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International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

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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:

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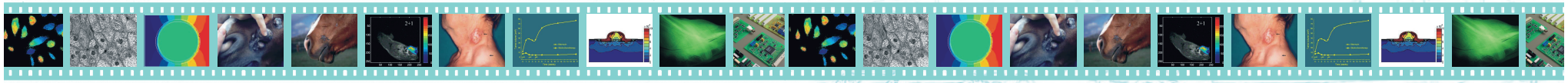

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Welcome note

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 706 participants coming from 39 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 the proceedings 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), to the Bioelectrochemical Society and to the International Society for Electroporation-Based Technologies and Treatments ISEBTT for supporting the Workshop and Course.

We also would like to thank Igea (Italy), Iskra PIO, Kemomed, Omega (Slovenia), Leroy Biotech (France), Orthofix, Pulse Bioscience, and Medtronic (USA) whose financial support allows us to assist several 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.

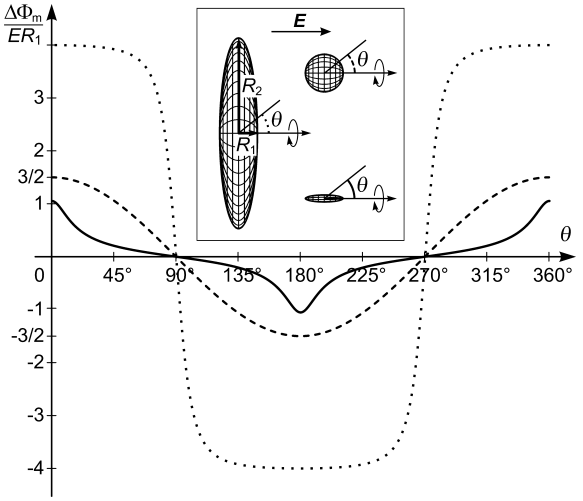

Figure 1: Normalized steady-state $\Delta\Phi_m$ as a function of the polar angle θ for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate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

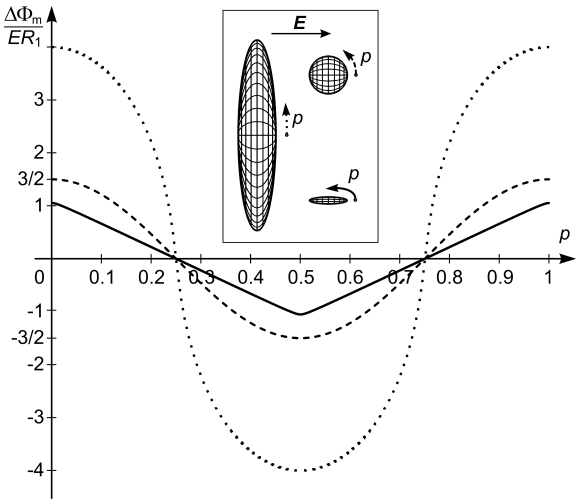

Figure 2: Normalized steady-state $\Delta\Phi_m$ as a function of the normalized arc length p for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: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

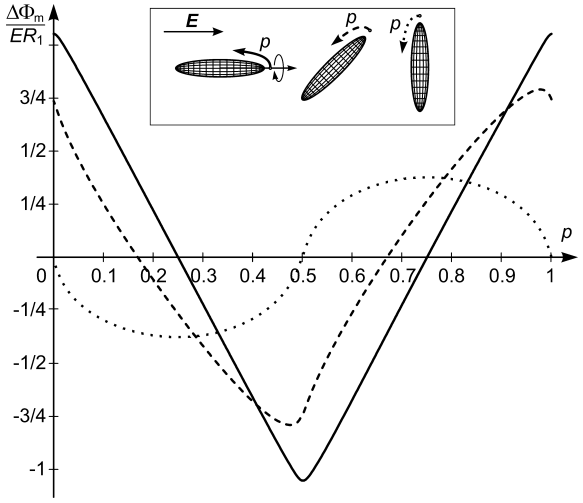

Figure 3: Normalized steady-state $\Delta\Phi_m(p)$ for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

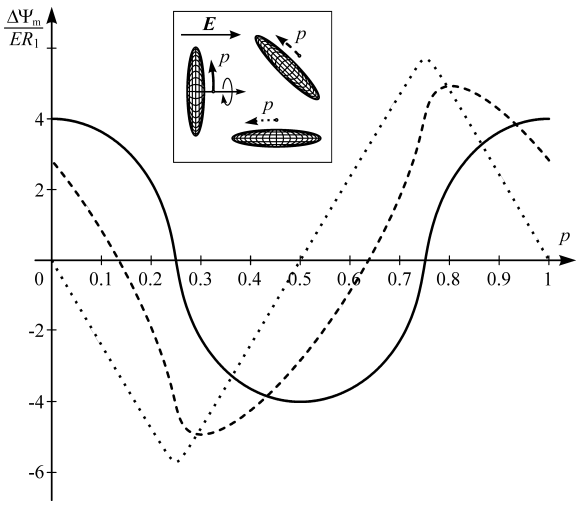

Figure 4: Normalized steady-state $\Delta\Phi_m(p)$ for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

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.

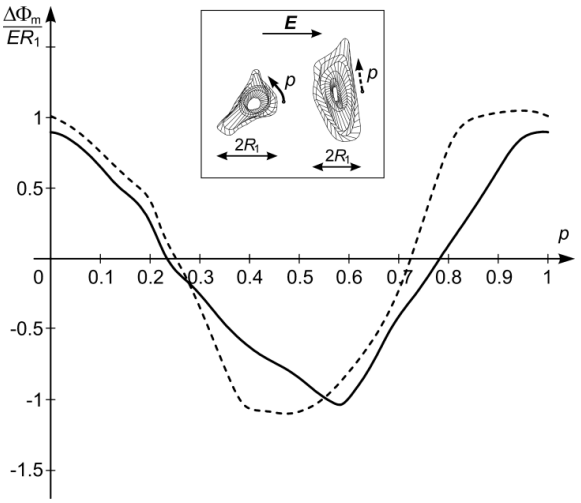

Figure 5: Normalized steady-state $\Delta\Phi_m(p)$ for two irregularly shaped cells growing on the flat surface of a Petri dish.

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.

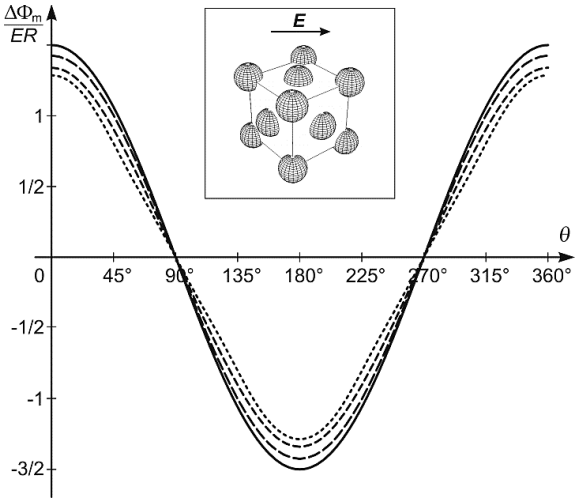

Figure 6: Normalized steady-state $\Delta\Phi_m(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by Eq. (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (with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

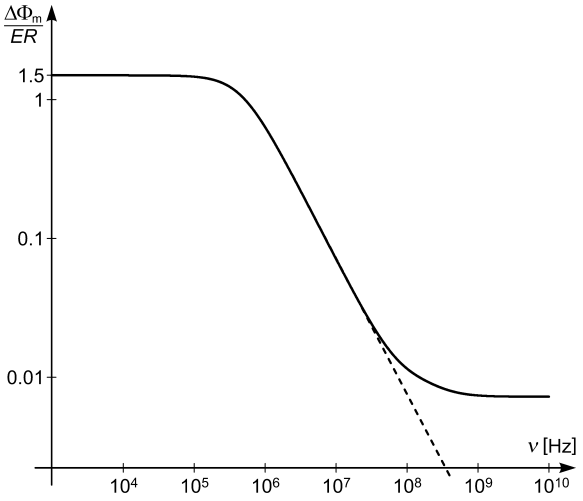

Figure 7: The amplitude of normalized steady-state $\Delta\Phi_m$ as a function of the frequency of the AC field. The dashed curve shows the first-order, and the solid curve the second-order Schwan's equation. Note that both axes are logarithmic.

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].

REFERENCES

[1] T. Kotnik, G. Pucihar, D. Miklavčič. Induced transmembrane voltage and its correlation with electroporation-mediated molecular transport. *J. Membrane Biol.* 236: 3-13, 2010.

[2] K.S. Cole. *Membranes, Ions and Impulses.* University of California Press, Berkeley, USA, 1972.

[3] H.L. Atwood, W.A. Mackay. *Essentials of Neurophysiology*. BC Decker, Toronto, Canada, 1989.

[4] H.P. Schwan. Electrical properties of tissue and cell suspensions. *Adv. Biol. Med. Phys.* 5: 147-209, 1957.

[5] H. Pauly, H.P. Schwan. Über die Impedanz einer Suspension von kugelförmigen Teilchen mit einer Schale. *Z. Naturforsch.* 14B: 125-131, 1959.

[6] T. Kotnik, F. Bobanović, D. Miklavčič. Sensitivity of transmembrane voltage induced by applied electric fields — a theoretical analysis. *Bioelectrochem. Bioenerg.* 43: 285-291, 1997.

[7] C. Grosse, H.P. Schwan. Cellular membrane potentials induced by alternating fields. *Biophys. J.* 63: 1632-1642, 1992.

[8] T. Kotnik, D. Miklavčič, T. Slivnik. Time course of transmembrane voltage induced by time-varying electric fields — a method for theoretical analysis and its application. *Bioelectrochem. Bioenerg.* 45: 3-16, 1998.

[9] T. Kotnik, D. Miklavčič. Second-order model of membrane electric field induced by alternating external electric fields. *IEEE Trans. Biomed. Eng.* 47: 1074-1081, 2000.

[10] T. Kotnik, D. Miklavčič. Analytical description of transmembrane voltage induced by electric fields on spheroidal cells. *Biophys. J.* 79: 670-679, 2000.

[11] J. Gimsa, D. Wachner. Analytical description of the transmembrane voltage induced on arbitrarily oriented ellipsoidal and cylindrical cells. *Biophys. J.* 81: 1888-1896, 2001.

[12] B. Valič, M. Golzio, M. Pavlin, A. Schatz, C. Faurie, B. Gabriel, J. Teissié, M.P. Rols, D. Miklavčič. Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment. *Eur. Biophys. J.* 32: 519-528, 2003.

[13] G. Pucihar, T. Kotnik, B. Valič, D. Miklavčič. Numerical determination of the transmembrane voltage induced on irregularly shaped cells. *Annals Biomed. Eng.* 34: 642-652, 2006.

[14] G. Pucihar, D. Miklavčič, T. Kotnik. A time-dependent numerical model of transmembrane voltage inducement and electroporation of irregularly shaped cells. *IEEE T. Biomed. Eng.* 56: 1491-1501, 2009.

[15] R. Susil, D. Šemrov, D. Miklavčič. Electric field induced transmembrane potential depends on cell density and organization. *Electro. Magnetobiol.* 17: 391-399, 1998.

[16] M. Pavlin, N. Pavšelj, D. Miklavčič. Dependence of induced transmembrane potential on cell density, arrangement, and cell position inside a cell system. *IEEE Trans. Biomed. Eng.* 49: 605-612, 2002.

[17] T. Kotnik, D. Miklavčič. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophys. J.* 90: 480-491, 2006.

[18] K.H. Schoenbach, S.J. Beebe, E.S. Buescher. Intracellular effect of ultrashort electrical pulses. Bioelectromagnetics 22: 440-448, 2001.

[19] S.J. Beebe, P.M. Fox, L.J. Rec, E.L. Willis, K.H. Schoenbach. Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells. *FASEB J.* 17: 1493-1495, 2003.

[20] E. Tekle, H. Oubrahim, S.M. Dzekunov, J.F. Kolb, K.H. Schoenbach, P. B. Chock. Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses. *Biophys. J.* 89: 274-284, 2005.

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NOTES

Electric Properties of Tissues and their Changes during Electroporation

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

INTRODUCTION

The electrical properties of biological tissues and cell suspensions have been of interest for over a century. They determine the pathways of current flow through the body and are thus very important in the analysis of a wide range of biomedical applications. On a more fundamental level, knowledge of these electrical properties can lead to the understanding of the underlying, basic biological processes. To analyze the response of a tissue to electric stimulus, data on the 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 [5].

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).

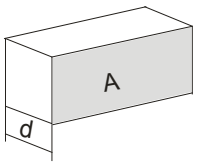

Figure 1: A considered theoretical small part of a material.

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 [6]. 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.

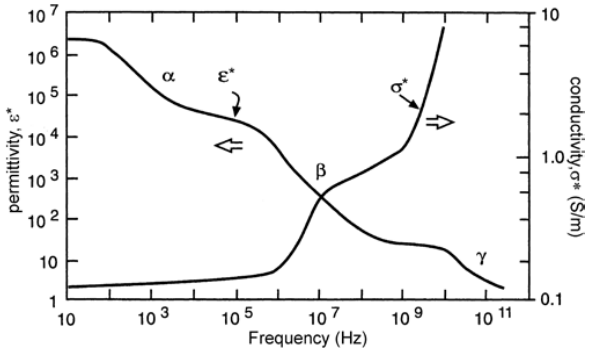

Figure 2: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.

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 [7]. 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 [8].

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 [9]. 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 [8].

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 role 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).

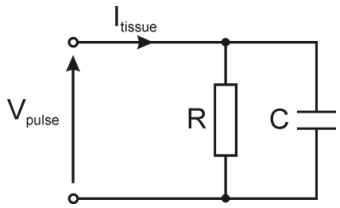

Figure 3: Parallel RC circuit: a theoretical representation of tissue response to electic pulses.

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) [10]. 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 [10].

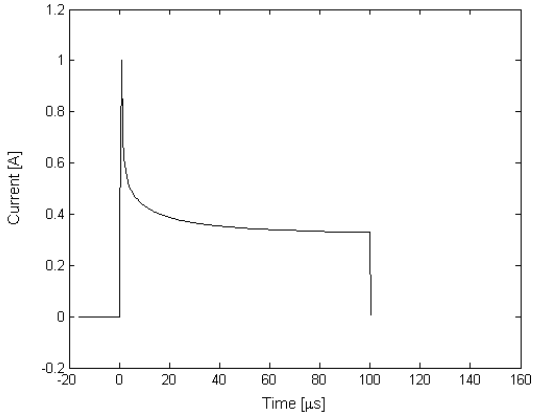

Figure 4: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs having the rise time of 1 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

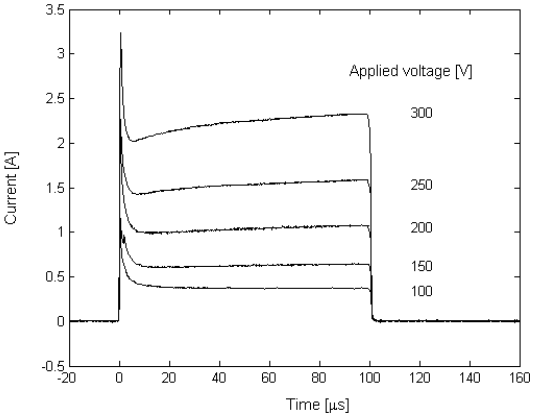

Figure 5: Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [10]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

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 [12]. In measuring ex vivo tissue and phantom tissue made of gel like material [17] 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 [18].

