of interstitial water, the H-bonds network is weaker because water is trapped in small interstices, water molecules fall more apart from each other, and thus hydrogen bonds are weaker favoring the O-H assymetric stretching.

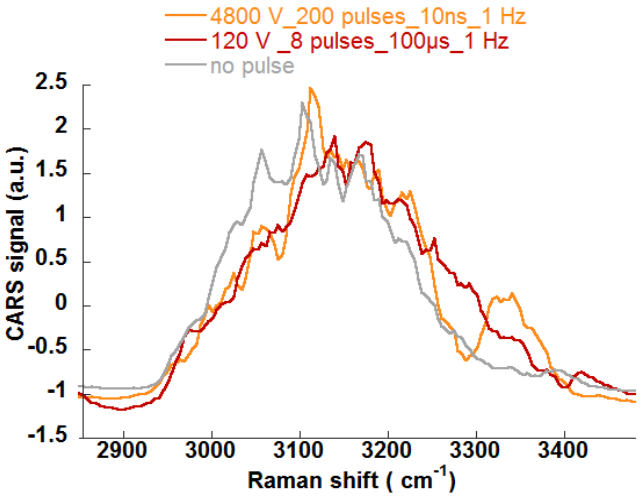

Figure 1: CARS spectra of DC-3F after the exposure to micro- and nano-second EPs

CONCLUSION

Fluorescence microscopy let us to detect the level of EPN on cells. However, CARS spectroscopy allows us to have a depth insight of chemical changes occurring at the level of the cell membranes under EPN. Both methods are useful to monitor cell EPN and the two techniques are evidently complementary in the understanding of the mechanisms involved in such phenomenon.

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Endogenous biological chemiluminescence as an indicator of oxidation

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INTRODUCTION

Pulsed electric fields have already wide use and further great potential for novel applications in biomedicine and food industry. Although not fully explored, one mechanism of the electric pulses action in biological samples is through the electric field induced oxidation [1]. In this context, our long term aim is to employ endogenous biological chemiluminescence (also termed us ultra-weak photon emission - UPE) for a label-free interrogation of the oxidative effects [2] of us-ns electric pulses in biosamples [3]. In this work we demonstrated the first step - that the UPE indeed reports oxidation rate. On the yeast cell culture as a model system we showed that UPE is generated both when oxidative substances are added to the sample or when the oxidation is produced during the normal metabolism during the growth of the cell culture.

METHODS

The culture of yeast (*Saccharomyces cerevisiae* (Euroscarf collection; genetic background BY4741, *MAT*a) was cultivated about 24 hour in standard YPD (Yeast extract-Peptone-Dextrose) medium in a laboratory shaker at 30°C. Then, the yeast culture was washed at least 2 times and transferred (3000 rpm, 5 min, centrifugation) to 3 mL of 2% sucrose. To generate oxidation, Fenton reaction ($FeSO_4$, H_2O_2) was used. Yeast in bioreactor was cultivated at 30°C, bubbled with the air and stirred (180 rpm). Initial concentration of yeast was $5 \cdot 10^5$ cells·ml^{-1} in 750 ml of YPD. Sample in a Petri dish or bioreactor was placed in a dark chamber and UPE was detected by a sensitive low noise photomultiplier tube (PMT) module Hamamatsu H7360-01. The average dark count of the system was cca. 15 counts per second and stable. The quantum efficiency of the PMT is in the range from 300 to 650 nm, the highest sensitivity is at the wavelength 375 nm. The signals showed are smoothed with a robust smooth function.

RESULTS

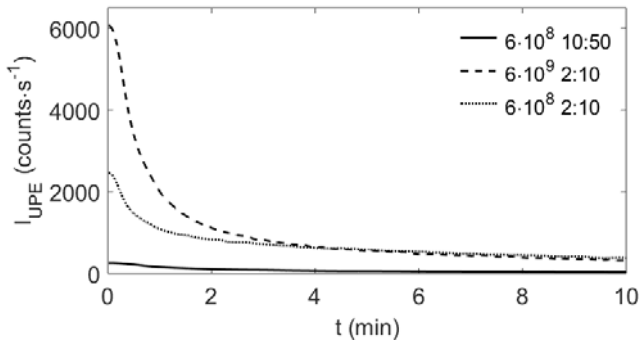

Figure 1: The higher the cell concentration or higher the oxidant concentration, the higher the UPE intensity. The first number in legend shows concentration of yeast (cells·ml^{-1}) in the sample (2% sucrose, 3 ml). The ratio gives final concentration in mM of $FeSO_4$ and H_2O_2 in the sample.

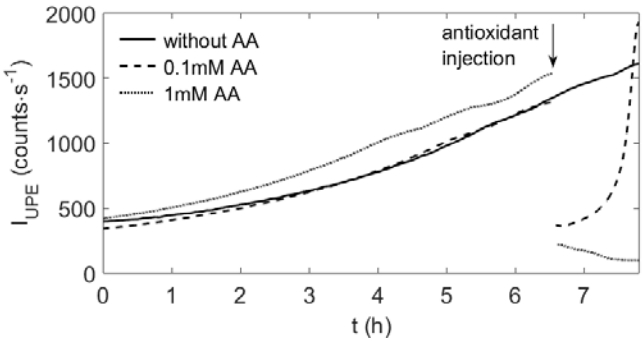

Figure 2: The course of ultra-weak photon emission (UPE) signal of yeast cultivated in a bioreactor (solid line) and the influence of the antioxidant ascorbic acid (AA) in final concentration 0.1 mM (dashed line) or 1 mM (dotted line) in the sample.

CONCLUSIONS

Our results provide the basis for label-free optical monitoring of the oxidative/radical processes in biosamples.

ACKNOWLEDGEMENTS

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Numerical study of electroporation of cells by helium atmospheric pressure plasma jet

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INTRODUCTION

Recently, atmospheric pressure plasma jet (APPJ) has gained a lot of attention, due to its low production cost and the wide range of applications in medicine, local surface modification, local sterilization etc. In particular, helium APPJ shows very promising results in selectively treating cancerous tissue while leaving the healthy tissue intact. However, the mechanism behind this is not very clear and is mainly attributed to the production of radicals which collide with the living tissue and the high electric field generated in the intracellular region [1]. In this study, the transmembrane voltage (TMV) and therefore the ability for electroporation [2] of cells in the presence of the electric field induced by a helium APPJ is investigated via numerical simulations. Different cell shapes are investigated since the shape is one of the main differences between normal and cancerous cells. Differences in the TMV could explain the ability of APPJ to selectively treat cancerous tissue.

THEORY AND METHODOLOGY

A two dimensional axi-symmetric model that considers a gas dynamic model (GDM) and a plasma fluid model (PFM) was developed [3] for the physical description of a helium plasma jet and its interaction with a dielectric surface that represents the average electrical properties of the tissue. The calculated voltage across the dielectric is subsequently used in a new model that considers the analytical geometry of normal and cancerous cells in order to investigate its effect on the TMV. For the calculations, a fixed area of 0.01 x 0.01 $(cm)^2$ containing a single cell in its centre is used. The voltage is also scaled down accordingly. In order to speed up and simplify the calculations, a 2D model for the cell is used. Cells consist of the cell membrane (10 nm thickness), the cytoplasm, the nucleus and the extracellular fluid. For the normal cells (NC), a circular geometry (10 μm radius) is considered, while for the cancer cells a larger and more irregular geometry is considered. Three different geometries that are becoming progressively more oval are used for the cancerous cells (CC1-3): a circular geometry (CC1, with 20 μm radius), and two elliptical geometries with minor and major axes radii of 10 μm and 20 μm for CC2 and 5 μm and 20 μm for CC3. Furthermore, for the elliptical cells, the induced voltage is applied on the major and minor axis of the ellipse in order to get the entire outcome range. The electrical properties of the cells are taken from [4].

RESULTS AND FIGURES

Figure 1 presents the maximum transmembrane voltage induced on normal and cancerous cell during the interaction with the applied voltage of an atmospheric pressure helium plasma jet. The key observation is that the TMV is higher for the larger cancerous cells (CC1) which could lead to electroporation and therefore treatment of cancerous cells while the normal cells remain unaffected. In addition, the move oval cells (CC3) tend to induce lower voltages.

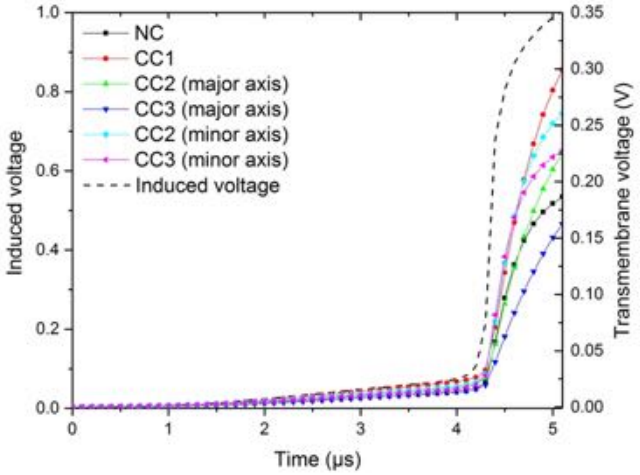

Figure 1: TMV of different cells in helium APPJ.