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 [19]. 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 [20]. 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.

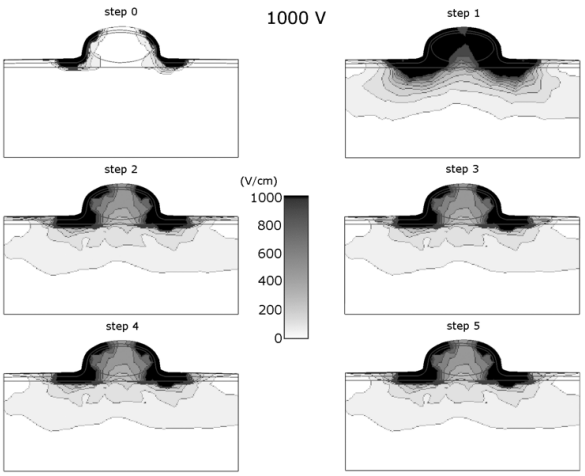

Figure 6: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [20]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact 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 [21]. 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 [22].

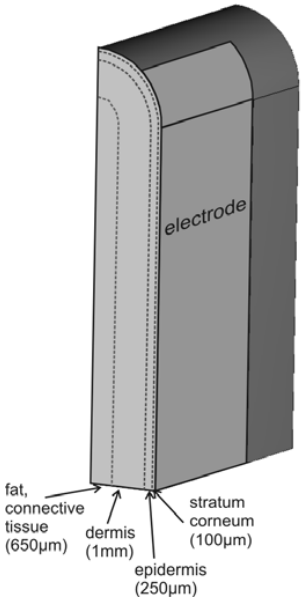

Figure 7: Schematics of a skinfold as described in a numerical model. Only one quarter of the skinfold is presented here.

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 [23]. Therefore, due effort needs to be invested into measurements of tissue electrical properties and their changes during electroporation [27].

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 [29], therefore thermal aspect in treatment planning and theoretical analysis of specific applications of electroporation-based treatments should be considered [30]. 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 [32].

REFERENCES

[1] D. Miklavčič, N. Pavšelj, FX Hart. Electric Properties of Tissues. Wiley Encyclopedia of Biomedical Engineering, John Wiley & Sons, New York, 2006.

[2] K.R. Foster and H.P. Schwan. Dielectric properties of tissues and biological materials: a critical review. *Critical Reviews in Biomedical Engineering* 17: 25-104, 1989.

[3] C. Gabriel, A. Peyman and E.H. Grant. Electrical conductivity of tissue at frequencies below 1 MHz. *Phys. Med. Biol.* 54(16): 4863-4878, 2009.

[4] Applied Bioelectricity, From Electrical Stimulation to Electropathology, J. Patrick Reilly, Springer-Verlag New York, 1998.

[5] Bioimpedance and Bioelectricity Basics, S. Grimnes, O. Martinsen, 3rd Edition, Academic Press 2015.

[6] Kyle C. Smith and James C. Weaver. Electrodiffusion of Molecules in Aqueous Media: A Robust, Discretized Description for Electroporation and Other Transport Phenomena. *IEEE Trans. Biomed. Eng.* 59(6), 1514-1522, 2012.

[7] S. Huclova, D. Erni and J. Frohlich. Modelling and validation of dielectric properties of human skin in the MHz region focusing on skin layer morphology and material composition. *J. Phys. D: Appl. Phys.* 45(2): 025301, 2012.

[8] F.X. Hart. Bioimpedance in the clinic. *Zdravniski vestnik-Slovenian Medical Journal* 78(12): 782-790, 2009.

[9] A. Peyman, B. Kos, M. Djokić, B. Trotovšek, C. Limbaeck-Stokin, G. Serša, D. Miklavčič. Variation in dielectric properties due to pathological changes in human liver. *Bioelectromagnetics* 36: 603-612, 2015.

[10] D. Cukjati, D. Batiuskaite, D. Miklavčič, L.M. Mir. Real time electroporation control for accurate and safe *in vivo* nonviral gene therapy. *Bioelectrochemistry* 70: 501-507, 2007.

[11] A. Ivorra and B. Rubinsky. In vivo electrical impedance measurements during and after electroporation of rat liver. *Bioelectrochemistry* 70: 287-295, 2007.

[12] M. Pavlin, D. Miklavčič. Effective conductivity of a suspension of permeabilized cells: A theoretical analysis. *Biophys. J.* 85: 719-729, 2003.

[13] M. Pavlin, M. Kanduser, M. Rebersek, G. Pucihar, F.X. Hart, R. Magjarevic and D. Miklavcic. Effect of cell electroporation on the conductivity of a cell suspension. *Biophys. J.* 88: 4378-4390, 2005.

[14] A. Ivorra, B. Al-Sakere B, B. Rubinsky and L.M. Mir. In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome. *Phys. Med. Biol.* 54(19):5949-5963, 2009.

[15] Y. Granot, A. Ivorra, E. Maor and B. Rubinsky. In vivo imaging of irreversible electroporation by means of electrical impedance tomography. *Phys. Med. Biol.* 54(16): 4927-4943, 2009.

[16] M. Essone Mezeme, G. Pucihar, M. Pavlin, C. Brosseau, D. Miklavčič. A numerical analysis of multicellular environment for modeling tissue electroporation. *Appl. Phys. Lett.* 100: 143701, 2012.

[17] M. Kranjc, F. Bajd, I. Serša, D. Miklavčič. Magnetic resonance electrical impedance tomography for measuring electrical conductivity during electroporation. *Physiol. Meas.* 35: 985-996, 2014.

[18] R.E. Neal, P.A. Garcia, J.L. Robertson, R.V. Davalos. Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning. *IEEE Trans. Biomed. Eng.* 59(4): 1076 – 1085, 2012.

[19] N. Pavšelj, D. Miklavčič. Numerical modeling in electroporation-based biomedical applications. *Radiol. Oncol.* 42:159-168, 2008.

[20] N. Pavšelj, Z. Bregar, D. Cukjati, D. Batiuskaite, L.M. Mir and D. Miklavčič. The course of tissue permeabilization studied on a mathematical model of a subcutaneous tumor in small animals. *IEEE Trans. Biomed. Eng.* 52(8):1373-1381, 2005.

[21] N. Pavšelj and V. Préat. DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse. *Journal of Controlled Release* 106:407-415, 2005.

[22] N. Pavšelj, V. Préat, D. Miklavčič. A numerical model of skin electropermeabilization based on in vivo experiments. *Annals Biomed. Eng.* 35:2138-2144, 2007.

[23] D. Miklavčič, M. Snoj, A. Županič, B. Kos, M. Čemažar, M. Kropivnik, M. Bračko, T. Pečnik, E. Gadžijev, G. Serša. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. Biomed. Eng. Online 9: 10, 2010.

[24] Edhemović I, Gadžijev EM, Brecelj E, Miklavčič D, Kos B, Županič A, Mali B, Jarm T, Pavliha D, Marčan M, Gašljevič G, Gorjup V, Mušič M, Pečnik Vavpotič T, Čemažar M, Snoj M, Serša G. Electrochemotherapy: A new technological approach in treatment of metastases in the liver. *Technol. Cancer Res. Treat*. 10: 475-485, 2011.

[25] A. Županič, B. Kos, D. Miklavčič- Treatment planning of electroporation-based medical interventions: electrochemotherapy, gene electrotransfer and irreversible electroporation. *Phys. Med. Biol.* 57: 5425-5440, 2012.

[26] J. Dermol-Černe, D. Miklavčič, From cell to tissue properties – Modeling skin electroporation with pore and local transport region formation. *IEEE Trans. Biomed. Eng.* 65(2): 458-468, 2018.

[27] J. Langus, M. Kranjc, B. Kos, M. Šuštar, D. Miklavčič. Dynamic finite-element model for efficient modelling of electric currents in electroporated tissue. *Sci. Rep.* 6: 26409, 2016.

[28] M. Pintar, J. Langus, I. Edhemović, E. Brecelj, M. Kranjc, G. Serša, T. Šuštar, T. Rodič, D. Miklavčič, T. Kotnik, B. Kos Time-dependent finite-element analysis of in vivo electrochemotherapy treatment. *Technol. Cancer. Res. Treat*. 17: 1-9, 2018.

[29] I. Lacković, R. Magjarević, D. Miklavčič. Three-dimensional finite-element analysis of joule heating in electrochemotherapy and in vivo gene electrotransfer. *IEEE T. Diel. El. Insul.* 15: 1338-1347, 2009.

[30] S. Bhonsle, M. Lorenzo, A. Safaai-Jazi, R.V. Davalos, Characterization of Nonlinearity and Dispersion in Tissue Impedance during High Frequency Electroporation. *IEEE Transactions on Biomedical Engineering*, 65(10), 2190-2201, 2017.

[31] B. Kos, P. Voigt, D. Miklavčič, M. Moche. Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE). *Radiol. Oncol.* 49: 234-241, 2015.

[32] Pavšelj N, Miklavčič D. Resistive heating and electropermeabilization of skin tissue during in vivo electroporation: A coupled nonlinear finite element model. *Int. J. Heat Mass Transfer* 54: 2294-2302, 2011.

[33] Županič A, Miklavčič D. Tissue heating during tumor ablation with irreversible electroporation. *Elektroteh. Vestn.* 78: 42-47, 2011.

[34] Robert E. Neal II , Paulo A. Garcia, John L. Robertson, Rafael V. Davalos, Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning *IEEE Trans. Biomed. Eng.* 59(4), 1076-1085, 2012.

[35] P.A. Garcia, R.V. Davalos, D. Miklavčič. A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue. *PLOS One* 9(8): e103083, 2014.

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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 $\Delta\Psi$ (i.e. the sum between the resting value of cell membrane $\Delta\Psi o$ and the electroinduced value $\Delta\Psi i$) reaches locally 250 mV, that part of the membrane becomes 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.

Coherent Anti-stokes Raman Scattering (CARS) is a label free spectroscopy. It was recently used to confirm that proteins were affected along electropermeabilization [23, 24].

CARS results are indicative of an alteration of the interfacial water molecules, a direct consequence of the fusogenicity of electropermeabilized membranes [25].

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 [27]. 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. Resealing is complex as permeabilization cancellation is obtained when bipolar pulses with a short interpulse delay are delivered [29,30].

C-3 Cellular responses

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) [27]. Reactive oxygen species (ROS) are generated at the permeabilized loci, depending on the electric field parameters [28]. 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 [31].

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 [32].

There is a loss of the bilayer membrane asymmetry of the phospholipids on erythrocytes [33] due to the induced osmotic swelling bringing hemolysis.

A mechanical stress is present during the pulse delivery when high fields are present as shown by the occurrence of shock waves [34]. and result in a biological response [35].

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 [36] as a recent review). Biochemical modifications such as lipid oxidation may be present as suggested by membrane blebbings formed just after the pulse delivery [37, 38]. 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) [39, 9]. SiRNA are only transferred by the electrophoretic drag present during the pulse [40]. 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 [41, 42].

Cell membrane electropermeabilization is a complex process. To improve our knowledge, one has to very careful in the description of the experimental protocol [43].

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REFERENCES

[1] Potter, H., *Application of electroporation in recombinant DNA technology, in Methods in Enzymology,* vol. 217, I. Academic Press, Editor. 1993.

[2] Orlowski, S. and L.M. Mir, *Cell electropermeabilization: a new tool for biochemical and pharmacological studies.* Biochim Biophys Acta, 1993. 1154(1): 51-63.

[3] Teissié, J. and M.P. Rols, *An experimental evaluation of the critical potential difference inducing cell membrane electropermeabilization.* Biophys J, 1993. 65(1): 409-13.

[4] Gross, D., L.M. Loew, and W.W. Webb, *Optical imaging of cell membrane potential changes induced by applied electric fields.* Biophys J, 1986. 50: 339-48.

[5] Lojewska, Z., et al., *Analysis of the effect of medium and membrane conductance on the amplitude and kinetics of membrane potentials induced by externally applied electric fields*. Biophys J, 1989. 56(1): 121-8.

[6] Hibino, M., et al., *Membrane conductance of an electroporated cell analyzed by submicrosecond imaging of transmembrane potential.* Biophys J, 1991. 59(1): 209-20.

[7] Pucihar, G., et al., *Electropermeabilization of dense cell suspensions.* Biophys J. 2007 36(3): 173-185

[8] Teissié, J. and T.Y. Tsong, *Electric field induced transient pores in phospholipid bilayer vesicles.* Biochemistry, 1981. 20(6): 1548-54.

[9] Rols, M.P. and J. Teissie, *Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon.* Biophys J, 1990. 58(5): 1089-98.

[10] Gabriel, B. and J. Teissie, *Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electropermeabilized cell membrane.* Biophys J, 1997. 73(5): 2630-7.

[11] Gabriel, B. and J. Teissie, *Time courses of mammalian cell electropermeabilization observed by millisecond imaging of membrane property changes during the pulse.* Biophys J, 1999. 76(4): 2158-65.

[12] Valič B, Golzio M, Pavlin M, Schatz A, Faurie C, Gabriel B, Teissié J, Rols MP, Miklavčič D. *Effect of electric field induced transmembrane potential on spheroidal cells: theory and experiment*. Eur. Biophys. J. **32**: 519-528, 2003

[13] Kinosita, K., Jr. and T.Y. Tsong, V*oltage-induced conductance in human erythrocyte membranes.* Biochim Biophys Acta, 1979. 554(2): 479-97.

[14] Sixou, S. and J. Teissie, *Specific electropermeabilization of leucocytes in a blood sample and application to large volumes of cells.* Biochim Biophys Acta, 1990. 1028(2): 154-60.

[15] Winterhalter M and Helfrich W *Deformation of spherical vesicles by electric fields* J. Colloid Interface Sci. 1988. 122 583–6

[16] Harbich W. and Helfrich W *Alignment and opening of giant lecithin vesicles by electric fields* Z Naturforsch 1991 34a, , 133-1335.

[17] Stulen G. *Electric field effects on lipid membrane structure*. Biochim Biophys Acta. 1981 ; 640(3):621-7

[18] Lopez A, Rols MP, Teissie J.*31P NMR analysis of membrane phospholipid organization in viable, reversibly electropermeabilized Chinese hamster ovary cells.* Biochemistry. 1988 ;27(4):1222-8

[19] Pillet F, Chopinet L, Formosa C, Dague E *Atomic Force Microscopy and pharmacology: From microbiology to cancerology* Biochimica et Biophysica Acta 1840 (2014) 1028–1050

[20] Chopinet L, Roduit C, Rols MP, Dague E *Destabilization induced by electropermeabilization analyzed by atomic force microscopy* Biochimica et Biophysica Acta 2013 1828 2223–2229

[21] Zalvidea D, Claverol-Tintur´e E *Second Harmonic Generation for time-resolved monitoring of membrane pore dynamics subserving electroporation of neurons* Biomedical Optics Express 2011 / Vol. 2, No. 2 / 305-314

[22] Moen, EK. Ibey, BL. Beier HT *Detecting Subtle Plasma Membrane Perturbation in Living Cells Using Second Harmonic Generation Imaging* Biophysical Journal 2014 106 L37–L40

[23] Azan A, Untereiner V, Gobinet C, Sockalingum GD, Breton M, Piot O, Mir LM. *Demonstration of the Protein Involvement in Cell Electropermeabilization using Confocal Raman Microspectroscopy*. Sci Rep. 2017 ;7:40448. doi: 10.1038/srep40448.

[24] Azan A, Untereiner V, Descamps L, Merla C, Gobinet C, Breton M, Piot O, Mir LM. *Comprehensive Characterization of the Interaction between Pulsed Electric Fields and Live Cells by Confocal Raman Microspectroscopy*. Anal Chem. 2017 ;89(20):10790-10797. doi: 10.1021/acs.analchem.7b02079.

[25] Azan A, Scherman M, Silve A, Breton M, Leray I, Dorval N, Attal-Trétout B, Mir LM *Interfacial water probing by CARS spectroscopy on biological samples exposed to intense pulsed electric fields* 2015 URSI AT-RASC Conference, ISBN: 9789090086286

[26] Gabriel, B. and J. Teissie, *Control by electrical parameters of short- and long-term cell death resulting from electropermeabilization of Chinese hamster ovary cells.* Biochim Biophys Acta, 1995. 1266(2): 171-8.

[27] Teissié, J. and M.P. Rols, *Manipulation of cell cytoskeleton affects the lifetime of cell membrane electropermeabilization.* Ann N Y Acad Sci, 1994. 720: 98-110.

[28] Gabriel, B. and J. Teissie, *Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability.* Eur J Biochem, 1994. 223(1): 25-33.

[29] Ibey BL, Ullery JC, Pakhomova ON, Roth CC, Semenov I, Beier HT, Tarango M, Xiao S, Schoenbach KH, Pakhomov AG, *Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses*, Biochem. Biophys. Res. Commun. 2014 443 568–573..

[30] Schoenbach KH, Pakhomov AG, Semenov I, Xiao S, Pakhomova ON, Ibey BL, *Ion transport into cells exposed to monopolar and bipolar nanosecond pulses,* Bioelectrochemistry 2015 103 44–51.

[31] Markelc B, Tevz G, Cemazar M, Kranjc S, Lavrencak J, Zegura B, Teissie J, Sersa G. *Muscle gene electrotransfer is increased by the antioxidant tempol in mice*. Gene Ther. 2011. doi: 10.1038

[32] Golzio, M., et al., *Control by osmotic pressure of voltage-induced permeabilization and gene transfer in mammalian cells.* Biophys J, 1998. 74(6): 3015-22.

[33] Haest, C.W., D. Kamp, and B. Deuticke, *Transbilayer reorientation of phospholipid probes in the human erythrocyte membrane. Lessons from studies on electroporated and resealed cells*. Biochim Biophys Acta, 1997. 1325(1): 17-33.

[34] Barnes RA, Roth CC, Beier HT, Noojin G, Valdez C, Bixler J, Moen E, Shadaram M, Ibey BL.*Probe beam deflection optical imaging of thermal and mechanical phenomena resulting from nanosecond electric pulse (nsEP) exposure in-vitro*. Opt Express. 2017;25(6):6621-6643. doi: 10.1364/OE.25.006621.

[35] Roth CC, Glickman RD, Martens SL, Echchgadda I, Beier HT, Barnes RA Jr, Ibey BL.*Adult human dermal fibroblasts exposed to nanosecond electrical pulses exhibit genetic biomarkers of mechanical stress*. Biochem Biophys 2017;9:302-309. doi: 10.1016/j.bbrep.2017.01.007

[36] Teissie J, Golzio M, Rols *MP Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge.*Biochim Biophys Acta, 2005 .1724(3): 270-80

[37] Gass GV, Chernomordik *LV Reversible large-scale deformations in the membranes of electrically-treated cells: electroinduced bleb formation*. Biochim Biophys Acta. 1990 1023(1):1-11

[38] Escande-Géraud ML, Rols MP, Dupont MA, Gas N, Teissié J. *Reversible plasma membrane ultrastructural changes*

correlated with electropermeabilization in Chinese hamster ovary cells. Biochim Biophys Acta. 1988 939(2):247-59

[39] Pucihar G, Kotnik T, Miklavcic D, Teissié J. *Kinetics of transmembrane transport of small molecules into electropermeabilized cells* Biophys J. 2008; **95**(6) :2837-48

[40] Paganin-Gioanni A, Bellard E, Escoffre JM, Rols MP, Teissié J, Golzio M. *Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells*. Proc Natl Acad Sci U S A. 2011;108(26): 10443-7.

[41] Wolf H, Rols MP, Boldt E, Neumann E, Teissié J.*Control by pulse parameters of electric field-mediated gene transfer in mammalian cells.* Biophys J. 1994;**66**(2):524-31.

[42] Golzio M, Teissie J, Rols MP. *Direct visualization at the single-cell level of electrically mediated gene delivery*.Proc Natl Acad Sci U S A. 2002; **99**(3): 1292-7.

[43] Cemazar M, Sersa G, Frey W, Miklavcic D, Teissié J *Recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples.* Bioelectrochemistry. 2018; 122:69-76. doi: 10.1016/j.bioelechem.2018.03.005.

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 $\Delta G=0$ 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

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

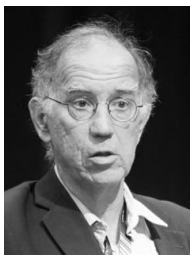
E_m is the transmembrane potential Pion is the permeability for that ion, $[ion]_{out}$ is the extracellular concentration of that ion, $[ion]_{in}$ is the intracellular concentration of that ion.

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

- https://www.khanacademy.org/test-prep/mcat/cells/transport-across-a-cell-membrane/a/passive-transport-and-active-transport-across-a-cell-membrane-article
- http://www.sumanasinc.com/webcontent/animations/content/vesiclebudding.html
- http://www.like2do.com/learn?s=Membrane_vesicle_trafficking
- Popescu I. Aurel, Biophysics. Current Status and Future Trends, Publishing House of the Romanian Academy, 2016



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

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.

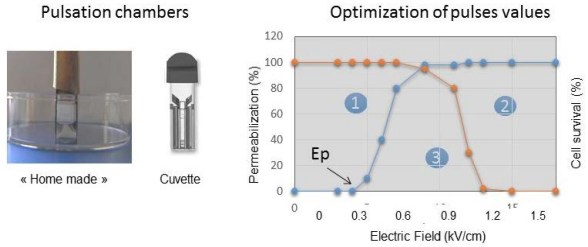

Fig. 1 Tips for your experiments. Cells are pulsed on Petri dish or on cuvettes. Permeabilized and viable cells are plotted to define the optimum conditions ((1) E < Ep or just above, poor permeabilization; (2) E>> Ep, viability loss; (3) best values).

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.

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).

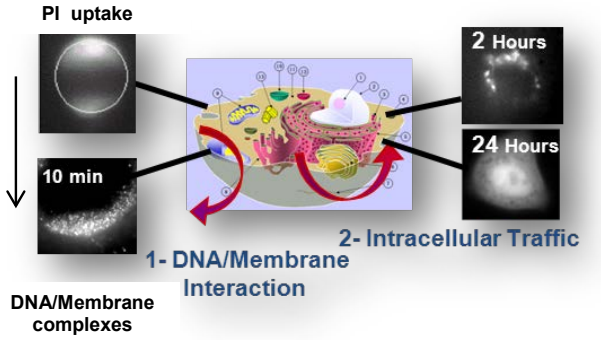

Figure 2: Molecule electrotransfer mechanisms. Left: During electric pulses application: Plasma membrane is electropermeabilized facing the 2 electrodes (PI uptake). DNA aggregates are formed. This interaction takes place only on the membrane facing the cathode. Right: About 2 h after electric pulses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

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

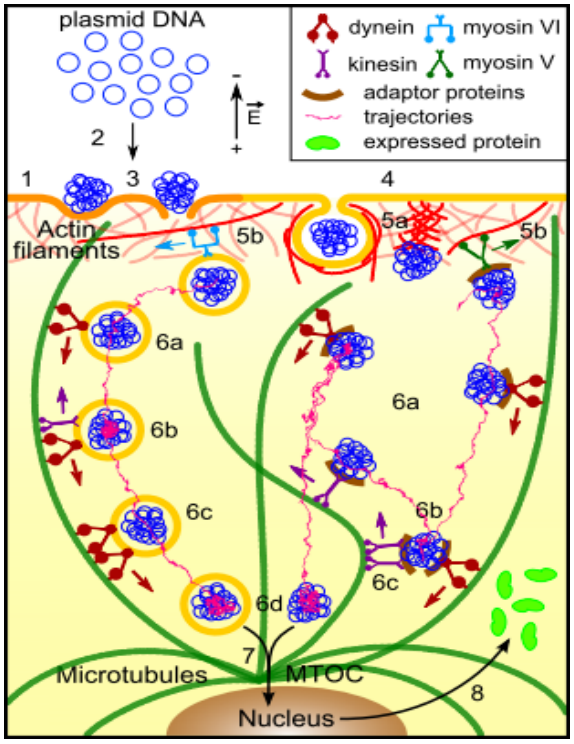

Figure 3: Schematic representation of the mechanism of DNA electrotransfer. During the electric pulses, (1) the plasma membrane is permeabilized, (2) DNA is electrophoretically pushed onto the cell membrane, which results in (3) DNA-membrane interactions. After resealing of the membrane, (4) DNA is internalized 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.

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)).

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

"Intracellular delivery of materials has become a critical component of genome-editing approaches, *ex vivo* cell-based therapies and a diversity of fundamental research applications. Limitations of current technologies motivate development of next-generation systems that can deliver a broad variety of cargo to diverse cell types. Every day in research institutes and clinical centres around the world, scientists use kits and protocols based on viral vectors, lipid transfection agents, and electroporation, among other options. The complex mechanisms of established methods and their often unpredictable impact on cell behaviour have dramatically limited the scope of biological experiments and reduced efficacy of potentially promising cell therapy concepts. The biomedical research community would benefit greatly from a more mechanistic and transparent understanding of intracellular delivery, both to further the development of more robust techniques and to realize key medical and industrial applications" (32). In this context, the so-called electroporation technology is probably the most promising one.

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.

REFERENCES

[1] Verma, I. M., and M. D. Weitzman. 2005. Gene therapy: twenty-first century medicine. *Annu Rev Biochem* 74:711-738.

[2] Yarmush, M. L., A. Golberg, G. Sersa, T. Kotnik, and D. Miklavcic. 2014. Electroporation-based technologies for medicine: principles, applications, and challenges. *Annual review of biomedical engineering* 16:295-320.

[3] Lambricht, L., A. Lopes, S. Kos, G. Sersa, V. Preat, and G. Vandermeulen. 2016. Clinical potential of electroporation for gene therapy and DNA vaccine delivery. *Expert Opin Drug Deliv* 13:295-310.

[4] Sersa, G., J. Teissie, M. Cemazar, E. Signori, U. Kamensek, G. Marshall, and D. Miklavcic. 2015. Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer. *Cancer immunology, immunotherapy : CII*.
[5] Daud, A. I., R. C. DeConti, S. Andrews, P. Urbas, A. I. Riker, V. K. Sondak, P. N. Munster, D. M. Sullivan, K. E. Ugen, J. L. Messina, and R. Heller. 2008. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J Clin Oncol* 26:5896-5903.
[6] Neumann, E., M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *Embo J* 1:841-845.
[7] Golzio, M., J. Teissie, and M. P. Rols. 2002. Direct visualization at the single-cell level of electrically mediated gene delivery. *Proc Natl Acad Sci U S A* 99:1292-1297.
[8] Paganin-Gioanni, A., E. Bellard, J. M. Escoffre, M. P. Rols, J. Teissie, and M. Golzio. 2011. Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells. *Proc Natl Acad Sci U S A* 108:10443-10447.
[9] Escoffre, J. M., E. Bellard, C. Faurie, S. C. Sebai, M. Golzio, J. Teissie, and M. P. Rols. 2014. Membrane disorder and phospholipid scrambling in electropermeabilized and viable cells. *Biochim Biophys Acta* 1838:1701-1709.
[10] Chopinet, L., C. Roduit, M. P. Rols, and E. Dague. 2013. Destabilization induced by electropermeabilization analyzed by atomic force microscopy. *Biochim Biophys Acta* 1828:2223-2229.
[11] Chopinet, L., C. Formosa, M. P. Rols, R. E. Duval, and E. Dague. 2013. Imaging living cells surface and quantifying its properties at high resolution using AFM in QI (TM) mode. *Micron* 48:26-33.
[12] Faurie, C., M. Rebersek, M. Golzio, M. Kanduser, J. M. Escoffre, D. Pavlin, J. Teissie, D. Miklavcic, and M. P. Rols. 2010. Electrically mediated gene transfer and expression are controlled by the life-time of DNA/Membrane complex formation. *Journal of Gene Medicine* 12:117-125.
[13] Escoffre, J. M., T. Portet, C. Favard, J. Teissie, D. S. Dean, and M. P. Rols. 2011. Electromediated formation of DNA complexes with cell membranes and its consequences for gene delivery. *Biochim Biophys Acta* 1808:1538-1543.
[14] Rosazza, C., J. M. Escoffre, A. Zumbusch, and M. P. Rols. 2011. The actin cytoskeleton has an active role in the electrotransfer of plasmid DNA in mammalian cells. *Mol Ther* 19:913-921.
[15] Rosazza, C., A. Buntz, T. Riess, D. Woll, A. Zumbusch, and M. P. Rols. 2013. Intracellular tracking of single plasmid DNA-particles after delivery by electroporation. *Mol Ther*.
[16] Rosazza, C., H. Deschout, A. Buntz, K. Braeckmans, M. P. Rols, and A. Zumbusch. 2016. Endocytosis and Endosomal Trafficking of DNA After Gene Electrotransfer In Vitro. *Molecular therapy. Nucleic acids* 5:e286.
[17] Lechardeur, D., and G. L. Lukacs. 2006. Nucleocytoplasmic Transport of Plasmid DNA: A Perilous Journey from the Cytoplasm to the Nucleus. *Hum Gene Ther* 17:882-889.
[18] Rosazza, C., S. H. Meglic, A. Zumbusch, M. P. Rols, and D. Miklavcic. 2016. Gene Electrotransfer: A Mechanistic Perspective. *Curr Gene Ther* 16:98-129.
[19] Rems, L., and A. Miklavcic. 2016. Titorial: electroporation of cells in complex materials and tissue. *J Appl Phys* 119:201101.
[20] Vaughan, E. E., and D. A. Dean. 2006. Intracellular trafficking of plasmids during transfection is mediated by microtubules. *Mol Ther* 13:422-428.
[21] Beebe, S. J., J. White, P. F. Blackmore, Y. Deng, K. Somers, and K. H. Schoenbach. 2003. Diverse effects of nanosecond pulsed electric fields on cells and tissues. *DNA Cell Biol* 22:785-796.
[22] Escoffre, J. M., K. Kaddur, M. P. Rols, and A. Bouakaz. 2010. In vitro gene transfer by electrosonoporation. *Ultrasound Med Biol* 36:1746-1755.
[23] Delalande, A., S. Kotopoulis, M. Postema, P. Midoux, and C. Pichon. 2013. Sonoporation: mechanistic insights and ongoing challenges for gene transfer. *Gene* 525:191-199.
[24] Riske, K. A., and R. Dimova. 2005. Electro-deformation and poration of giant vesicles viewed with high temporal resolution. *Biophys J* 88:1143-1155.
[25] Portet, T., C. Favard, J. Teissie, D. Dean, and M. P. Rols. 2011. Insights into the mechanisms of electromediated gene delivery and application to the loading of giant vesicles with negatively charged macromolecules. *Soft Matter* 7:3872-3881.
[26] Sutherland, R. M. 1988. Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* 240:177-184.
[27] Gibot, L., and M. P. Rols. 2013. Progress And Prospects: The Use Of 3D Spheroid Model As A Relevant Way To Study And Optimize Dna Electrotransfer. *Curr Gene Ther*.
[28] Marrero, B., and R. Heller. 2012. The use of an in vitro 3D melanoma model to predict in vivo plasmid transfection using electroporation. *Biomaterials*.
[29] Madi, M., M. P. Rols, and L. Gibot. 2015. Efficient In Vitro Electropermeabilization of Reconstructed Human Dermal Tissue. *J Membr Biol*.
[30] Madi, M., M. P. Rols, and L. Gibot. 2016. Gene Electrtransfer in 3D Reconstructed Human Dermal Tissue. *Curr Gene Ther* 16:75-82.
[31] Pillet, F., L. Gibot, M. Madi, M. P. Rols, and E. Dague. 2017. Importance of endogenous extracellular matrix in biomechanical properties of human skin model. *Biofabrication* 9:025017.
[32] Stewart, M. 2016. In vitro and ex vivo strategies for intracellular delivery. *Nature* 538:183-192.