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Influence of Pulsed Electric Fields (PEF) pre-treatment on the drying process of onions

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INTRODUCTION

The cell disruption of different food materials can be initiated by the pre-treatment with **Pulsed Electric Fields (PEF)** resulting in higher moisture release during the drying process. The study shows the effect of PEF treatment and the influence of treatment parameters, namely the **electrical field strength *E*** and **specific energy *W***, on the cell disruption of onions and detailing the impacts on the convective drying process at various drying temperatures.

MATERIAL AND METHODS

Characterisation of the cell disintegration by impedance measurement

The electrical conductivity σ is a characteristic factor for the cell disintegration of vegetable and animal cellular products following a PEF treatment. With rising cell disintegration, the electrical conductivity increases, because of the decreasing insulating effect of the cell membranes [1]. The conductivity disintegration index *Z* was determined using an impedance measurement system (DIL, German Institute of Food Technologies, Germany) and describes the relationship between the electrical conductivity of the PEF treated sample, intact material and totally destroyed material.

$$Z=\frac{(\sigma-\sigma_i)}{(\sigma_d-\sigma_i)}\ [-] \qquad 0 \le Z \le 1$$

Z: conductivity disintegration index
σ: electrical conductivity (measured at 0.5 kHz)
i: intact material (Z = 0)
d: totally destroyed material (Z = 1)

Drying trials

The drying curves of the drying process were created by recording the periodic change of the samples moisture load M_t [g/g] during drying under defined conditions (Tab. 1). The target residual moisture M_r [%] was set to 7 %.

Table 1: Drying conditions and experimental setup.

Drying type	Convection
Temperature [°C]	45 - 75
Air velocity [m/s]	0.2
Duration [h]	5 h or the necessary time to reach Mr <7 %
Sample preparation	Circular, r= 1.6 cm
Determination of initial moisture [%]	Moisture analyser
Determination of moisture load [g/g]	Gravimetric
Target residual moisture [%]	7

RESULTS AND FIGURES

The previous cell disintegration trials showed an increasing conductivity disintegration index *Z* at rising electrical field strengths. At the highest electrical field strength of 1.07 kV/cm and a specific energy of 16 kJ/kg the highest conductivity disintegration index of 1.0 was determined.

According to the course of the curves (Fig.1) a higher residual moisture for the PEF treated samples dried at 60 and 75 °C is present during the first drying phase. Followed by a rapid decrease in the second part of the drying process, resulting in a reduced drying time.

By varying the drying temperature the PEF treatment shows an accelerating effect on the drying speed of onions at all drying temperatures, whereby this effect increases with decreasing drying temperature. The highest accelerating effect of the PEF treatment on the drying process of 30 % could be determined at a drying temperature of 45 °C.

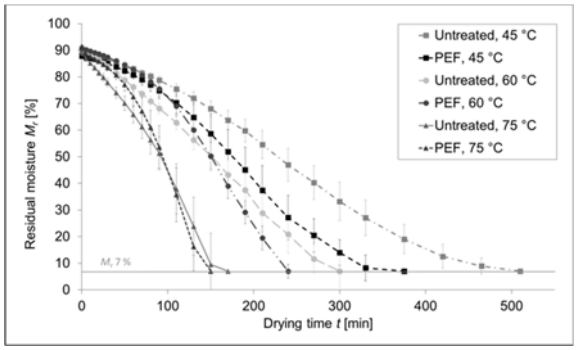

Figure 1: Residual moisture of untreated and PEF treated (E = 1.07 kV/cm, W = 4 kJ/kg) onions at different drying temperatures for a total drying period of 300 minutes or the necessary time to dry the samples to a M_r of 7 %.

CONCLUSION

- Rising electric field strength and increasing specific energy result in a **higher cell disintegration** of onions
- **Reduced time** of the convective drying of onions due to a PEF pre-treatment
- Maximum accelerating effect at low drying temperatures leads to a more **gentle drying process**
- Change in the **residual moisture distribution** throughout the drying process after applying PEF

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Effect of pulsed electric fields (PEF) application on bioaccumulation of magnesium in *Lactobacillus rhamnosus* B 442 cells

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INTRODUCTION

In the cells treated with PEF the induced transmembrane potential causes pore formation in the membrane and leads to an increase of its permeability [1]. At the optimized parameters of the process membrane electroporation is reversible [2]. Yeast or bacteria biomass enriched in Mg^{2+}, Zn^{2+}, Ca^{2+} or Se^{4+} becomes an alternative to cationic deficiency-related pharmacological supplementation. Bioelements provided in the form of metalloproteins are better assimilated by the body in comparison to the pharmacological preparations Food products containing bacteria enriched with the above mentioned elements could constitute in the future an additional source of them in a diet [3].

MATERIALS AND METHODS

Optimization of PEF parameters and ion concentrations in the medium in order to maximize Mg accumulation in *Lactobacillus rhamnosus* B 442 cells was carried out according to the scheme below:

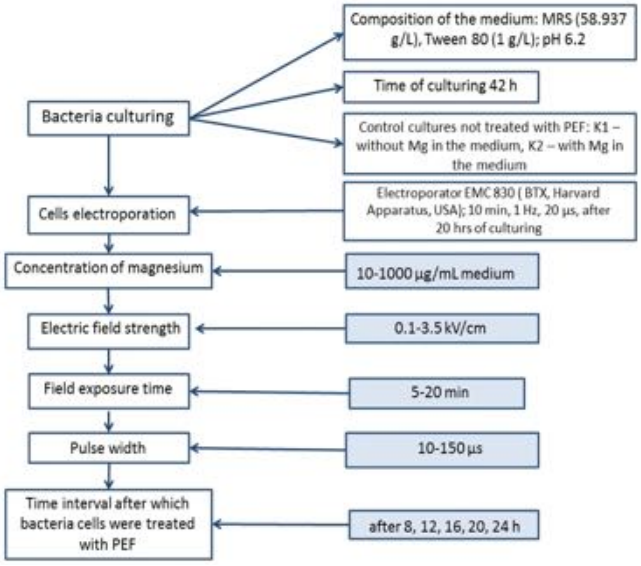

Figure 1: Scheme of the experiment

Ion concentration in the cells was determined using an electrothermal atomic absorption spectrophotometer (F-AAS), crop of bacteria biomass was estimated spectrophotometrically based on the standard curve, and the total number of microorganisms was determined by plate dilution method.

RESULTS AND FIGURES

The application of PEF increased the accumulation of Mg^{2+} in *Lactobacillus rhamnosus* B 442 cells. Maximum accumulation of this element (4.28 mg/g s.m.) was obtained at the following optimal parameters: concentration of magnesium ions 400 µg/mL medium, electric field strength 2.0 kV/cm, pulse width 20 µs, electroporation time 15 min after culturing for 20 h. Optimization of PEF parameters caused an increase of magnesium concentration in the cells by 220% in comparison to the control not treated with PEF (K2).

Bacteria biomass varied in the range from 0.201 to 0.281 g/g. Electroporation influenced also total number of microorganisms. Its value oscillated between 10^7 and 10^{10} cfu/ml.

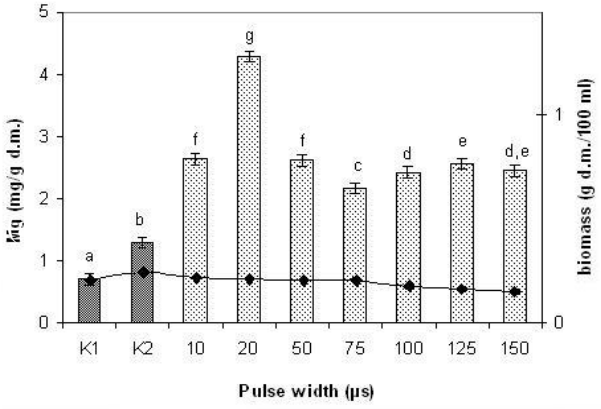

Figure 2: Effect of pulse width PEF treatment upon the magnesium accumulation in *Lb. rhamnosus* B 442.The same letters mean no significant differences between the results.

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The Epstein Barr Virus promotes nuclear reorganization in B lymphocytes

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INTRODUCTION

Burkitt's lymphoma (BL) is a highly proliferative B-cell tumor, in which the MYC allele located on chromosome 8, becomes translocated upstream of the IGH locus positioned on chromosome 14. Once MYC is juxtaposed to IGH, rearrangements involving MYC drive cells into lymphomagenesis through its deregulated expression (Gruhne et al., 2009). Numerous studies have shown that IGH and CMYC occupy different core regions within the nuclear space of naïve B lymphocytes (Roix et al., 2003). The drive from a lytic to latent EBV infectious cycle is primarily regulated by the immediate-early transcriptional transactivator *BZLF1* and its gene product, ZEBRA (Gruhne et al., 2009). Up to now, it is unclear how EBV induces this specific translocation in B lymphocytes.

Given that spatial proximity correlates with translocation frequencies, the study aimed to understand if EBV and its early lytic protein ZEBRA is capable of perturbing the nuclear organization in B lymphocytes and induce spatial proximity between the MYC and IGH loci.