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NOTES

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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.

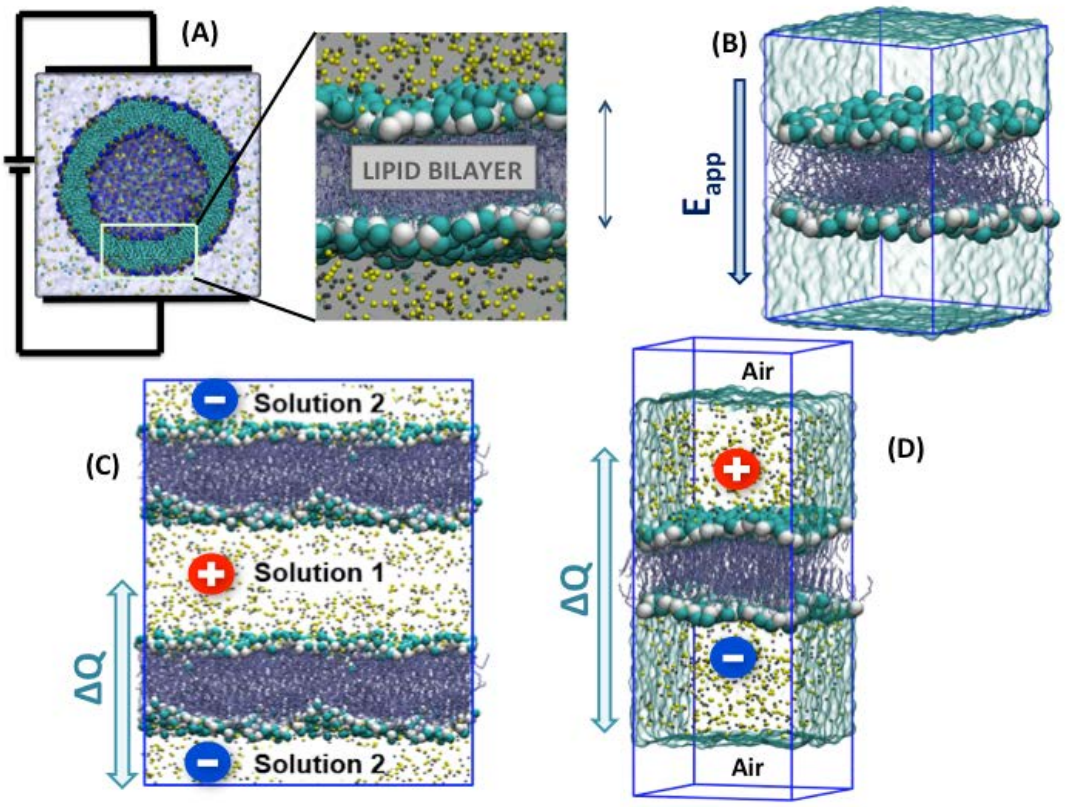

Figure. 1 Protocols for atomistic modelling of cell membranes or liposomes lipid bilayers (A) electroporation; (B) nsPEFs protocol: the system is modeled in absence of salt, and subject to an electric field E_{app} perpendicular to the bilayer (z axis). Note that in some studies ions were also considered; (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:

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

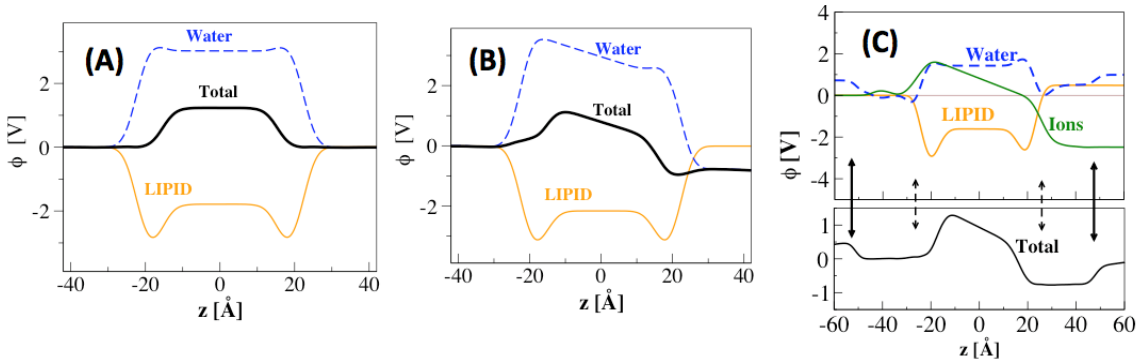

Figure. 2 Electrostatic potential profiles ϕ(z) along the membrane normal (z axis) of a POPC lipid bilayer. Bilayer (A) at rest, (B) subject to a transverse electric field (nsPEF protocol), and (C) bilayer set with a charge imbalance (μs-msPEF protocol). z=0 represents the center of the lipid bilayer. 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].

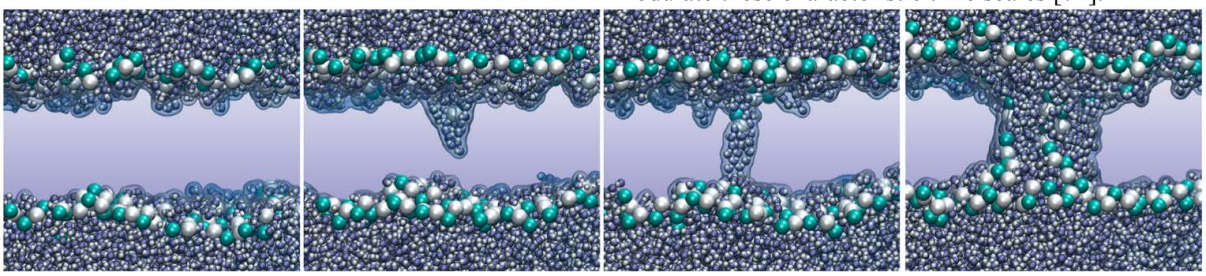

Figure. 3 Pore evolution in a POPC bilayer: The POPC headgroups are shown as cyan and white beads, the lipids tails are not show for clarity. The pore creation, in MD simulations, takes places in the range of nanoseconds.

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 $\Delta \boldsymbol{V}$ 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 $\Delta \boldsymbol{V}$. Quite interestingly, the profiles show clearly that, in contrast to the electric field case where the TM voltage is mainly due to the water dipole reorientation, most of the voltage drop in the charge imbalance method is due to the contribution from the ions. Indeed the sole collapse of the electrostatic potential due to the charge imbalance separation by the membrane lipid core accounts for the largest part of $\Delta \boldsymbol{V}$.

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 $\Delta \boldsymbol{V}$ from which the capacitance can be estimated as $C = Q_s . \Delta \boldsymbol{V}^{-1}$. The capacitance values extracted from simulations are

expected to depend on the lipid composition (charged or not) and on the force field parameters used and as such 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.

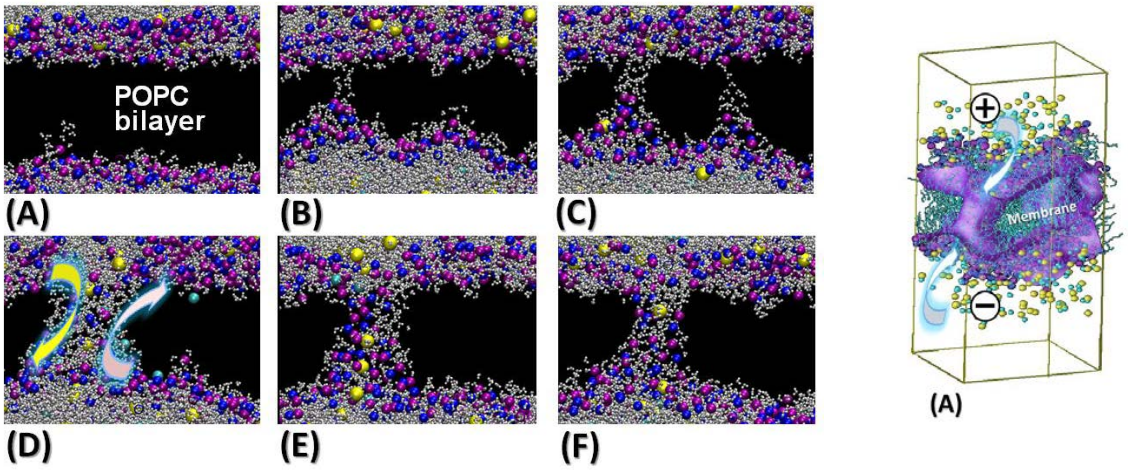

Figure. 4 *Left* Sequence of events following the application of a TM voltage to a POPC lipid bilayer using the charge imbalance method (panels A to F). Note the migration of Na+ (yellow) and Cl- (cyan) ions through the formed hydrophilic pores that are lined with lipid phosphate (magenta) and nitrogen (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.

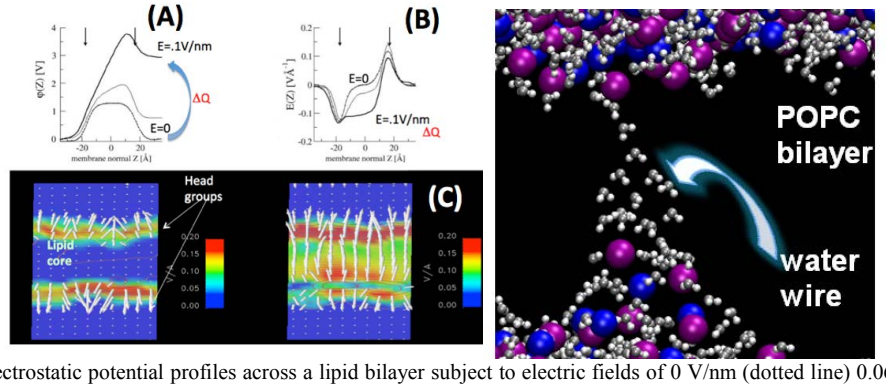

Figure. 5 (A) Electrostatic potential profiles across a lipid bilayer subject to electric fields of 0 V/nm (dotted line) 0.06 V/nm (thin line) and 0.30 V/nm (bold line), or to a charge imbalances ΔQ. (B) Corresponding electric field profiles. (C) 2d (out of plane) maps of the electric field distribution. 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].

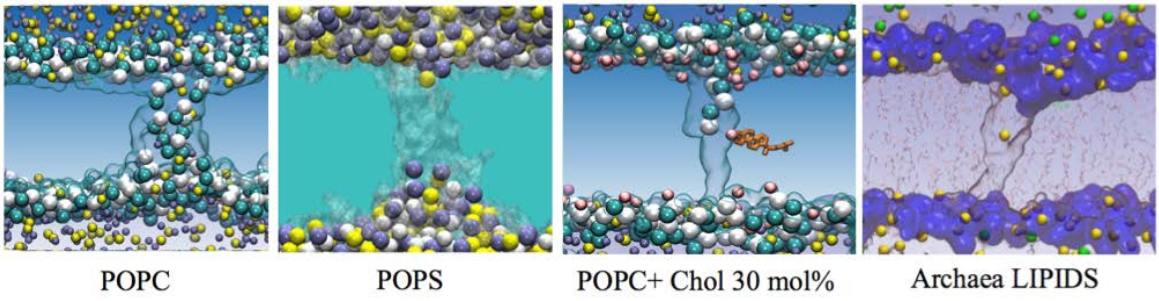

Figure 6. Various morphologies of conducting pores revealed by MD simulations. Note that beside the POPC zwitterionic lipids, pores formed in POPS, a negatively charged lipid, with addition of cholesterol, or in the complex Archaea lipids (sugar like head groups), the electropores are not stabilized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

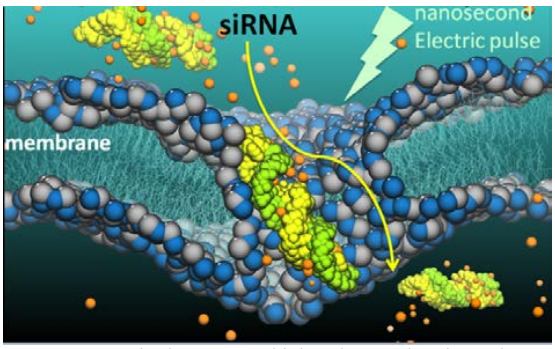

Figure 7: A single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance 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.

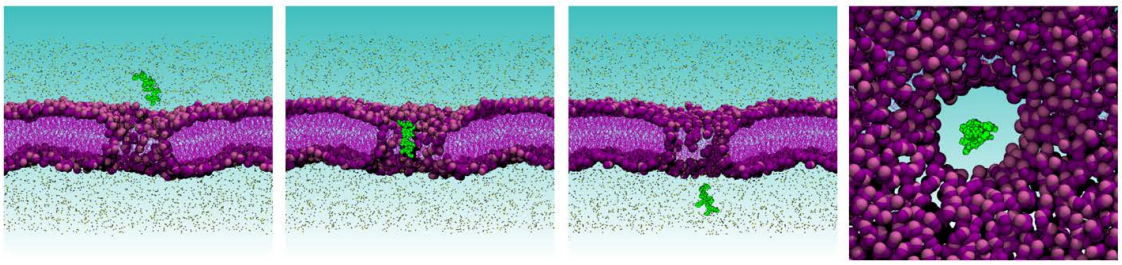

Figure. 8 The process of Tat_{11} transport in three frames corresponding to 0, 8, and 12 ns. In the right panel the top view clearly shows no interactions between the molecule and the pore walls. The POPC headgroups are shown as mauve and violet beads, the tails as purple lines; sodium and chloride 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.

REFERENCES

[1] N. Eberhard, A. E. Sowers, and C. A. Jordan, *Electroporation and electrofusion in cell lbiology*. New York: Plenum Press, 1989.

[2] J. A. Nickoloff, *Animal cell electroporation and electrofusion protocols*, vol. 48. Totowa, NJ: Humana Press, 1995.

[3] S. Li, *Electroporation protocols: preclinical and clinical gene medecine*, vol. 423. Totowa, NJ.: Humana press, 2008.

[4] R. B. Gennis, *Biomembranes: molecular structure and function*. Heidelberg: Spring Verlag, 1989.

[5] I. G. Abidor, V. B. Arakelyan, L. V Chernomordik, Y. A. Chizmadzhev, V. F. Pastushenko, and M. P. Tarasevich, "Electric breakdown of bilayer lipid membranes. I. The main experimental facts and their qualitative discussion," *J. Electroanal. Chem.*, vol. 104, no. C, pp. 37–52, 1979.

[6] R. Benz, F. Beckers, and U. Zimmerman, "Reversible electrical breakdown of lipid bilayer membranes - Charge-pulse relaxation study," *J. Membr. Biol.*, vol. 48, pp. 181–204, 1979.

[7] J. C. Weaver and Y. A. Chizmadzhev, "Theory of electroporation: A review," *Bioelectrochemistry and Bioenergetics*, vol. 41, no. 2. Elsevier Science S.A., pp. 135–160, 1996.

[8] J. C. Weaver, "Electroporation of biological membranes from multicellular to nano scales," *IEEE Trans. Dielectr. Electr. Insul.*, vol. 10, pp. 754–768, 2003.

[9] C. Chen, S. W. Smye, M. P. Robinson, and J. A. Evans, "Membrane electroporation theories: A review," *Medical and Biological Engineering and Computing*, vol. 44, no. 1–2. pp. 5–14, 2006.

[10] G. Pucihar, T. Kotnik, B. Valic, and D. Miklavcic, "Numerical determination of transmembrane voltage induced on irregularly shaped cells," *Ann. Biomed. Eng.*, vol. 34, pp. 642–652, 2006.

[11] G. Pucihar, T. Kotnik, D. Miklavcic, and T. J., "Kinetics of transmembrane transport of small molecules into electropermeabilized cells," *Biophys. J.*, vol. 95, pp. 2837–2848, 2008.

[12] D. C. Chang, "Structure and dynamics of electric field-induced membrane pores as revealed by rapid-freezing electron microscopy," in *Guide to Electroporation and Electrofusion*, Orlando, Florida: Academic Press, 1992, pp. 9–27.

[13] D. P. Tieleman, S.-J. Marrink, and H. J. C. Berendsen, "A Computer Perspective of Membranes: Molecular Dynamics Studies of Lipid Bilayer Systems," *Biochim. Biophys. Acta*, vol. 1331, no. 3, pp. 235–270, 1997.

[14] D. J. Tobias, K. Tu, and M. L. Klein, "Assessment of all--atom potentials for modeling membranes: Molecular dynamics simulations of solid and liquid alkanes and crystals of phospholipid fragments," *J. Chim. Phys.*, vol. 94, pp. 1482–1502, 1997.

[15] L. R. Forrest and M. S. P. Sansom, "Membrane simulations: bigger and better," *Curr. Opin. Struct. Biol.*, vol. 10, pp. 174–181, 2000.

[16] S. E. Feller, "Molecular dynamics simulations of lipid bilayers," *Curr. Opin. Coll In.*, vol. 5, pp. 217–223, 2000.

[17] C. Chipot, M. L. Klein, M. Tarek, and S. Yip, "Modeling lipid membranes.," in *Handbook of Materials Modeling*, S. Yip, Ed. Dordrecht, The Netherland: Springer, 2005, pp. 929–958.

[18] S. J. Marrink, A. H. de Vries, and D. P. Tieleman, "Lipids on the move: Simulations of membrane pores, domains, stalks and curves," *Biochim. Biophys. Acta. Biomembr.*, vol. 1788, pp. 149–168, 2009.

[19] Q. Hu, S. Viswanadham, R. P. Joshi, K. H. Schoenbach, S. J. Beebe, and P. F. Blackmore, "Simulations of transient membrane behavior in cells subjected to a high-intensity ultrashort electric pulse," *Phys. Rev. E - Stat. Nonlinear, Soft Matter Phys.*, vol. 71, no. 3, p. 31914, 2005.

[20] D. P. Tieleman, "The molecular basis of electroporation.," *BMC Biochem.*, vol. 5, no. 1, p. 10, Jan. 2004.

[21] M. Tarek, "Membrane electroporation: a molecular dynamics simulation.," *Biophysical journal*, vol. 88, no. 6. pp. 4045–4053, 2005.

[22] R. A. Böckmann, B. L. de Groot, S. Kakorin, E. Neumann, and H. Grubmüller, "Kinetics, statistics, and energetics of lipid membrane electroporation studied by molecular dynamics simulations.," *Biophys. J.*, vol. 95, no. 4, pp. 1837–1850, 2008.

[23] M. J. Ziegler and P. T. Vernier, "Interface water dynamics and porating electric fields for phospholipid bilayers," *J. Phys Chem. B*, vol. 112, pp. 13588–13596, 2008.

[24] M. P. Allen and D. J. Tildesley, *Computer simulation of liquids*. Oxford: Clarendon Press, 1987.

[25] A. R. Leach, *Molecular modelling: principles and applications*, Second Edi. Prentice Hall, 2001.

[26] L. D. Schuler, X. Daura, and W. F. van Gunsteren, "An improved GROMOS96 force field for aliphatic hydrocarbons in the condensed phase," *J. Comp. Chem*, vol. 22, pp. 1205–1218, 2001.

[27] A. D. MacKerell Jr., D. Bashford, M. Bellott, R. L. Dunbrack Jr., J. Evanseck, M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, D. Joseph-McCarthy, L. Kuchnir, K. Kuczera, F. T. K. Lau, C. Mattos, S. Michnick, T. Ngo, D. T. Nguyen, B. Prodhom, W. E. Reiher III, B. Roux, M. Schlenkrich, J. C. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorkiewicz-Kuczera, D. Yin, and M. Karplus, "All-atom empirical potential for molecular modeling and dynamics studies of proteins.," *J. Phys. Chem. B*, vol. 102, pp. 3586–3616, 1998.

[28] D. A. Case, D. A. Pearlman, J. W. Caldwell, T. E. Cheatham III, W. S. Ross, C. L. Simmerling, T. A. Darden, K. M. Merz, R. V Stanton, A. L. Cheng, J. J. Vincent, M. Crowley, V. Tsui, R. J. Radmer, Y. Duan, J. Pitera, I. Massova, G. L. Seibel, and U. C. Singh, *AMBER6*. San Francisco: University of California, 1999.

[29] K. Vanommeslaeghe, E. Hatcher, C. Acharya, S. Kundu, S. Zhong, J. Shim, E. Darian, O. Guvench, P. Lopes, I. Vorobyov, and A. and Mackerell, "CHARMM General Force Field: A Force Field for Drug-Like Molecules Compatible With the CHARMM All-Atom Additive Biological Force Fields," *J. Comp. Chem.*, vol. 31, no. 4, pp. 671–690, 2010.

[30] A. Warshel, M. Kato, and A. V Pisliakov, "Polarizable force fields: history, test cases, and prospects," *J. Chem. Theory Comput.*, vol. 3, pp. 2034–2045, 2007.

[31] T. A. Halgren and W. Damm, "Polarizable force fields," *Curr. Opin. Struct. Biol.*, vol. 11, pp. 236–242, 2001.

[32] E. Lindahl and O. Edholm, "Mesoscopic undulations and thickness fluctuations in lipid bilayers from molecular dynamics simulations," *Biophys. J.*, vol. 79, pp. 426–433, 2000.

[33] S. J. Marrink and A. E. Mark, "Effect of undulations on surface tension in simulated bilayers.," *J. Phys. Chem. B*, vol. 105, pp. 6122–6127, 2001.

[34] S.W. Chiu, M. Clark, E. Jakobsson, S. Subramaniam, and H. L. Scott, "Optimization of hydrocarbon chain interaction parameters: Application to the simulation of fluid phase lipid bilayers," *J. Phys. Chem. B*, vol. 103, pp. 6323–6327, 1999.

[35] T. Rög, K. Murzyn, and M. Pasenkiewicz-Gierula, "The dynamics of water at the phospholipid bilayer: A molecular dynamics study," *Chem. Phys. Lett.*, vol. 352, pp. 323–327, 2002.

[36] L. Saiz and M. L. Klein, "Computer simulation studies of model biological membranes," *Acc. Chem. Res.*, vol. 35, pp. 482–489, 2002.

[37] S. E. Feller, K. Gawrisch, and A. D. MacKerell, "Polyunsaturated fatty acids in lipid bilayers: intrinsic and environmental contributions to their unique physical properties," *J. Am. Chem. Soc.*, vol. 124, pp. 318–326, 2002.

[38] M. L. Berkowitz and M. J. Raghavan, "Computer simulation of a water/membrane interface," *Langmuir*, vol. 7, pp. 1042–1044, 1991.

[39] K. V Damodaran and K. M. Merz, "A comparison of dmpc and dlpe based lipid bilayers," *Biophys. J.*, vol. 66, pp. 1076–1087, 1994.

[40] J. J. L. Cascales, J. G. a de la Torre, S. J. Marrink, and H. J. C. Berendsen, "Molecular dynamics simulation of a charged biological membrane," *J. Chem. Phys.*, vol. 104, pp. 2713–2720, 1996.

[41] P. Mukhopadhyay, L. Monticelli, and D. P. Tieleman, "Molecular dynamics simulation of a palmitoyl-oleoyl phosphatidylserine bilayer with Na+ Counterions and NaCl," *Biophys. J.*, vol. 86, pp. 1601–1609, 2004.

[42] S. W. Chiu, S. Vasudevan, E. Jakobsson, R. J. Mashl, and H. L. Scott, "Structure of sphingomyelin bilayers: A simulation study," *Biophys. J.*, vol. 85, pp. 3624–3635, 2003.

[43] A. S. Pandit, D. Bostick, and M. L. Berkowitz, "Molecular dynamics simulation of a dipalmitoylphosphatidylcholine bilayer with NaCl.," *Biophys. J*, vol. 84, pp. 3743–3750, 2003.

[44] R. Y. Patel and P. V Balaji, " Characterization of symmetric and asymmetric lipid bilayers composed of varying concentrations of ganglioside GM1 and DPPC," *J. Phys Chem. B*, vol. 112, pp. 3346–3356, 2008.

[45] M. Dahlberg and A. Maliniak, "Molecular dynamics simulations of cardiolipin bilayers," *J. Phys Chem. B*, vol. 112, pp. 11655–11663, 2008.

[46] A. A. Gurtovenko and I. Vattulainen, "Effect of NaCl and KCl on phosphatidylcholine and phosphatidylethanolamine lipid membranes: Insight from atomic-scale simulations for understanding salt-induced effects in the plasma membrane ," *J. Phys Chem. B*, vol. 112, pp. 1953–1962, 2008.

[47] R. Vacha, M. L. Berkowitz, and P. Jungwirth, "Molecular model of a cell plasma membrane with an asymmetric multicomponent composition: Water permeation and ion effects," *Biophys J*, vol. 96, pp. 4493–4501, 2009.

[48] T. Rog, H. Martinez-Seara, N. Munck, M. Oresic, M. Karttunen, and I. Vattulainen, "Role of cardiolipins in the inner mitochondrial membrane: Insight gained through atom-scale simulations," *J. Phys Chem. B*, vol. 113, pp. 3413–3422, 2009.

[49] Z. Li, R. M. Venable, L. A. Rogers, D. Murray, and R. W. Pastor, "Molecular dynamics simulations of PIP2 and PIP3 in lipid bilayers: Determination of ring orientation, and the effects of surface roughness on a poisson-boltzmann description," *Biophys J*, vol. 97, pp. 155–163, 2009.

[50] M. Tarek, D. J. Tobias, S. H. Chen, and M. L. Klein, "Short waverlength collective dynamics in phospholipid bilayers: a molecular dynamics study," *Phys. Rev . Lett.*, vol. 87, p. 238101, 2001.

[51] C. Anézo, A. H. de Vries, H. D. Höltje, D. P. Tieleman, and S. J. Marrink, "Methodological issues in lipid bilayer simulations," *J. Phys. Chem. B*, vol. 107, pp. 9424–9433, 2003.

[52] S. J. Beebe and K. H. Schoenbach, "Nanosecond pulsed electric fields: A new stimulus to activate intracellular signaling," *j. Biomed. Biotech.*, vol. 4, pp. 297–300, 2005.

[53] Z. Vasilkoski, A. T. Esser, T. R. Gowrishankar, and J. C. Weaver, "Membrane electroporation: The absolute rate equation and nanosecond time scale pore creation," *Phys. Rev. E - Stat. Nonlinear, Soft Matter Phys.*, vol. 74, no. 2, 2006.

[54] R. Sundararajan, "Nanosecond electroporation: another look," *Mol. Biotech.*, vol. 41, pp. 69–82, 2009.

[55] J. Deng, K. H. Schoenbach, E. Stephen Buescher, P. S. Hair, P. M. Fox, and S. J. Beebe, "The Effects of Intense Submicrosecond Electrical Pulses on Cells," *Biophys. J.*, vol. 84, no. 4, pp. 2709–2714, Apr. 2003.