MATERIALS AND METHODS

1. B lymphocytes isolated from healthy donors were infected with either EBV wild type or ZEBRA knock-out EBV viral copies at 10000 viral copies/million cells.
2. Infected B lymphocytes were processed for 3D Fluorescent In Situ Hybridization. Each hybridized sample was analysed using a Leica SP8 high resolution confocal microscopy whereby a multicolour excitation was captured, namely: DAPI using the UV laser at 458 nm to observe the nuclear space, Green emittance at 514 nm to observe IGH probes and Red/Orange emittance at 633 nm to observe CMYC probes.
3. The images were analysed with Bitplane Imaris software capable of measuring inter-genetic distances. Allelic CMYC/IGH regions of 1 µm or less were considered colocalized. At least 100 nuclei were analysed and subjected to statistical quantification. Each scan consisted of 15-20 layers (z-stacks) through the nuclear space.
4. The screening of *ZEBRA* was validated using cDNA amplified alongside *ZEBRA* oligonucleotides through Real Time Quantitative PCR (RTqPCR). The results were normalized by the gene expression for *GAPDH* and calibrated by the naïve B lymphocyte controls.

RESULTS AND CONCLUSION

The measurement of two loci located less than than 1 µm, is a vital event in the formation of cancer inducing translocations. We successfully visaulized the relocalization of *CMYC* towards the *IGH* locus region in EBV infected B lymphocytes. Here we confirmed an EBV driven recruitment of CMYC toward the centre of the nucleus. Preliminary investigations using a mutated EBV-Zebra knock-out encouraged our hypothesis of the direct involvement ZEBRA played in promoting *IGH* and *CMYC* loci proximity. Thus it is plausible that inappropriate activity due to EBV infection in B lymphocytes can lead to pathogenic rearrangements.

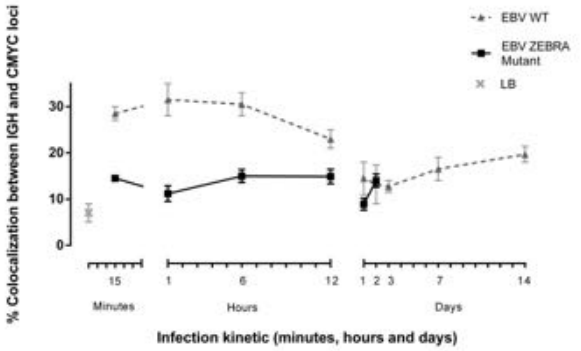

Figure 1: Frequency of colocalization between *IGH* and *CMYC* loci during EBV WT and EBV Zebra Mutant infection. *LB: Lymphocyte B*

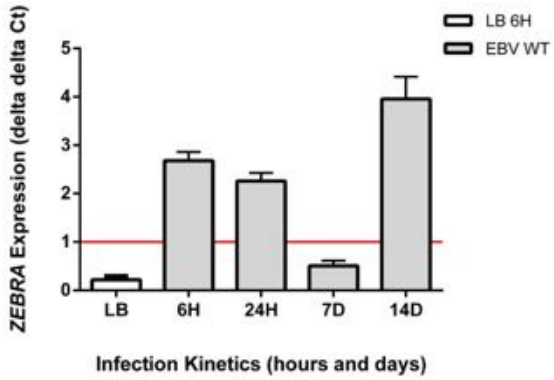

Figure 2: An over expression of ZEBRA (*BZLF1*) is observed in EBV WT infected B lymphocytes.

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Electrotransfection of plasmid DNA in human adherent cells

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INTRODUCTION

Electrotransfection is a physical method for gene delivery with pulsed electric field. Many clinical trials using electroporation are underway; however the basic mechanisms are not fully identified [1] [2].

My project consists in evaluating different parameters such as cell density, adherent cell type (shape), cycle phase, which could positively influence the transfection of plasmid, in order to define the exact role of these parameters, in order to improve gene delivery.

METHODS

Different cell types will be tested: keratinocyte, fibroblast, melanoma and HCT 116. The influence of the shapes and the cell density on the efficacy of electrotransfection of a GFP plasmid will be analysed by real time microscopy (Incucyte, Essenbio).

To identify the phase of the cell cycle most sensitive to electroporation I will use HCT 116 cells containing a plasmid Fucci (invitrogen), which emits a red fluorescence in G1 phase and green in G2 / M phase. In addition, I will quantify the percentage of transfected cells by flow cytometry.

RESULTS

In a preliminary study, different electric pulse parameters were tested: 0-100-200-300-400-500 V/cm 10 pulses of 5 ms at 1 Hz. In addition, two different concentrations of DNA plasmid GFP were tested: 5 or 10 µg/ml.

Micrographs were taken by the Incucyte microscope every 2 hours (at mag x 10) during 72 hours.

Based on tested parameters and conditions, the optimal electric field intensity is 400 V/cm and 10 µg/ml DNA plasmid. We obtain 5% of transfected cells, which is low in comparison with the study of Chopinet *et al.*, who obtained 10 % of transfected cells in a spheroid (3D cell model) and 20% in cell suspension [3]. Our working hypothesis to improve our transfection yield is to further evaluate the impact of cell division cycle and cell density on DNA plasmid transfection.

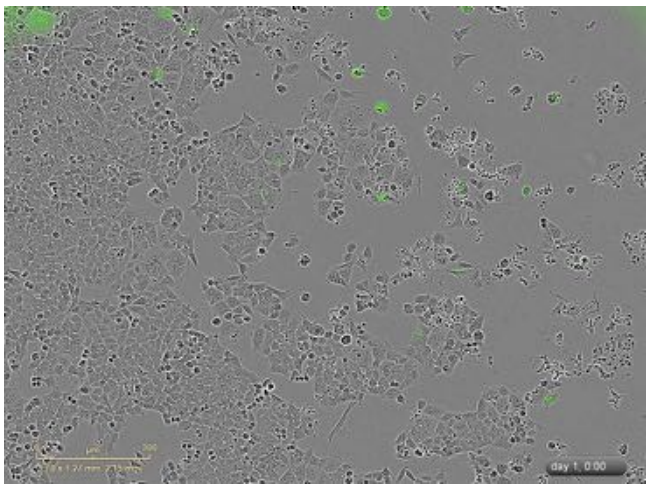

Figure 1: HCT 116 electrotranfection with DNA plasmid GFP [10µg/ml]; 400 V/cm 10 pulses of 5 ms at 1 Hz, 24h post-transfection.

ACKNOWLEDGEMENTS

LEA EBAM, COST TD1104

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The influence of microsecond electroporation on human glioblastoma cells– *in vitro* study

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INTRODUCTION

Glioblastoma is a malignant brain tumor which stem cells are resistant to chemotherapy and radiotherapy. Even with current treatments, patients with glioblastomas tend to have poor survival rates [1]. Nowadays, the greatest hopes for improving life expectancy of sick patients are connected with experimental therapies such as the microsecond electroporation. Our study aims to investigate the influence of different concentrations of calcium on the cytotoxicity of glioblastoma cells. In order to extend the treatment efficacy in this study we used calcium combined with electroporation [2,3].

METHODS

The experiments were performed on the SNB19 cell line (human glioblastoma cell line). Cells were grown under sterile conditions in monolayer culture bottles. SNB19 cells were electroporated in the presence and absence of calcium ions. Evaluation of mitochondrial activity was performed by the MTT cytotoxicity assay (3- (4,5-Dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide) after 24 h and 72 h incubation. Further, cells were incubated with or without calcium ions. Additionally, immunocytochemical evaluation of the MGMT (O6- alkylguanine DNA alkyltransferase) and apoptosis markers: caspase-3, caspase-8, caspase-12.

RESULTS

here was no significant impact of EP in a range of 400 to 1000 V/cm on cells' viability. A 4-fold drop in mitochondrial anctivity was observed following the enhancement of Ca transport with electroporation. The results show that the appropriately selected electroporation parameters (electric field strength and calcium concentration) can efficiently eliminate the glioma cells. The results of immunocytochemical staining indicate elevated expression of proteins involved in the apoptosis process. As the intensity of the electric field increases, we observed a strong coloration in the microscope images showing a higher cell death rate. With the μsPEF method, glioblastoma cells are killed by apoptosis, as demonstrated by the activation of caspases. The results suggest the final stage of activation of caspase 12 as a result of stress induced by electroporation with excessive influx of calcium ions. Caspase 12 and Caspase 8, in the case of stronger expression, are at the activation stage of caspase 3. Activation of this pathway is most likely due to the effect of external electrical field and rapid increase of concentration of Ca^{2+} ions in the cytoplasm. As a result of such oxidative stress, electrolyte transport disorders and DNA damage occur.

Calcium can enhance the effectiveness of CaEP. Calcium chloride, compared to standardized cytostatics, at appropriately selected concentrations exhibits low toxicity to healthy cells, which was confirmed in other studies [2,3]. In addition, it is generally cheaper than cytostatics, widely available and easier to administer to patients. Moreover it can potentially reduce the number of side effects when compared to standard cytostatic drugs such as cisplatin or bleomycin.

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The potential role of catechin in the electrochemotherapy of sensitive and resistant pancreatic cancer – *in vitro* study

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INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies and of increasing incidence. Nowadays, the greatest hopes for improving treatment efficacy are mainly associated with experimental therapies, including electroporation. Unfortunately, due to intrinsic and acquired drug resistance even the enhancement of the intracellular drug transport may be insufficient. Therefore, many studies currently focus on the modulation of drug resistance. It has been shown before that green tea polyphenols exhibit strong anticancer activity by targeting several cells signaling pathways that are relevant to cancer development [1]. In our study we investigate the role of green tea catechin preincubation on the effectiveness of electroporation with common cytostatic drugs. We focused particularly on the disturbances in the functioning of one of the major proteins responsible for multi-drug resistance – ABCB1 (also known as P-glycoprotein or MDR1).