[56] H. Pauly and H. P. Schwan, "Uber Die Impedanz Einer Suspension Von Kugelformigen Teilchen Mit Einer Schale - Ein Modell Fur Das Dielektrische Verhalten Von Zellsuspensionen Und Von Proteinlosungen," *Z Naturforsch B*, vol. 14, no. 2, pp. 125–131, 1959.

[57] T. Kotnik, D. Miklavcic, and T. Slivnik, "Time course of transmembrane voltage induced by time-varying electric fields - a method for theoretical analysis and its application ," *Bioelectrochem. Bioenerg.*, vol. 45, no. 1, pp. 3–16, 1998.

[58] T. Kotnik and D. Miklavcic, "Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields," *Biophys J*, vol. 90, no. 2, pp. 480–491, 2006.

[59] Q. Zhong, Q. Jiang, P. B. Moore, D. M. Newns, and M. L. Klein, "Molecular dynamics simulation of a synthetic ion channel," *Biophys. J.*, vol. 74, pp. 3–10, 1998.

[60] Y. Yang, D. Henderson, P. Crozier, R. L. Rowley, and D. D. Busath, "Permeation of ions through a model biological channel: effect of periodic boundary condition and cell size. ," *Molec. Phys*, vol. 100, pp. 3011–3019, 2002.

[61] D. P. Tieleman, J. H. C. Berendsen, and M. S. P. Sansom, "Voltage-dependent insertion of alamethicin at phospholipid/water and octane water interfaces.," *Biophys. J.*, vol. 80, pp. 331–346, 2001.

[62] P. S. Crozier, D. Henderson, R. L. Rowley, and D. D. Busath, "Model channel ion currents in NaCl extended simple point charge water solution with applied-field molecualr dynamics," *Biophys. J.*, vol. 81, pp. 3077–3089, 2001.

[63] B. Roux, "The membrane potential and its representation by a constant electric field in computer simulations," *Biophys J*, vol. 95, pp. 4205–4216, 2008.

[64] P. T. Vernier, M. J. Ziegler, Y. Sun, W. V Chang, M. A. Gundersen, and D. P. Tieleman, "Nanopore formation and phosphatidylserine externalization in a phospholipid bilayer at high transmembrane potential," *J. Am. Chem. Soc.*, vol. 128, no. 19, pp. 6288–6289, 2006.

[65] W. Treptow, B. Maigret, C. Chipot, and M. Tarek, "Coupled motions between pore and voltage-sensor domains: a model for Shaker B, a voltage-gated potassium channel.," *Biophys. J.*, vol. 87, pp. 2365–2379, 2004.

[66] A. Aksimentiev and K. Schulten, "Imaging a-hemolysin with molecular dynamics: ionic conductance, osmotic permeability, and the electrostatic potential map," *Biophys. J.*, vol. 88, pp. 3745–3761, 2005.

[67] F. Khalili-Araghi, E. Tajkhorshid, and K. Schulten, "Dynamics of K+ ion conduction through Kv1.2. ," *Biophys. J.*, vol. 91, pp. L72–L74, 2006.

[68] M. Sotomayor, V. Vasquez, E. Perozo, and K. Schulten, "Ion conduction through MscS as determined by electrophysiology and simulation," *Biophys. J.*, vol. 92, pp. 886–902, 2007.

[69] C. Chimerel, L. Movileanu, S. Pezeshki, M. Winterhalter, and U. Kleinekathofer, "Transport at the nanoscale: temperature dependence of ion conductance," *Eur. Biophys. J. Biophy. Lett.*, vol. 38, pp. 121–125, 2008.

[70] D. J. Tobias, "Electrostatic calculations: recent methodological advances and applications to membranes," *Curr. Opin. Struct. Biol.*, vol. 11, pp. 253–261, 2001.

[71] K. Gawrisch, D. Ruston, J. Zimmerberg, V. Parsegian, R. Rand, and N. Fuller, "Membrane dipole potentials, hydration forces, and the ordering of water at membrane surfaces," *Biophys. J.*, vol. 61, pp. 1213–1223, 1992.

[72] P. T. Vernier and M. J. Ziegler, "Nanosecond field alignment of head group and water dipoles in electroporating phospholipid bilayers," *J. Phys. Chem. B*, vol. 111, no. 45, pp. 12993–12996, 2007.

[73] Z. A. Levine and P. T. Vernier, "Life cycle of an electropore: Field-dependent and field-independent steps in pore creation and annihilation," *J. Membr. Biol.*, vol. 236, no. 1, pp. 27–36, 2010.

[74] Z. A. Levine and P. T. Vernier, "Calcium and phosphatidylserine inhibit lipid electropore formation and reduce pore lifetime," *J. Membr. Biol.*, vol. 245, no. 10, pp. 599–610, 2012.

[75] M. Golzio, J. Teissie, and M.-P. Rols, "Direct visualization at the single-cell level of electriclly mediated gene delivery," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 99, pp. 1292–1297, 2002.

[76] a Paganin-Gioanni, E. Bellard, J. M. Escoffre, M. P. Rols, J. Teissié, and M. Golzio, "Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 26, pp. 10443–10447, 2011.

[77] M. Breton, L. Delemotte, A. Silve, L. M. Mir, and M. Tarek, "Transport of siRNA through lipid membranes driven by nanosecond electric pulses: an experimental and computational study.," *J. Am. Chem. Soc.*, vol. 134, no. 34, pp. 13938–13941, Aug. 2012.

[78] J. N. Sachs, P. S. Crozier, and T. B. Woolf, "Atomistic simulations of biologically realistic transmembrane potential gradients," *J. Chem. Phys.*, vol. 121, pp. 10847–10851, 2004.

[79] L. Delemotte, F. Dehez, W. Treptow, and M. Tarek, "Modeling membranes under a transmembrane potential," *J. Phys. Chem. B*, vol. 112, no. 18, pp. 5547–5550, 2008.

[80] L. Delemotte and M. Tarek, "Molecular dynamics simulations of lipid membrane electroporation," *J. Membr. Biol.*, vol. 245, no. 9, pp. 531–543, 2012.

[81] B. Roux, "Influence of the membrane potential on the free energy of an intrinsic protein," *Biophys. J.*, vol. 73, pp. 2980–2989, 1997.

[82] A. A. Gurtovenko and I. Vattulainen, "Pore Formation Coupled to Ion Transport through Lipid Membranes as Induced by Transmembrane Ionic Charge Imbalance: Atomistic Molecular Dynamics Study," *J. Am. Chem. Soc.*, vol. 127, no. 50, pp. 17570–17571, 2005.

[83] S. K. Kandasamy and R. G. Larson, "Cation and anion transport through hydrophilic pores in lipid bilayers," *J. Chem. Phys.*, vol. 125, p. 74901, 2006.

[84] Kutzner C, Grubmüller H, de Groot BL, and Z. U., "Computational electrophysiology: the molecular dynamics of ion channel permeation and selectivity in atomistic detail," *Biophys J*, vol. 101, pp. 809–817, 2011.

[85] Y. A. Liberman and V. P. Topaly, "Permeability of biomolecular phospholipid membranes for fat-soluble ions," *Biophys. USSR*, vol. 14, p. 477, 1969.

[86] S. J. Marrink, F. Jähniga, and H. J. Berendsen, "Proton transport across transient single-file water pores in a lipid membrane studied by molecular dynamics simulations," *Biophys J.*, vol. 71, pp. 632–647, 1996.

[87] M.-C. Ho, Z. A. Levine, and P. T. Vernier, "Nanoscale, Electric Field-Driven Water Bridges in Vacuum Gaps and Lipid Bilayers," *J. Membr. Biol.*, vol. 246, no. 11, pp. 793–801, May 2013.

[88] Z. A. Levine and P. T. Vernier, "Life cycle of an electropore: Field-dependent and field-independent steps in pore creation and annihilation," *J. Membr. Biol.*, vol. 236, no. 1, pp. 27–36, Jul. 2010.

[89] W. F. D. Bennett and D. P. Tieleman, "The importance of membrane defects-lessons from simulations.," *Acc. Chem. Res.*, vol. 47, no. 8, pp. 2244–51, 2014.

[90] U. Pliquett, R. P. Joshi, V. Sridhara, and K. H. Schoenbach, "High electrical field effects on cell membranes.," *Bioelectrochemistry*, vol. 70, no. 2, pp. 275–282, 2007.

[91] D. P. Tieleman, H. Leontiadou, A. E. Mark, and S. J. Marrink, "Simulation of pore formation in lipid bilayers by mechanical stress and electric fields," *J. Am. Chem. Soc.*, vol. 125, no. 21, pp. 6382–6383, 2003.

[92] S. Koronkiewicz and S. Kalinowski, "Influence of cholesterol on electroporation of bilayer lipid membranes: chronopotentiometric studies," *Biochim. Biophys. Acta - Biomembr.*, vol. 1661, no. 2, pp. 196–203, Mar. 2004.

[93] M. Casciola, D. Bonhenry, M. Liberti, F. Apollonio, and M. Tarek, "A molecular dynamic study of cholesterol rich lipid membranes: comparison of electroporation protocols.," *Bioelectrochemistry*, vol. 100, pp. 11–17, Dec. 2014.

[94] S. Kakorin, U. Brinkmann, and E. Neumann, "Cholesterol reduces membrane electroporation and electric deformation of small bilayer vesicles," *Biophys. Chem.*, vol. 117, no. 2, pp. 155–171, 2005.

[95] M. L. Fernández, M. Risk, R. Reigada, and P. T. Vernier, "Size-controlled nanopores in lipid membranes with stabilizing electric fields," *Biochem. Biophys. Res. Commun.*, vol. 423, no. 2, pp. 325–330, 2012.

[96] A. Polak, D. Bonhenry, F. Dehez, P. Kramar, D. Miklavčič, and M. Tarek, "On the electroporation thresholds of lipid bilayers: molecular dynamics simulation investigations.," *J. Membr. Biol.*, vol. 246, no. 11, pp. 843–850, Nov. 2013.

[97] A. Polak, M. Tarek, M. Tomšič, J. Valant, N. P. Ulrih, A. Jamnik, P. Kramar, and D. Miklavčič, "Electroporation of archaeal lipid membranes using MD simulations," *Bioelectrochemistry*, vol. 100, pp. 18–26, 2014.

[98] A. A. Gurtovenko and A. S. Lyulina, "Electroporation of asymmetric phospholipid membranes," *J. Phys. Chem. B*, vol. 118, no. 33, pp. 9909–9918, 2014.

[99] T. J. Piggot, D. A. Holdbrook, and S. Khalid, "Electroporation of the E. coli and S. aureus membranes: Molecular dynamics simulations of complex bacterial membranes," *J. Phys. Chem. B*, vol. 115, no. 45, pp. 13381–13388, 2011.

[100] F. Dehez, L. Delemotte, P. Kramar, D. Miklavčič, and M. Tarek, "Evidence of conducting hydrophobic nanopores across membranes in response to an electric field," *J. Phys. Chem. C*, vol. 118, no. 13, pp. 6752–6757, 2014.

[101] M. Casciola, M. A. Kasimova, L. Rems, S. Zullino, F. Apollonio, and M. Tarek, "Properties of lipid electropores I: Molecular dynamics simulations of stabi- lized pores by constant charge imbalance Properties of lipid electropores I: Molecular dy- namics simulations of stabilized pores by constant charge imbalance," *Bioelectrochemistry*, vol. 109, pp. 108–116, 2016.

[102] P. Kramar, L. Delemotte, A. Maček Lebar, M. Kotulska, M. Tarek, and D. Miklavčič, "Molecular-level characterization of lipid membrane electroporation using linearly rising current.," *J. Membr. Biol.*, vol. 245, no. 10, pp. 651–659, Oct. 2012.

[103] M. C. Ho, M. Casciola, Z. A. Levine, and P. T. Vernier, "Molecular dynamics simulations of ion conductance in field-stabilized nanoscale lipid electropores," *J. Phys. Chem. B*, vol. 117, no. 39, pp. 11633–11640, 2013.

[104] H. Leontiadou, A. E. Mark, and S.-J. Marrink, "Ion transport across transmembrane pores.," *Biophys. J.*, vol. 92, no. 12, pp. 4209–4215, Jun. 2007.

[105] A. A. Gurtovenko and I. Vattulainen, "Ion leakage through transient water pores in protein-free lipid membranes driven by transmembrane ionic charge imbalance.," *Biophys. J.*, vol. 92, no. 6, pp. 1878–1890, 2007.

[106] A. A. Gurtovenko and I. Vattulainen, "Molecular mechanism for lipid flip-flops," *J. Phys. Chem. B*, vol. 111, no. 48, pp. 13554–13559, 2007.

[107] A. A. Gurtovenko, J. Anwar, and I. Vattulainen, "Defect-Mediated Trafficking across Cell Membranes: Insights from in Silico Modeling," 2010.

[108] V. Sridhara and R. P. Joshi, "Numerical study of lipid translocation driven by nanoporation due to multiple high-intensity, ultrashort electrical pulses," *Biochim. Biophys. Acta - Biomembr.*, vol. 1838, no. 3, pp. 902–909, 2014.

[109] F. Salomone, M. Breton, I. Leray, F. Cardarelli, C. Boccardi, D. Bonhenry, M. Tarek, L. M. Mir, and F. Beltram, "High-yield nontoxic gene transfer through conjugation of the CM 18-Tat11 chimeric peptide with nanosecond electric pulses," *Mol. Pharm.*, vol. 11, no. 7, pp. 2466–2474, 2014.

[110] P. T. Vernier, M. J. Ziegler, Y. Sun, M. A. Gundersen, and D. P. Tieleman, "Nanopore- facilitated, voltage-driven phosphatidylserine translocation in lipid bilayers- in cells and in silico," *Phys. Biol*, vol. 3, pp. 233–247, 2006.

[111] M. Casciola and M. Tarek, "A molecular insight into the electro-transfer of small molecules through electropores driven by electric fields," *Biochim. Biophys. Acta - Biomembr.*, 2016.

[112] J. Teissie and M. P. Rols, "An Experimental Evaluation of the Critical Potential Difference Inducing Cell-Membrane Electropermeabilization," *Biophys. J.*, vol. 65, no. 1, pp. 409–413, 1993.

[113] L. C. Benov, P. A. Antonov, and S. R. Ribarov, "Oxidative damage of the membrane-lipids after electroporation," *Gen. Physiol. Biophys.*, vol. 13, no. 2, pp. 85–97, Apr. 1994.

[114] B. Gabriel and J. Teissie, "Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their consequence on cell viability," *Eur. J. Biochem.*, vol. 223, no. 1, pp. 25–33, Jul. 1994.

[115] M. Maccarrone, M. R. Bladergroen, N. Rosato, and A. F. Agro, "Role of Lipid Peroxidation in Electroporation-Induced Cell Permeability," *Biochem. Biophys. Res. Commun.*, vol. 209, no. 2, pp. 417–425, Apr. 1995.

[116] M. Maccarrone, N. Rosato, and A. F. Agrò, "Electroporation enhances cell membrane peroxidation and luminescence.," *Biochem. Biophys. Res. Commun.*, vol. 206, no. 1, pp. 238–245, Jan. 1995.

[117] Y. Zhou, C. K. Berry, P. A. Storer, and R. M. Raphael, "Peroxidation of polyunsaturated phosphatidyl-choline lipids during electroformation.," *Biomaterials*, vol. 28, no. 6, pp. 1298–1306, Feb. 2007.

[118] O. N. Pakhomova, V. A. Khorokhorina, A. M. Bowman, R. Rodaitė-Riševičienė, G. Saulis, S. Xiao, and A. G. Pakhomov, "Oxidative effects of nanosecond pulsed electric field exposure in cells and cell-free media.," *Arch. Biochem. Biophys.*, vol. 527, no. 1, pp. 55–64, Nov. 2012.

[119] M. Breton, M. Amirkavei, and L. M. Mir, "Optimization of the Electroformation of Giant Unilamellar Vesicles (GUVs) with Unsaturated Phospholipids.," *J. Membr. Biol.*, vol. 248, no. 5, pp. 827–835, Oct. 2015.

[120] P. Jurkiewicz, A. Olżyńska, L. Cwiklik, E. Conte, P. Jungwirth, F. M. Megli, and M. Hof, "Biophysics of Lipid Bilayers Containing Oxidatively Modified Phospholipids: Insights from Fluorescence and EPR Experiments and from MD Simulations," *Biochim. Biophys. Acta*, vol. 1818, no. 10, pp. 2388–2402, 2012.

[121] P. T. Vernier, Z. A. Levine, Y.-H. Wu, V. Joubert, M. J. Ziegler, L. M. Mir, and D. P. Tieleman, "Electroporating fields target oxidatively damaged areas in the cell membrane.," *PLoS One*, vol. 4, no. 11, p. e7966, Jan. 2009.

[122] L. Beranova, L. Cwiklik, P. Jurkiewicz, M. Hof, and P. Jungwirth, "Oxidation changes physical properties of phospholipid bilayers: fluorescence spectroscopy and molecular simulations.," *Langmuir*, vol. 26, no. 9, pp. 6140–6144, May 2010.

[123] S. Kalghatgi, C. S. Spina, J. C. Costello, M. Liesa, J. R. Morones-Ramirez, S. Slomovic, A. Molina, O. S. Shirihai, and J. J. Collins, "Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells.," *Sci. Transl. Med.*, vol. 5, no. 192, p. 192ra85, 2013.

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NOTES

NOTES

Nanoscale and Multiscale Membrane Electrical Stress and Permeabilization

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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.

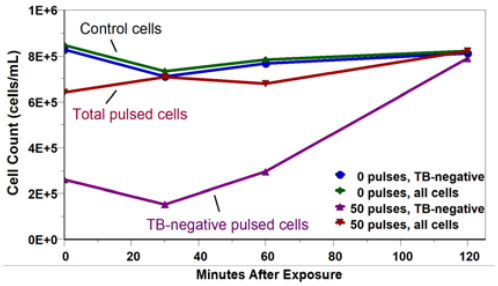

Figure 1. Nanoelectropulsed Jurkat T lymphoblasts recover over 2 hrs from initial Trypan blue permeabilization after exposure to 50, 20 ns, 4 MV/m pulses at 20 Hz.

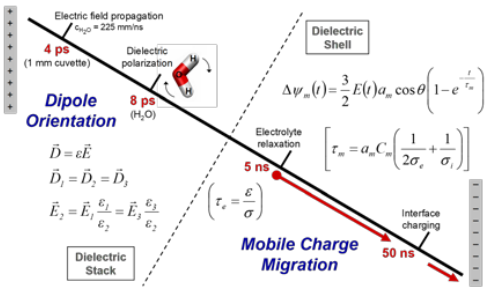

Figure 2. Timeline representing the sequence of events following electrical polarization of a biological tissue or aqueous suspension of cells. The dielectric properties of the system are important in the sub-nanosecond regime. For longer times the distribution of fields and potentials is dominated by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

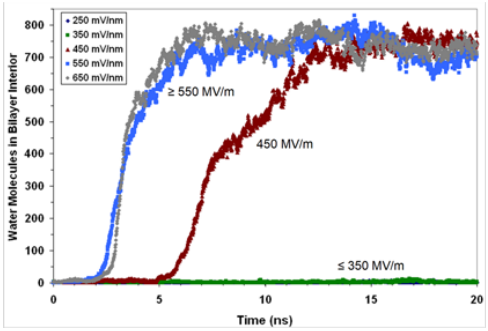

Figure 3. Electric field-driven intrusion of water into a simulated lipid bilayer.

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, MODELS

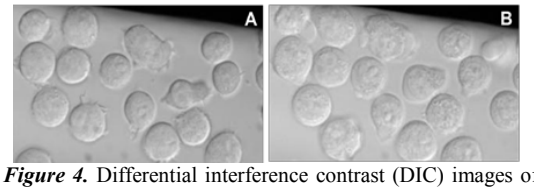

Figure 4. Differential interference contrast (DIC) images of Jurkat T lymphoblasts before (A) and 30 s after (B) exposure to 5 ns, 10 MV/m electric pulses (30 pulses, 1 kHz). Note swelling, blebbing, and intracellular granulation and vesicle expansion, results of the osmotic imbalance caused by electropermeabilization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

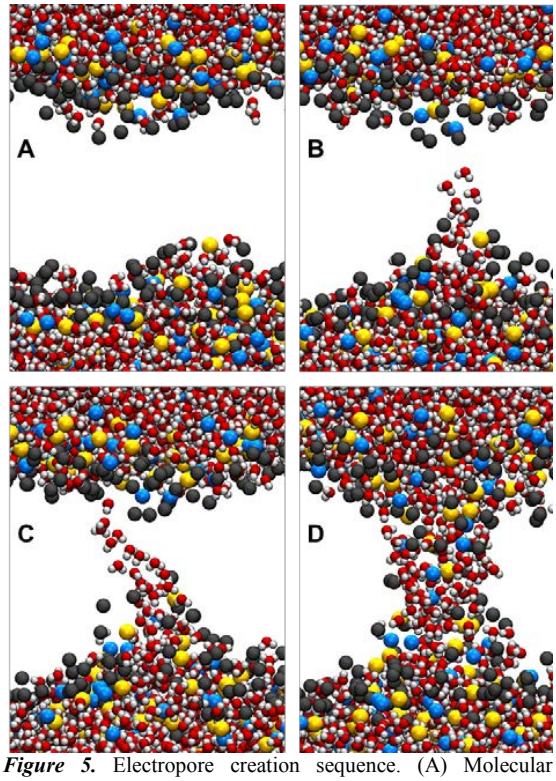

Figure 5. Electropore creation sequence. (A) Molecular dynamics representation of a POPC lipid bilayer. Small red and white spheres at the top and bottom of the panel are water oxygen and hydrogen atoms. Gold and blue spheres are head group phosphorus and nitrogen, respectively, and grey spheres are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

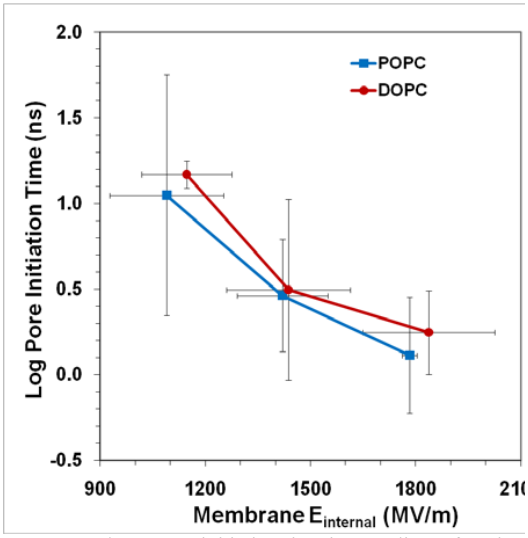

Figure 6. Electropore initiation time is a nonlinear function of the magnitude of the porating electric field. Pore initiation time (time required to form the water bridge shown in Fig. 1C) is exponentially dependent on the applied electric field, expressed here as the electric field observed in the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

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

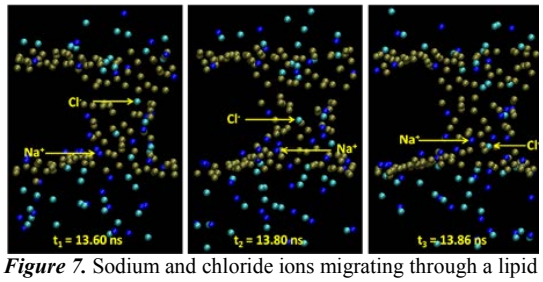

Figure 7. Sodium and chloride ions migrating through a lipid nanopore in the presence of an external electric field.

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

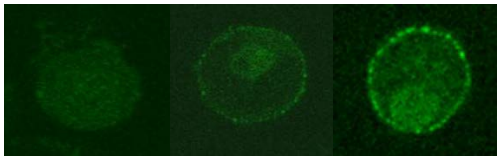

Figure 8. Immunocytochemical labeling of dopamine-β-hydroxylase (DβH) using an anti-DβH antibody coupled with a fluorescently-tagged 2° antibody. DβH is externalized by exocytotic fusion of vesicles with plasma membrane. Left panel, control. Center panel, 2 min after treatment with the pharmacological 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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REFERENCES

[1] Sher, L. D., E. Kresch, and H. P. Schwan. 1970. On the possibility of nonthermal biological effects of pulsed electromagnetic radiation. Biophys. J. 10:970-979.

[2] Drago, G. P., M. Marchesi, and S. Ridella. 1984. The frequency dependence of an analytical model of an electrically stimulated biological structure. Bioelectromagnetics 5:47-62.

[3] Plonsey, R., and K. W. Altman. 1988. Electrical stimulation of excitable cells - a model approach. Proceedings of the IEEE 76:1122-1129.

[4] Schoenbach, K. H., R. P. Joshi, J. F. Kolb, N. Y. Chen, M. Stacey, P. F. Blackmore, E. S. Buescher, and S. J. Beebe. 2004. Ultrashort electrical pulses open a new gateway into biological cells. Proceedings of the IEEE 92:1122-1137.

[5] Hofmann, F., H. Ohnimus, C. Scheller, W. Strupp, U. Zimmermann, and C. Jassoy. 1999. Electric field pulses can induce apoptosis. J. Membr. Biol. 169:103-109.

[6] Schoenbach, K. H., S. J. Beebe, and E. S. Buescher. 2001. Intracellular effect of ultrashort electrical pulses. Bioelectromagnetics 22:440-448.

[7] Benz, R., and U. Zimmermann. 1980. Pulse-length dependence of the electrical breakdown in lipid bilayer membranes. Biochim. Biophys. Acta 597:637-642.

[8] Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen. 2004. Nanoelectropulse-induced phosphatidylserine translocation. Biophys. J. 86:4040-4048.

[9] Gowrishankar, T. R., and J. C. Weaver. 2006. Electrical behavior and pore accumulation in a multicellular model for conventional and supra-electroporation. Biochem. Biophys. Res. Commun. 349:643-653.