METHODS

The research material consisted of 3 pancreatic cancer cell lines: EPP85-181P (sensitive to daunorubicin), EPP85-181RNOV (multi-drug resistant) and primary cell culture derived from pulmonary metastasis from pancreatic cancer. Prior to experiments we assessed the level of ABCB1 fluorescence in each cell line using confocal microscopy. We also evaluated catechin cytotoxicity and the influence of catechin preincubation on the expression levels of caspase-3 as an early apoptotic marker and superoxide dismutase to detect oxidative stress response. Within the experiment cells were preincubated in medium containing catechin of concentration 10 μM for 24 h or 50 μM for 2 h. Following chemotoxicity tests we performed electroporation with EP buffer with 5 and 10 μM concentration of cisplatin (CisEP), using the electric field strength in range 600÷1000 V/cm. Additionally, we performed electroporation with 25 and 50 μM catechin (CatEP). Cell viability was measured via MTT assay after 24 and 72 hours of incubation. The expression of ABCB1 protein was evaluated with immunocytochemical approach. In order to study the potential effect of catechin on drug uptake we performed electroporation with propidium iodide (2 μM) and daunorubicin (2 μM) by flow cytometry method.

RESULTS

There was no effect of CatEP on cells viability. After CisEP we observed a significant drop of mitochondrial activity in preincubated cells, especially when cisplatin was administered simultaneously with 10 μM catechin. However, this effect was noticeable only for drug-resistant cell line. There was a visible decrease in the expression of ABCB1 protein following the incubation with 50 μM catechin for 2 h or 10 μM for 24 h. We found no increase in the uptake of propidium iodide and daunorubicin resulting from catechin preincubation. Catechin preincubation had also no impact on the expression levels of caspase 3 and superoxide dismutase.

CONCLUSIONS

Catechin can enhance the effectiveness of CisEP. Significant response in drug-resistant cells and lack of increased fluorescence after EP with daunorubicin and propidium iodide suggest that the effect may be attributed to the drop in the levels of MDR1 protein which is usually overexpressed in resistant tumors. Rapid change of MDR1 levels after incubation suggests that catechin acts presumable via non-genomic, competitive mechanism.

Acknowledgments: This work was supported by the grant STM.A040.17.039 and Statutory Funds of UMED No. ST.C010.16.086.

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Endoscopic assisted electrochemotherapy for sigmoid colon cancer: a phase I clinical trial

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INTRODUCTION

The aim of this phase I, clinical trial is to establish the safety and efficacy of treating patients with sigmoid colon cancer with electrochemotherapy as an additive treatment prior to intended curative surgery, using an endoscopic electroporation device (EndoVE®).

BACKGROUND

Electrochemotherapy (ECT) involves application of brief electrical pulses which reversibly destabilize the cell membrane, allowing intracellular access of highly immunogenic chemotherapeutic drugs that otherwise would not penetrate the cell membrane [1].

Increasing evidence suggests that ECT may induce favorable immunogenic changes in the tumor tissue [2,3]. We aim to investigate the direct treatment effects across potential curative tumor stages in the sigmoid colon. We hypothesize that in situ tumor ablation by endoscopic ECT will cause tumor regression and downsizing due to direct ECT mediated tumor elimination. Furthermore, we aim to provide the evidence that ECT may provide the foundation for treatment of micrometastatic disease due to release of tumor-associated antigens and dissemination of immunogenic tumor response prior to surgical removal of the primary tumor. Our vision is that ECT based neoadjuvant treatment will improve the oncological outcome for patients undergoing treatment for colorectal cancer by reducing the risk of disease recurrence.

STUDY DESIGN

The study involves recruitment of 9 patients undergoing elective surgery for histologically verified, sigmoid colon cancer, UICC stage I-III. In this study we intend to provide the ECT treatment within one week after recruitment. Surgery will be scheduled approximately 14 days after ECT treatment.

Intraluminal biopsies from the sigmoid tumor will be collected just before bleomycin is induced. Furthermore, after the elective surgery, samples will be collected from the tumor. Tissue collected before the ECT treatment will be characterized and compared with tumor tissue collected after elective resection of the sigmoid tumor.

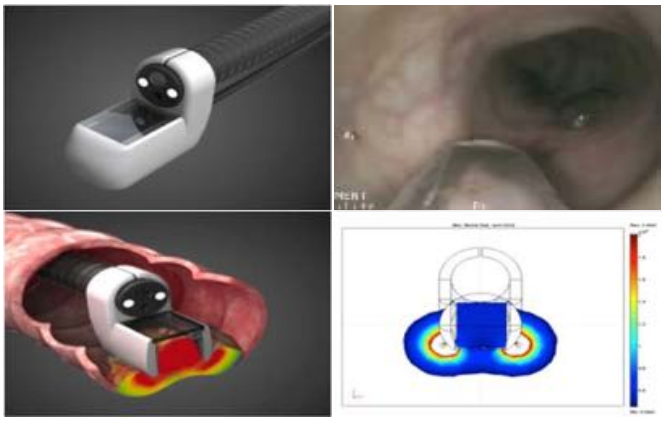
Additionally, blood samples will be collected before and after the ECT treatment. Systemic immunological response will be analyzed. Furthermore, circulating levels of ctDNA and cfDNA will be measured.

Any adverse events reported will be evaluated, including tissue perforation and EndoVE® device related complications. The efficacy of the treatment will be evaluated according to the pathological examination of the surgical specimen. As such, final tumor staging, T-cell infiltration of the primary tumor, and PD1/PDL-1 status after treatment are considered endpoints parameters. Furthermore, systemic response will be analyzed. As such, immunological response and ctDNA and cfDNA will be analyzed.

STUDY TREATEMNT

A concomitant bolus injection of bleomycin (15,000 IU Bleomycin x Body Surface Area) will be administered intravenously. Eight minutes after intravenous administration is completed the endoscopic procedure can commence with permeabilization of the tumor tissue with electroporation pulses. The electrical pulses will be delivered in a series of eight wave pulses of 100 µsec, 4 Hz, 1000 v/cm.

The endoscopic device (EndoVE) is designed as a single-use, vacuum assisted electrode attachment for a standard endoscope. By applying vacuum suction, cancer tissue is drawn into the treatment chamber and subjected to electroporation through an external pulse generator (Cliniporator).



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Our experience with electrochemotherapy of cutaneous and subcutaneous tumors in the head and neck region

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INTRODUCTION

Surgical treatments of malignant tumors of the head and neck region, especially in case of locally advanced tumors, may cause considerable morbidity, particularly in visible areas, such as the face, with consequent unacceptable cosmetic outcomes and/or functional impairments. Alternatives to surgery may be preferred under certain circumstances. Electrochemotherapy (ECT) is an effective and safe method for local control of cutaneous and subcutaneous tumors. The goal of ECT is complete eradication of the tumor with preservation of the surrounding structures in an aesthetically acceptable manner. [1,2]

MATERIALS AND METHODS

Between 2007 and 2017, 22 patients (15 male and 7 female; mean age: 61-year) with head and neck localized tumors were treated with ECT in our department. Most of the patients (15) suffered from non-melanoma skin cancer (NMSC), 3 patients were treated because of cutaneous metastases of malignant melanoma and 4 of other histological type of tumors (1 Merkel-cell carcinoma, 1 angiosarcoma, 1 malignant Schwannoma and 1 hypopharynx SCC). Treatments were performed according to the ESOPE (European Standard Operating Procedures of Electrochemotherapy) guideline. All treated patients received Bleomycin, the administration of the drug was intravenous for 13 patients and intratumoral for 7 patients. Patients were treated under general anesthesia. All treatments were carried out using the Cliniporator TM device. If necessary ECT sessions were repeated at 2-monthly intervals. [3]

RESULTS

Overall, 156 tumors were treated in the head and neck area. Response rate was determined at least 60 days following ECT in each case. Objective response was over 96% (151/156), complete response was achieved in 50% (78/156) of the treated tumors. No relapse was observed during follow-up period, which was at least 3 months (3-46 months). Excellent cosmetic and/or functional results were achieved meeting the expectations of our patients. Partial response was seen in 46.7% (73/156) of cases, there was no change in 3.2% (5/156) of the lesions, and we experienced progression in 0.6 % (1/156) of the treated tumors. Progression and stable disease usually were seen in case of locally advanced tumors (>3 cm) (Figure 1) and neoplasms with aggressive biological behaviour (malignant melanoma, Merkell cell cc.). In treated lesions > 3cm in diameter we experienced high-grade ulceration, which required special wound management. Moreover, mild and transient side-effects were noted and the treatment was well tolerated by the patients.

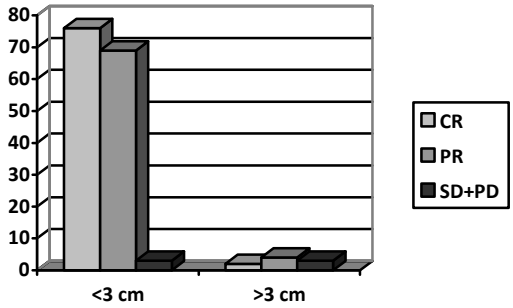

Figure 1: Size distribution of the tumors and their responses

CONCLUSION

In our experience, in case of multifocal or extensive tumors, which are not amenable to simple surgical treatment in cosmetically and functionally sensitive areas, ECT is the treatment of choice. ECT can provide good local control of the tumors with preservation of organ function and acceptable aesthetic result. After treatment of bigger (> 3cm) lesions both patient and allied health personnel should be prepared of prolonged wound healing.

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Confirmatory Trial in the Evaluation of Ca electroporation for the Treatment of Cutaneous Metastases, Preliminary results

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INTRODUCTION

Bleomycine based electrochemotherapy (ECT) became a widespread method in the treatment of cutaneous/subcutaneous tumours, and has been recently added in the clinical practice. [1].

In vitro and *in vivo* studies have shown that the combination of calcium and electroporation is an effective method in killing cancer cells without serious side effects [2]. This new combination opens the possibility of replacing bleomycin with calcium in treatments with electroporation.

The aim of this randomized confirmatory trial going on since November 2016 is to evaluate and compare response rate of calcium electroporation and electrochemotherapy with bleomycin on cutaneous metastases.

METHODS

Six patients with cutaneous metastases of malignant melanoma (5), and breast cancer (1) were treated according to the protocol. It was a once only treatment and the patients were followed up with regular clinical controls.

Altogether 39 metastases were randomized, than bleomycin or calcium was administered intratumoraly, followed by electric pulses, which were generated using a Cliniporator device according to the ESOPE criteria (European Standard Operating Procedure of Electrochemotherapy) [3].In 11 cases linear, in 28 cases hexagonal electrodes were used. According to the protocol 180 days after treatment the randomization code was revealed and biopsies were taken from both calcium and bleomycin electroporation treated metastases. Response evaluation was performed according to RECIST-like guidelines [4].

RESULTS AND FIGURES

Here we report the results of the first 4 patients who are through the 180 day visit with a total of 23 cutaneous metastases from malignant melanoma and breast cancer. Altogether 16 metastases were evaluated for response, another 7 metastases were used for biopsy.

9 of them were treated with Ca-electroporation and 7 had ECT with bleomycin. Results are shown in Figure 1.

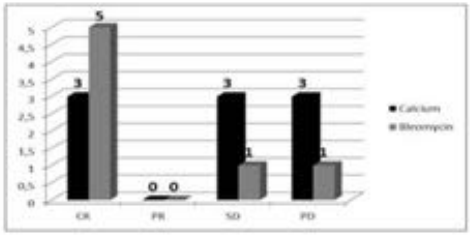

Figure 1: Tumor response

Altogether 8 tumours (50%) presented complete response (CR), 4 (25%) had no change (SD), and we experienced on 4 (25%) metastases progressive disease (PD) after treatment.

180 days after treatment biopses were taken, and proved to be negative in half of the calcium- (2/4), and more than half of the bleomycin cases (2/3). Figure 2.

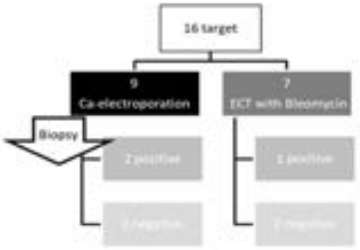

Figure 2: results of the biopsies

CONCLUSIONS

Calcium electroporation is a simple and inexpensive cancer treatment that does not involve any administration of chemotherapeutic drugs therefore it would be useful in cases when chemotherapy is contradicted (e.g. severe lung functions impairment, pregnancy etc.)

The final result of this study will provide further evidence about the effectiveness of Ca-electroporation for metastatic cutaneous cancer.

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Electrochemotherapy Induced Tissue Changes in Porcine Liver: Study Protocol

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INTRODUCTION

Electrochemotherapy (ECT) is a novel method of cancer treatment which combines the use of electroporation and cytotoxic drugs [1]. It has recently been used to treat colorectal liver metastases, which were not amenable to surgery or local ablation techniques [2]. Despite promising early results and no reports of ECT related serious adverse effects, there is scarce data regarding histological changes caused by ECT in both normal and tumour tissue.

In a recently published follow-up analysis of liver tissue specimens from patients included in the first clinical trial, tissue damage with destruction of liver parenchyma, tumour cells, small vessels and bile ducts was observed near electrodes which were used to deliver electrical pulses. However, functionality of blood vessels larger than 5 mm in diameter and biliary structures was mostly preserved which has important clinical implications for the use of ECT for treatment of inoperable tumours adjacent to major hepatic blood vessels and other vital structures [3].

In this abstract we present the design of an animal model study to further evaluate the effects of ECT on normal liver tissue, especially major blood vessels and bile ducts.

AIMS

The main aim of our proposed animal model study is to study the effects of ECT on normal liver tissue using histological analysis. We will compare tissue changes after using different types of electrodes (linear, hexagonal) at different time intervals as well as compare the ECT induced changes to a control group in which only electrical pulses without cytotoxic drugs will be used. The secondary aims of our study are also to evaluate ECT induced changes using radiological analysis, laboratory testing of blood samples and measurement of electrical parameters during and after the procedure.

METHODS

The proposed study will be carried out on a total of seven female domestic pigs (*sus scrofa domesticus)* (Table 1). We chose domestic pigs as our test animals because porcine liver closely relates in size, anatomy and physiology to human liver. Pigs are also often used to test novel treatment methods for hepatic tumours [4]. Our study was approved by the Ethics Committee at the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection.

Table 1: Test animals.

Number	Time to euthanasia	Electrode type	Treatment type
Pre-Study	2 days	Linear	ECT
1	7 days	Linear	ECT
2	7 days	Hexagonal	ECT
3	2 days	Linear	ECT
4	2 days	Hexagonal	ECT
5	7 days	Linear	Pulses only
6	2 days	Linear	Pulses only

ECT will be performed *in vivo* on test animals under general anaesthesia in four different anatomical locations on the liver (caudal vein, hepatic veins, portal veins and liver parenchyma) using bleomycin and different electrode types. Electrode location will be confirmed using intraoperative ultrasound guidance. After a predetermined time, the animals will be euthanised and the liver explanted for histological analysis. Contrast enhanced computed tomography of the liver will be performed immediately after the procedure to look for possible changes in liver blood flow. Blood samples will also be collected to study biochemical changes due to ECT. Electrical parameters will be validated using real time voltage, current and impedance measurements.

EXPECTED RESULTS

We expect to demonstrate that ECT of the liver is safe and feasible even for treatment of tumours in close proximity or in direct contact with major hepatic blood vessels and bile duct. Furthermore, we hope to elucidate the amount and type of normal liver tissue damage in regard to the type of electrodes used for ECT.

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Electro-Thermal Modeling and Characterization of Irreversible Electroporation of Large Tissues for Cancer Treatment

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INTRODUCTION

This research will focus on: Irreversible Electroporation (IRE) as one of the most promising therapies for cancer treatment. Although it is a relatively new technique [1], a significant number of tests have already been performed.

IRE requires of extensive experimentation in order to optimize the process and maximize the desired cell destruction. Besides, unlike purely thermal treatments like radiofrequency or microwaves ablation, it requires in vivo experiment to assess its biological effects [2].

The aim of this research is therefore to propose a finite element analysis (FEA) based model of the irreversible electroporation process taking into account both electrical and thermal effects. The accuracy of the proposed model is verified by comparing with experimental results during on in vivo IRE experiment [3].

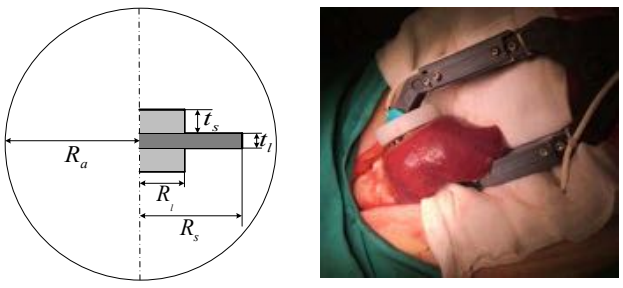
METHODS

The proposed FEA (**Napaka! Vira sklicevanja ni bilo mogoče najti.**(a)) model is based on a 2D geometry with symmetry of revolution. The parameters that define the geometry are summarized in **Napaka! Vira sklicevanja ni bilo mogoče najti.**. The parameters described in **Napaka! Vira sklicevanja ni bilo mogoče najti.** are those representing the laboratory conditions (**Napaka! Vira sklicevanja ni bilo mogoče najti.**(b)).

The geometry is composed of a sphere of air; inside of them, two cylindrical steel electrodes will be found. Between the two electrodes is placed a geometry representing the tissue. The model has been developed with the software COMSOL Multiphysics.

Table 1: Geometric parameters

Symbol	Quantity	Value
t_l	Thickness (Liver)	1.5 cm
t_s	Thickness (Steel)	0.8 cm
R_a	Radius (Air)	10 cm
R_l	Radius (Liver)	5 cm
Rs	Radius (Steel)	1.5 cm



(a) (b)

Figure 1: Images representing the treatment: (a) 2D model outline, (b) real application.

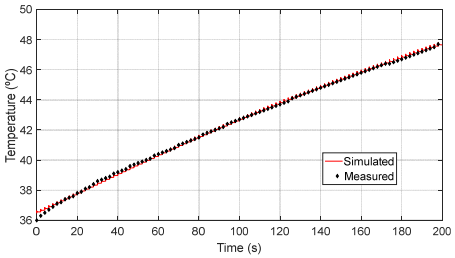


Figure 2: Comparison of experimental and simulated thermal evolution in the center of the liver.

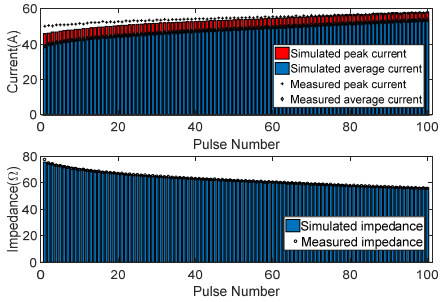


Figure 3: Comparison of experimental and simulated electrical signals.

RESULTS

The experimental and simulation results are compared for a treatment with 100 symmetrical unipolar 3000-V pulses, with a separation of 2 s between pulses. Voltage and current were monitorized using a high performance digital oscilloscope, and temperature was measured with a thermocouple. The impedance is calculated by using the average current and voltage of each pulse $Z_{avg}=V_{avg}/I_{avg}$.

The results obtained are shown in figures Figure 2 and **Napaka! Vira sklicevanja ni bilo mogoče najti.**. With the proposed methodology, it has been possible to adequately reproduce both the average impedance, Z_{avg} and temperature values over a complete treatment.

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Numerical reconstruction of electrochemotherapy treatment in the spine

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INTRODUCTION

Metastases in the vertebral column are a common complication in cancer patients which greatly decreases the patients' quality of life. It has been demonstrated in preclinical and clinical studies that bone metastases can be efficiently treated by electrochemotherapy (ECT). For ECT to be successful an adequate concentration of chemotherapeutic drug and sufficiently high electric field need to be established in the whole tumor volume.

The case presented by Gasbarrini *et al* [1] is the first reported clinical case using ECT to treat spinal metastases. No ECT-related adverse events were observed during the procedure and overall improvement in pain outcome and global function was excellent. We have numerically reconstructed the case presented by Gasbarrini *et al* to determine the electric fields during the treatment and extract dielectric properties of the treated tissues. The aim of our study was to develop a numerical framework suited for planning electroporation (EP) based treatments in the spine.

METHODS

An anatomically accurate 3D numerical model of the vertebra was built using pre- and intra-operative images and measurements gathered during the procedure. All relevant anatomical structures (tissues) were segmented using 3D slicer (https://www.slicer.org) and a 3D model was built in Matlab (Mathworks, Natick, MA) computing environment.

Four virtual needle electrodes were inserted into the 3D model, forming six active electrode pairs. Intraoperative photographs, fluoroscopic control images and measured distances between electrode pairs were used to reconstruct electrode positions. The anatomical model with inserted electrodes is presented in Figure 1.The electrical properties of included tissues – thresholds for reversible/irreversible EP, electrical conductivity before and after EP of tissue – were determined from available literature and through numerical fitting algorithms.

A previously designed numerical framework for treatment planning of deep seated tumors was used for the numerical computations [3]. All numerical computations were performed in Comsol Multiphysics 5.2 (COMSOL Inc., Sweden) finite element analysis software. Electric field distribution and electric currents were calculated for each electrode pair. Calculated electric currents were then used for fitting of tumor tissue conductivity. We designed an algorithm for fitting of tumor tissue conductivity increase after EP based on the mean square error between numerically calculated and measured electric currents. Bone tissue conductivity was also fitted, using data gathered from previous studies [2].

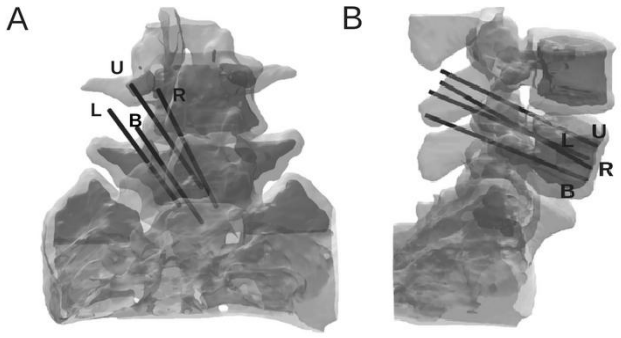

Figure 3: A 3D model developed for the numerical reconstruction of the clinical case presented in **[1]**.

RESULTS AND CONCLUSIONS

At the end of tissue conductivity fitting the mean square error between calculated and measured electric current was 2,61 A, while the maximum relative error was 16 %. Numerical reconstruction of the case predicted 100 % coverage of tumor tissue with electric field above the reversible EP threshold indicating a successful treatment. Complete coverage of tumor tissue was achieved already after the third delivered pulse sequence out of six sequences total, but delivery to all electrode pairs increases treatment robustness by ensuring electroporation of potential micrometastases around the macroscopic target metastasis. Further studies on electrical properties of tissues and effects of EP on vital anatomical structures are still needed to fully understand the potential of ECT to treat spinal tumors.

ACKNOWLEDGEMENT

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Effects of calcium electroporation on normal and tumour vasculature

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INTRODUCTION

Calcium electroporation (EP) is gaining on its value as anti-cancer treatment. Recently, it was demonstrated that calcium EP effectively reduces survival and growth of different cells, spheroids and tumours. This effect is supposed to be differential for cancer and normal cells and can therefore be less toxic for surrounding normal tissue *in vivo* [1–3]. Anti-tumour properties of calcium EP have already been demonstrated [4], however its effects on the vasculature were not investigated yet, which was the aim of our study.

METHODS

The anti-vascular effect of calcium EP was evaluated by intravital microscopy of normal and tumour vasculature in dorsal window chamber (DWC) model in C57Bl/6 mice. DWC was implanted surgically onto the extended double layer of skin. To evaluate effect on tumour blood vessels B16F1 GFP tumours were induced (7×10^7 cells/ml) by intradermal injection into the skin in DWC. Normal, as well as tumour blood vessels were imaged from day 5 after the implantation of DWC (day 0). The treatment was performed by injection of either 5 µl of MiliQ water, $CaCl_2$ (50 mM, 168 mM, 250 mM) or bleomycin (1.4 mM) into tumours or dermis of intact skin and then electric pulses (EP: 8 square-wave pulses, 1300 V/cm voltage to distance ratio, 100 µs, 1 Hz) were applied. After treatment, DWC were imaged for 3 days, then Rhodamine B labelled dextran (70 kDa) was injected into the orbital plexus and images of fluorescent vessels were taken. The experimental procedures were performed in compliance with the guidelines for animal experiments of the EU directive (2010/63/EU) and the permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. U34401-1/2015/16).

RESULTS

Calcium EP affects normal blood vessels *in vivo*, as it disrupts the vessels and caused tissue damage, similarly to electrochemotherapy with bleomycin. In all cases, larger vessels appear to be less affected. After injection of 250 mM calcium, vessels were damaged even without electroporation, whereas 168 mM and 50 mM caused no apparent damage. This effect was amplified in combination with electroporation for all tested calcium concentrations, where severe damage of vessels and skin was observed. Anti-tumour and anti-vascular effects were also visible in B16F1 GFP tumours, where calcium EP caused almost immediate tumour necrosis (within 1 h), in contrast to electrochemotherapy with bleomycin, which caused gradual tumour death (within 24 h).

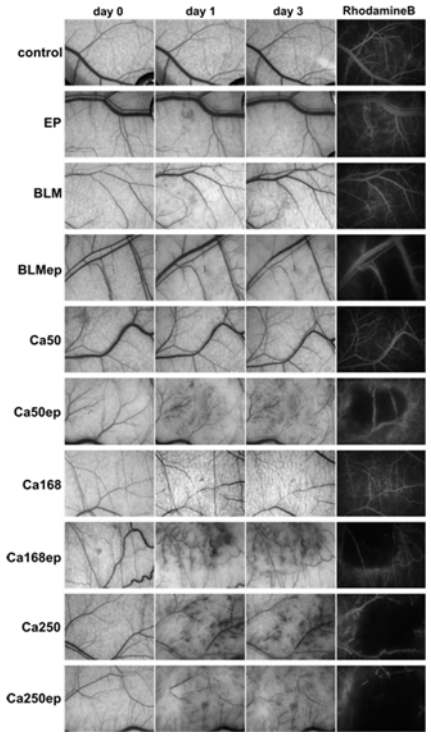

Figure 1: Damage of normal vessels after calcium EP.

CONCLUSIONS

Calcium electroporation affects both, normal and tumour blood vessels. The effect is more pronounced in small vessel and it is dependent on the calcium dose.

ACKNOWLEDGEMENTS

Research was funded by the Slovenian Research Agency and conducted in the scope of EBAM European Associated Laboratory (LEA).

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Anthropogenic carbon nanotubes: from tailpipes to human lungs

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INTRODUCTION

Fine particulate matter (PM) of less than 2.5 μm in diameter was ranked as one of the leading causes of death and disability worldwide. Yet, the components of the particulate mix, which penetrate the lungs and could be responsible for deleterious health effects, have not been comprehensively examined. The objective of this work was to characterize the ambient PM and to compare it with PM found in the lungs of Parisian children [1].

METHODS

Transmission electron microscopy (TEM), high-resolution TEM (HRTEM), energy dispersive X-ray spectroscopy (EDX), and near infrared fluorescence microscopy (NIRFM) were used to characterize PM. Particulate matter was characterized in vehicle tailpipes, in outdoor air, and in 69 biological samples obtained from asthmatic Parisian children (the samples involved either broncho-alveolar lavage fluid-BALF extracts or alveolar macrophages).

RESULTS

The PM similar to that found in car exhaust (Figure 1A) and ambient air (Figure 1B), was found in all BALF samples. The EDX microanalysis confirmed the carbonaceous origin of airborne and inhaled nanoparticles and while nano-spherules were mainly made of amorphous carbon, fibrous structures corresponded to organized carbon nanostructures.

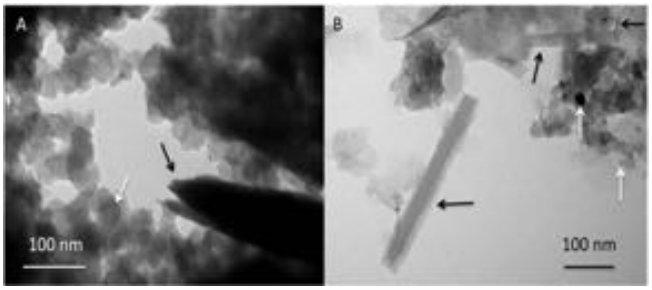

Figure 1: Representative transmission electron microscopy micrographs of particulate matter (PM) found in A) Tailpipe of a diesel-engine car and B) Dusts collected in ambient air in the Paris urban area. (White arrows point to spherical and black arrows to fibrous PM.)

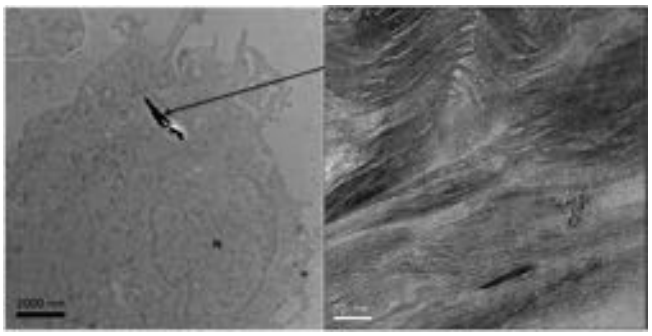

Figure 2: Representative TEM micrograph of alveolar macrophage (left) containing particulate matter (PM) (right). (Black arrow and magnified view on the right hand side show fibrous PM. The "N" denotes the cell's nucleus).

Inside the cells, PM was rare (Figure 2). While both carbonaceous spherules and fibres could be found, the fibres were prevailing. The parallel fringes observed by HRTEM (Figure 2- right) had an average spacing of 0.33 nm, which is characteristic for multi walled carbon nanotubes. In addition (not shown on figure), some fibrous structures exhibited an average interlayer spacing of 0.70 nm, which can be attributed to single walled carbon nanotubes (SWNTs) bundles. The presence of semiconducting individualized SWNTs was confirmed by NIRFM.

CONCLUSIONS

Humans are routinely exposed to anthropogenic carbon nanotubes. The remaining challenge is to determine if carbon nanotubes are the markers of atmospheric pollution or if they are active contributors, directly or indirectly [2] involved in the onset of different pathological conditions.

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Effect of electrochemotherapy in combination with BRAF inhibitors on melanoma cell lines *in vitro*

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INTRODUCTION

Electrochemotherapy (ECT) is increasingly being used as a therapy for skin tumors of various histological origins.[1] Among the newest treatment approaches for melanoma are target drugs that target mutated BRAF gene.[2] ECT could be used in patients who have already received or are receiving therapy with drugs that target mutated BRAF gene.

METHODS

ECT with bleomycin was performed on two human melanoma cell lines, with (SK-MEL-28) or without (CHL-1) BRAF V600E mutation. Cell survival was determined using clonogenic assay. Furthermore, the effectiveness of ECT in concomitant treatment with BRAF inhibitor vemurafenib was also determined with clonogenic assay.

RESULTS

ECT effectively reduced survival of both cell lines with IC_{90} 3.8 x 10^{-10} M and without BRAF V600E mutation IC_{90} 7.7 x 10^{-10} M. ECT was even more effective on BRAF mutated cells that required 2 times lower concentration of BLM at IC_{90}, compared to non-mutated cells (Figure 1).

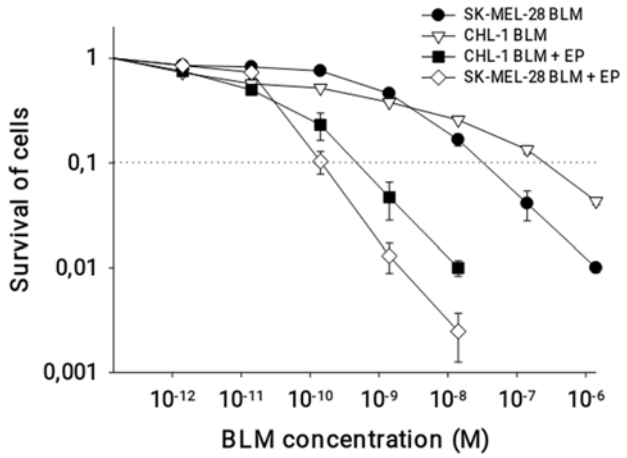

Figure 1: Survival of BRAF V600E mutated SK-MEL-28 melanoma cells and non-mutated CHL-1 cells after ECT with bleomycin; BLM + EP (ECT with bleomycin). Dotted line represents the IC_{90} value.

Concomitant treatment with ECT and vemurafenib was simulated *in vitro* on cells in the way, that ECT treated cells were seeded into dishes containing 0.5 μM vemurafenib. The vemurafenib treatment decreased survival of BRAF mutated cells for 50%. A 4.5 times lower concentration of BLM was needed at the IC_{90} for cells treated with vemurafenib IC_{90} value 8.5 x 10^{-11} M compared to cells without vemurafenib treatment IC_{90} value 3.8 x 10^{-10} M (Figure 2). The potentiation was more than additive, in fact, according to the method developed by Spector *et al.* was synergistic.[3]

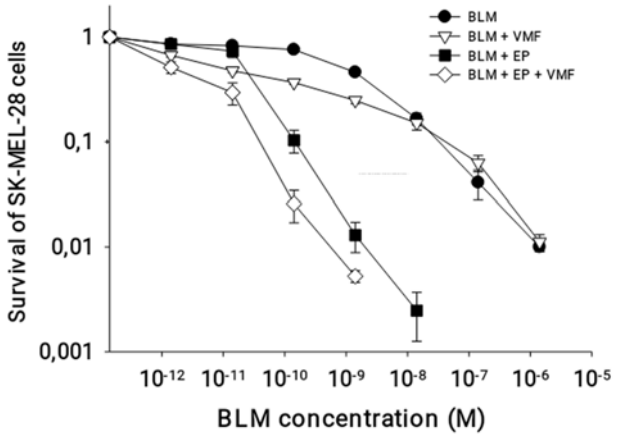

Figure 2: Survival of BRAF V600E mutated SK-MEL-28 melanoma cells after concomitant treatment with ECT and vemurafenib; BLM + VMF (bleomycin and 0.5 μM vemurafenib); BLM + EP (ECT with bleomycin) BLM + EP + VMF (ECT with bleomycin and 0.5 μM vemurafenib). Dotted line represents the IC_{90} value.

CONCLUSIONS

The effectiveness of ECT in BRAF mutated melanoma cells as well as potentiation of its effectiveness during the treatment with vemurafenib *in vitro* implies on clinical applicability of ECT in melanoma patients with BRAF mutation and/or during the treatment with BRAF inhibitors.

ACKNOWLEDGEMENTS

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Electroporation Enhanced by Gold Nanoparticles

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INTRODUCTION

Electroporation is a way of increasing membrane permeability and allowing different molecules to enter the cell [1]. It has been shown, that gold nanoparticles (Au NPs) enhanced transfection by electroporation [2]. It has been predicted that highly conductive NPs locally enhance electric field [3] and enable electroporation at lower electric fields. In order to test this hypothesis, we incubated CHO cells with Au NPs of two different sizes (5 nm and 20 nm) and then exposed them to high voltage electric pulses.

MATERIAL AND METHODS

A stock solution of Au NPs (Sigma Aldrich) was diluted in electroporation buffer to 10, 25 and 50 µg/ml. Cells were re-suspended in nanoparticles and incubated for 1 hour at 4°C before the start of the experiment. Cells without nanoparticles represented control.

Pulse generator made at the University of Ljubljana, Faculty of Electrical Engineering was applied. Before treatment sample was mixed Propidium Iodide (PI) or YO-PRO®-1 (YP). Square pulses 8x100 µs, 1 Hz repetition frequency of different voltages was applied between stainless steel plate electrodes (d = 2 mm).

Cells were analyzed using flow cytometer. Cell survival was assessed via metabolic activity MTS assay.

ANOVA was used as a statistical tool with $p<0.05$.

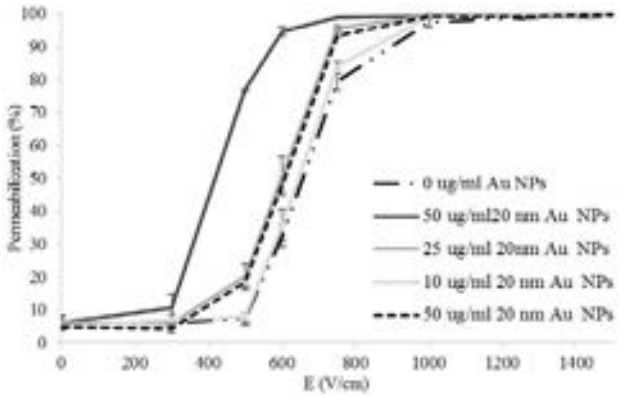

Figure 1: Percentage of permeabilized cells by PI with PEF treatment at different concentration of Au NPs, N=3

RESULTS

In Figure 1 we can see that cells with the 50 µg/ml of 20 nm Au NPs are statically significant more PI permeabilized than cells without NPs at the same electric field. Results with YP are similar, although threshold is at a higher electric field in comparison to PI (data not shown). Difference in permeabilization to PI between 10 µg/ml and 25 µg/ml NPs is statically significant. There is no significant difference between control and 10 µg/ml NPs. The effect of 5 nm NPs is less obvious than 20 nm NPs (50 µg/ml), but the statically significant difference with respect to control is still obtained.

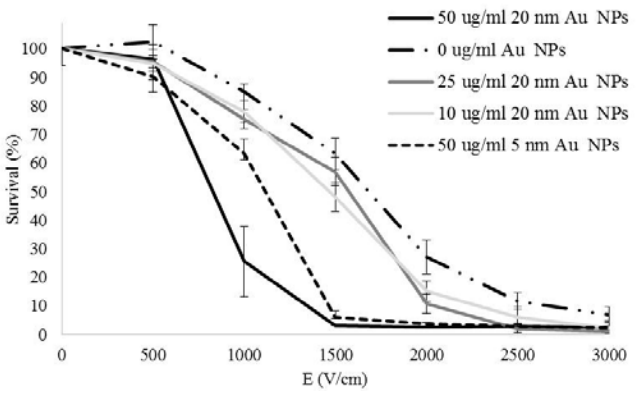

Figure 2: Survival of PEF treated cells at different concentration of Au NPs, N=3

NPs also affect cell survival, when combined with high voltage electric pulses (Figure 2). Higher concentrations of NPs cause cell death at a lower electric field in comparison to lower concentration and control. Size of NPs statically significantly influences survival rate only at 1 kV/cm. There is no statistical difference between 10 µg/ml, 25 µg/ml and control up to 2 kV/cm, where all cells with NPs are dead and 30% of cells without NPs is still alive. Cells without NPs can survive higher electric field.

CONCLUSION

According to the result we can confirm our hypothesis. NPs enhance electric field and enable electroporation at lower electric fields.

ACKNOWLEDGMENT

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Electrical Effects of Electroporation on the Cardiac Electrogram

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INTRODUCTION

Use of direct current (DC) to cause Irreversible Electroporation (IRE) is a novel, non-thermal method for the ablation and the treatment of cardiac arrhythmias. It confers significant advantages over radiofrequency (RFA) due to its non-thermal nature and potential for tissue specific effects. Attenuation of the cardiac electrogram (EGM) amplitude with application of RFA is a marker of tissue destruction. To date, there is a lack of information on the impact of IRE on the EGM. This information could be crucial in identifying optimal IRE protocols and providing realtime feedback during IRE treatment. Our aim is to provide preliminary data on the impact of cardiac IRE ablation on the EGM.

METHODS

We performed IRE in three canine experiments, where pulmonary vein myocardium was targeted. DC was delivered in a bipolar fashion between two electrodes integrated into a novel balloon catheter prototype. Energy was delivered using the NanoKnife System (Angiodynamics, New York). EGMs were recorded from the balloon catheter using the Prucka recording system (General Electric, Chicago). Mean amplitude was calculated by measuring and averaging the EGM over 5 beats. The polarity post IRE was analyzed for change after the first set of pulses (between 10-100) delivered at a given site. The energy of electric pulses delivered was calculated using impendance measurements at the time of delivery.

RESULTS

In three chronic canine experiments, bipolar DC was delivered on 8 separate occasions to the pulmonary veins (PV). The delivery was targeted at 4 sites [Left inferior PV (n=6), Left middle PV (n=1) and right superior PV (n=1)]. Electroporation protocols included voltage of 1000-2000, with pulse duration of 100 microseconds, and total pulse number of 10-200. Electroporation pulse delivery was ECG-gated in all cases. Mean change in EGM amplitude was 39% from baseline. The change was a decrease in all cases except one. Polarity of the EGM changed with the first delivery at every site. There was no recovery of EGM signal, despite a mean waiting time of 5 mins after energy delivery (range 2-13).

Figure 1: Example of change in local EGM.

CONCLUSIONS

Changes in EGM amplitude and polarity have the potential to be employed as an realtime indicator of optimal IRE delivery and subsequently guide optimal DC delivery towards reliable IRE. These findings merit further study in a larger cohort of animals and at different locations in the heart.

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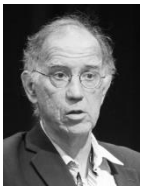

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In vitro Studies of the Antiviral Effects of Marine Active Ingredients

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INTRODUCTION

A wide spectrum of cell surface molecules, including glycoproteins, glycolipids, and proteoglycans participate in cell–virion interaction and recognition. Many viruses gain access to the cell via human intercellular adhesion molecule 1 (ICAM-1), or low-density lipoprotein receptor (LDLR), or bound to heparan sulphate proteoglycans (HSPG), which are present ubiquitously on the surface of almost all eukaryotic cell types and in the extracellular matrix where it has been implicated in numerous biological functions. Based on their capacity to mediate virus attachment to the host cell surface by binding to proteins of different viruses, HSPGs have been considered an attractive target for the development of multitarget antiviral drugs to prevent viral infections that cannot be eliminated through classical antiviral treatment or protective vaccines. Hence, one of the strategies to block this cell-viruses binding via HSPGs is to use polysaccharides as competitors (HSPG antagonists) [1]. In general, polysaccharides exhibiting antiviral potential are highly sulfated. Here we chose to present some preliminary results on the rhabdomyosarcoma (RD) cells interaction with jellyfish collagen, a model of virus-feeder layer, in the presence of our studied Fucoidan-derived compound, namely FAV, a highly sulphated polysaccharide found in the extracellular matrix of brown algae, an easily available natural polymer, nontoxic, inexpensive, safe, biodegradable, and biocompatible.

EXPERIMENTAL METHODS

Based on an established cell adhesion assay [2], a cell binding inhibition assay was performed with different marine active ingredients, along with the FAV, to quantify living cells that have adhesion to a virus feeder layer. ICAM-1 negative RD cells were used as model. Since fibrillar collagens are the more abundant proteins of the extracellular matrix, Mediterranean jellyfish collagen developed in our laboratory was used to promote cell adhesion, comparing to the cell adherence to the viruses.

Fucoidan of Sigma® (exogenous competitors of cell surface proteoglycan-mediated events) was used as positive control, together with other studied extracts namely CSAV (chondroitin sulphate derived compound) and PNSAV (poly-non-sulfated polysaccharide derived compound) as negative controls. Cells were mixed with or without active ingredients before plating on wells previously coated with jellyfish collagen at optimal adhesion concentration. The buoyancy method was used to remove non-adherent cells and to fix adherent cells. Cell attachment was quantitated by measuring the absorbance at 560 nm in a microplate reader, as described in [2].

RESULTS

Dose-response studies of four compounds on RD cells adhesion to jellyfish collagen were performed. The interaction of jellyfish collagen and RD cells was almost completely inhibited by Fucoidan (Sigma®) and FAV at only 10 µg/mL, while CSAV and PNSAV did not show any inhibition effect.

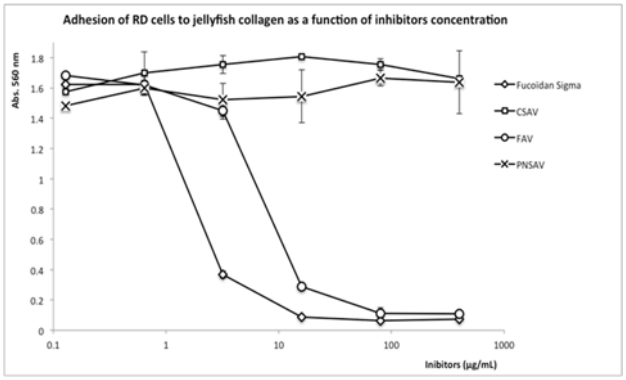

Figure 1: **Dose-response profiles of different active ingredients for adhesion of RD cells to jellyfish collagen.** Before the inhibition of adhesion assay, RD cells were incubated with increasing concentrations of CSAV, FAV, PNSAV and control inhibitor Fucoidan (Sigma®). Values shown were the mean of triplicates minus non-specific binding on BSA.

CONCLUSIONS & PERSPECTIVES

At a very low dose, FAV showed a potential *in vitro* inhibition activity on RD cells adhesion to Jellyfish collagen, as effective as Fucoidan (Sigma®). Those results have encouraged trials on live human rhinoviruses and variants, of which our laboratory also established an adapted lab-scale protocol of production, amplification and purification. We studied the influence of various concentrations of the purified virus on the cell adhesion, as well as those of the putative inhibitors on the viral infection. Along with this marine active ingredient, several studies also focus on non-virus-specific compounds such as Carrageenan, a sulphated galactose polymer derived from Rhodophyceae seaweeds that is broadly active against respiratory viruses *in vitro* and has an excellent safety profile. In 2015, a nasal spray containing iota-carrageenan has been developed and registered as a medical device, and has been licensed to Boehringer Ingelheim Pharma GmbH & Co KG [3].

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