[10] Grosse, C., and H. P. Schwan. 1992. Cellular membrane potentials induced by alternating fields. Biophys. J. 63:1632-1642.
[11] Gowrishankar, T. R., and J. C. Weaver. 2003. An approach to electrical modeling of single and multiple cells. Proc. Natl. Acad. Sci. U. S. A. 100:3203-3208.
[12] Kotnik, T., and D. Miklavcic. 2000. Second-order model of membrane electric field induced by alternating external electric fields. IEEE Trans. Biomed. Eng. 47:1074-1081.
[13] Timoshkin, I. V., S. J. MacGregor, R. A. Fouracre, B. H. Crichton, and J. G. Anderson. 2006. Transient electrical field across cellular membranes: pulsed electric field treatment of microbial cells. Journal of Physics D-Applied Physics 39:596-603.
[14] Schoenbach, K. H., S. Xiao, R. P. Joshi, J. T. Camp, T. Heeren, J. F. Kolb, and S. J. Beebe. 2008. The effect of intense subnanosecond electrical pulses on biological cells. IEEE Trans. Plasma Sci. 36:414-422.
[15] Frey, W., J. A. White, R. O. Price, P. F. Blackmore, R. P. Joshi, R. Nuccitelli, S. J. Beebe, K. H. Schoenbach, and J. F. Kolb. 2006. Plasma membrane voltage changes during nanosecond pulsed electric field exposure. Biophys. J. 90:3608-3615.
[16] Vernier, P. T., Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen. 2004. Nanosecond pulsed electric fields perturb membrane phospholipids in T lymphoblasts. FEBS Lett. 572:103-108.
[17] Tieleman, D. P. 2004. The molecular basis of electroporation. BMC Biochem 5:10.
[18] Hu, Q., R. P. Joshi, and K. H. Schoenbach. 2005. Simulations of nanopore formation and phosphatidylserine externalization in lipid membranes subjected to a high-intensity, ultrashort electric pulse. Phys Rev E Stat Nonlin Soft Matter Phys 72:031902.
[19] Vernier, P. T., M. J. Ziegler, Y. Sun, W. V. Chang, M. A. Gundersen, and D. P. Tieleman. 2006. Nanopore formation and phosphatidylserine externalization in a phospholipid bilayer at high transmembrane potential. J. Am. Chem. Soc. 128:6288-6289.
[20] Vernier, P. T., M. J. Ziegler, Y. Sun, M. A. Gundersen, and D. P. Tieleman. 2006. Nanopore-facilitated, voltage-driven phosphatidylserine translocation in lipid bilayers - in cells and in silico. Physical Biology 3:233-247.
[21] Vernier, P. T., Y. Sun, and M. A. Gundersen. 2006. Nanoelectropulse-driven membrane perturbation and small molecule permeabilization. BMC Cell Biol. 7:37.
[22] Idziorek, T., J. Estaquier, F. De Bels, and J. C. Ameisen. 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J. Immunol. Methods 185:249-258.
[23] Pakhomov, A. G., J. F. Kolb, J. A. White, R. P. Joshi, S. Xiao, and K. H. Schoenbach. 2007. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). Bioelectromagnetics 28:655-663.
[24] Pakhomov, A. G., R. Shevin, J. A. White, J. F. Kolb, O. N. Pakhomova, R. P. Joshi, and K. H. Schoenbach. 2007. Membrane permeabilization and cell damage by ultrashort electric field shocks. Arch. Biochem. Biophys. 465:109-118.
[25] Pakhomov, A. G., A. M. Bowman, B. L. Ibey, F. M. Andre, O. N. Pakhomova, and K. H. Schoenbach. 2009. Lipid nanopores can form a stable, ion channel-like conduction pathway in cell membrane. Biochem. Biophys. Res. Commun. 385:181-186.
[26] Vernier, P. T., Y. Sun, M. T. Chen, M. A. Gundersen, and G. L. Craviso. 2008. Nanosecond electric pulse-induced calcium entry into chromaffin cells. Bioelectrochemistry 73:1-4.
[27] Wang, S., J. Chen, M. T. Chen, P. T. Vernier, M. A. Gundersen, and M. Valderrabano. 2009. Cardiac myocyte excitation by ultrashort high-field pulses. Biophys. J. 96:1640-1648.
[28] Rogers, W. R., J. H. Merritt, J. A. Comeaux, C. T. Kuhnel, D. F. Moreland, D. G. Teltschik, J. H. Lucas, and M. R. Murphy. 2004. Strength-duration curve for an electrically excitable tissue extended down to near 1 nanosecond. IEEE Trans. Plasma Sci. 32:1587-1599.
[29] DeBruin, K. A., and W. Krassowska. 1998. Electroporation and shock-induced transmembrane potential in a cardiac fiber during defibrillation strength shocks. Ann. Biomed. Eng. 26:584-596.
[30] F. M. Andre, M. A. Rassokhin, A. M. Bowman, and A. G. Pakhomov, "Gadolinium blocks membrane permeabilization induced by nanosecond electric pulses and reduces cell death," *Bioelectrochemistry,* vol. 79, pp. 95-100, Aug 2010.
[31] O. M. Nesin, O. N. Pakhomova, S. Xiao, and A. G. Pakhomov, "Manipulation of cell volume and membrane pore comparison following single cell permeabilization with 60- and 600-ns electric pulses," *Biochim Biophys Acta,* vol. 1808, pp. 792-801, Dec 20 2010.
[32] Z. A. Levine and P. T. Vernier, "Life cycle of an electropore: field-dependent and field-independent steps in pore creation and annihilation," *J Membr Biol,* vol. 236, pp. 27-36, Jul 2010.
[33] R. J. Clarke, "The dipole potential of phospholipid membranes and methods for its detection," *Adv Colloid Interface Sci,* vol. 89-90, pp. 263-81, Jan 29 2001.
[34] I. P. Sugar and E. Neumann, "Stochastic model for electric field-induced membrane pores. Electroporation," *Biophys Chem,* vol. 19, pp. 211-25, May 1984.
[35] S. A. Freeman, M. A. Wang, and J. C. Weaver, "Theory of electroporation of planar bilayer membranes: predictions of the aqueous area, change in capacitance, and pore-pore separation," *Biophys J,* vol. 67, pp. 42-56, Jul 1994.
[36] R. W. Glaser, S. L. Leikin, L. V. Chernomordik, V. F. Pastushenko, and A. I. Sokirko, "Reversible electrical breakdown of lipid bilayers: formation and evolution of pores," *Biochim Biophys Acta,* vol. 940, pp. 275-87, May 24 1988.
[37] J. M. S. McQuade, J. H. Merritt, S. A. Miller, T. Scholin, M. C. Cook, A. Salazar, O. B. Rahimi, M. R. Murphy, and P. A. Mason, "Radiofrequency-radiation exposure does not induce detectable leakage of albumin across the blood-brain barrier," *Radiation Research,* vol. 171, pp. 615-621, May 2009.
[38] N. D. Volkow, D. Tomasi, G. J. Wang, P. Vaska, J. S. Fowler, F. Telang, D. Alexoff, J. Logan, and C. Wong, "Effects of cell phone radiofrequency signal exposure on brain glucose metabolism," *JAMA,* vol. 305, pp. 808-13, Feb 23 2011.
[39] Stämpfli, R., and M. Willi. 1957. Membrane potential of a Ranvier node measured after electrical destruction of its membrane. *Experientia* 13:297-298.
[40] Coster, H. G. L. 1965. A quantitative analysis of the

voltage-current relationships of fixed charge membranes and the associated property of "punch-through". *Biophys. J.* 5:669-686.

[41] Chernomordik, L. V., S. I. Sukharev, S. V. Popov, V. F. Pastushenko, A. V. Sokirko, I. G. Abidor, and Y. A. Chizmadzhev. 1987. The electrical breakdown of cell and lipid membranes: the similarity of phenomenologies. *Biochim. Biophys. Acta* 902:360-373.

[42] Kalinowski, S., G. Ibron, K. Bryl, and Z. Figaszewski. 1998. Chronopotentiometric studies of electroporation of bilayer lipid membranes. *Biochim. Biophys. Acta* 1369:204-212.

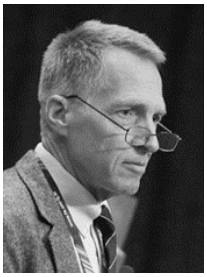
[43] Melikov, K. C., V. A. Frolov, A. Shcherbakov, A. V. Samsonov, Y. A. Chizmadzhev, and L. V. Chernomordik. 2001. Voltage-induced nonconductive pre-pores and metastable single pores in unmodified planar lipid bilayer. *Biophys. J.* 80:1829-1836.

[44] Koronkiewicz, S., S. Kalinowski, and K. Bryl. 2002. Programmable chronopotentiometry as a tool for the study of electroporation and resealing of pores in bilayer lipid membranes. *Biochim. Biophys. Acta* 1561:222-229.

[45] Fernandez, M. L., M. Risk, R. Reigada, and P. T. Vernier. 2012. Size-controlled nanopores in lipid membranes with stabilizing electric fields. *Biochem. Biophys. Res. Commun.* 423:325-330.

[46] G. L. Craviso, P. Chatterjee, G. Maalouf, A. Cerjanic, J. Yoon, I. Chatterjee, and P. T. Vernier, "Nanosecond electric pulse-induced increase in intracellular calcium in adrenal chromaffin cells triggers calcium-dependent catecholamine release," *Ieee Transactions on Dielectrics and Electrical Insulation,* vol. 16, pp. 1294-1301, Oct 2009.

[47] G. L. Craviso, S. Choe, P. Chatterjee, I. Chatterjee, and P. T. Vernier, "Nanosecond electric pulses: a novel stimulus for triggering Ca^{2+} influx into chromaffin cells via voltage-gated Ca^{2+} channels," *Cell Mol Neurobiol,* vol. 30, pp. 1259-65, Nov 2010.



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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].

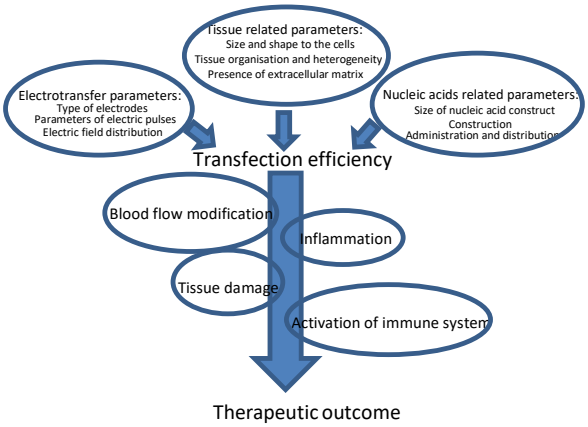

Figure 1: Different parameters can influence the transfection efficiency and therapeutic outcome of gene electrotransfer.

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].

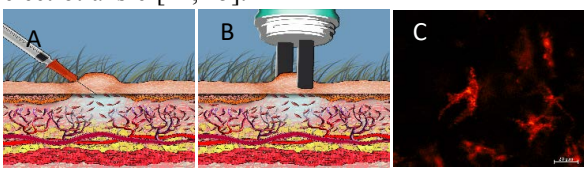

Figure 2: Gene electrotransfer to skin. **A** injection of plasmid DNA subcutaneously. A bubble on the skin will be formed. **B** If using plate electrodes, they are positioned in a way that the bubble is encompassed between the two plates. **C** Intravital confocal microscopy of cells in mouse skin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

REFERENCES

[1] E. Neumann, M. Schaeferridder, Y. Wang, and P. H. Hofschneider, “GENE-TRANSFER INTO MOUSE LYOMA CELLS BY ELECTROPORATION IN HIGH ELECTRIC-FIELDS,” *Embo J.*, vol. 1, no. 7, pp. 841–845, 1982.

[2] S. Orlowski, J. Belehradek J., C. Paoletti, and L. M. Mir, “Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs,” *Biochem Pharmacol*, vol. 37, no. 24, pp. 4727–4733, 1988.

[3] A. V Titomirov, S. Sukharev, and E. Kistanova, “In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA.,” *Biochim Biophys Acta*, vol. 1088, no. 1, pp. 131–134, 1991.

[4] T. Nishi, K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, I. Kitamura, T. Yoshimura, H. Saya, J. Kuratsu, and Y. Ushio, “High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation.,” *Cancer Res*, vol. 56, no. 5, pp. 1050–1055, 1996.

[5] R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, and C. Nicolau, “In vivo gene electroinjection and expression in rat liver.,” *FEBS Lett*, vol. 389, no. 3, pp. 225–228, 1996.

[6] M. P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros, and J. Teissie, “In vivo electrically mediated protein and gene transfer in murine melanoma.,” *Nat Biotechnol*, vol. 16, no. 2, pp. 168–171, 1998.

[7] L. M. Mir, M. F. Bureau, R. Rangara, B. Schwartz, and D.

Scherman, "Long-term, high level in vivo gene expression after electric pulse-mediated gene transfer into skeletal muscle.," *C R Acad Sci III*, vol. 321, no. 11, pp. 893–899, 1998.

[8] H. Aihara and J. Miyazaki, "Gene transfer into muscle by electroporation in vivo.," *Nat Biotechnol*, vol. 16, no. 9, pp. 867–870, 1998.

[9] L. Lambricht, A. Lopes, S. Kos, G. Sersa, V. Préat, and G. Vandermeulen, "Clinical potential of electroporation for gene therapy and DNA vaccine delivery.," *Expert Opin. Drug Deliv.*, vol. 13, no. 2, pp. 295–310, 2016.

[10] R. Heller and L. C. Heller, "Gene electrotransfer clinical trials.," *Adv. Genet.*, vol. 89, pp. 235–62, 2015.

[11] E. Kinnear, L. J. Caproni, and J. S. Tregoning, "A Comparison of Red Fluorescent Proteins to Model DNA Vaccine Expression by Whole Animal In Vivo Imaging.," *PLoS One*, vol. 10, no. 6, p. e0130375, 2015.

[12] A. Gothelf, J. Eriksen, P. Hojman, and J. Gehl, "Duration and level of transgene expression after gene electrotransfer to skin in mice.," *Gene Ther.*, vol. 17, no. 7, pp. 839–45, Jul. 2010.

[13] A. Gothelf and J. Gehl, "Gene electrotransfer to skin; review of existing literature and clinical perspectives," *Curr Gene Ther*, vol. 10, no. 4, pp. 287–299, 2010.

[14] C. Trollet, D. Scherman, and P. Bigey, "Delivery of DNA into muscle for treating systemic diseases: advantages and challenges.," *Methods Mol. Biol.*, vol. 423, pp. 199–214, 2008.

[15] M. Cemazar, M. Golzio, G. Sersa, M. P. Rols, and J. Teissié, "Electrically-assisted nucleic acids delivery to tissues in vivo: where do we stand?," *Curr Pharm Des*, vol. 12, no. 29, pp. 3817–3825, 2006.

[16] F. M. Andre, J. Gehl, G. Sersa, V. Preat, P. Hojman, J. Eriksen, M. Golzio, M. Cemazar, N. Pavselj, M. P. Rols, D. Miklavcic, E. Neumann, J. Teissie, and L. M. Mir, "Efficiency of High- and Low-Voltage Pulse Combinations for Gene Electrotransfer in Muscle, Liver, Tumor, and Skin," *Hum. Gene Ther.*, vol. 19, no. 11, pp. 1261–1271, 2008.

[17] N. Pavselj and V. Préat, "DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse.," *J. Control. Release*, vol. 106, no. 3, pp. 407–15, Sep. 2005.

[18] M. Cemazar, M. Golzio, G. Sersa, P. Hojman, S. Kranjc, S. Mesojednik, M. P. Rols, and J. Teissie, "Control by pulse parameters of DNA electrotransfer into solid tumors in mice," *Gene Ther.*, vol. 16, no. 5, pp. 635–644, 2009.

[19] V. Todorovic, U. Kamensek, G. Sersa, and M. Cemazar, "Changing electrode orientation, but not pulse polarity, increases the efficacy of gene electrotransfer to tumors in vivo," *Bioelectrochemistry*, vol. 100, pp. 119–127, 2014.

[20] R. Heller, Y. Cruz, L. C. Heller, R. A. Gilbert, and M. J. Jaroszeski, "Electrically mediated delivery of plasmid DNA to the skin, using a multielectrode array.," *Hum. Gene Ther.*, vol. 21, no. 3, pp. 357–62, Mar. 2010.

[21] S. Kos, T. Blagus, M. Cemazar, U. Lampreht Tratar, M. Stimac, L. Prosen, T. Dolinsek, U. Kamensek, S. Kranjc, L. Steinstraesser, G. Vandermeulen, V. Préat, and G. Sersa, "Electrotransfer parameters as a tool for controlled and targeted gene expression in skin.," *Mol. Ther. Nucleic Acids*, vol. 5, no. 8, p. e356, Aug. 2016.

[22] M. Donà, M. Sandri, K. Rossini, I. Dell'Aica, M. Podhorska-Okolow, and U. Carraro, "Functional in vivo gene transfer into the myofibers of adult skeletal muscle.," *Biochem. Biophys. Res. Commun.*, vol. 312, no. 4, pp. 1132–8, Dec. 2003.

[23] L. Zhang, E. Nolan, S. Kreitschitz, and D. P. Rabussay, "Enhanced delivery of naked DNA to the skin by non-invasive in vivo electroporation.," *Biochim. Biophys. Acta*, vol. 1572, no. 1, pp. 1–9, Aug. 2002.

[24] S. Babiuk, M. E. Baca-Estrada, M. Foldvari, L. Baizer, R. Stout, M. Storms, D. Rabussay, G. Widera, and L. Babiuk, "Needle-free topical electroporation improves gene expression from plasmids administered in porcine skin.," *Mol. Ther.*, vol. 8, no. 6, pp. 992–8, Dec. 2003.

[25] L. Daugimont, N. Baron, G. Vandermeulen, N. Pavselj, D. Miklavcic, M. C. Jullien, G. Cabodevila, L. M. Mir, and V. Préat, "Hollow microneedle arrays for intradermal drug delivery and DNA electroporation," *J Membr Biol*, vol. 236, no. 1, pp. 117–125, 2010.

[26] S. Mazères, D. Sel, M. Golzio, G. Pucihar, Y. Tamzali, D. Miklavcic, and J. Teissié, "Non invasive contact electrodes for in vivo localized cutaneous electropulsation and associated drug and nucleic acid delivery," *J Control Release*, vol. 134, no. 2, pp. 125–131, 2009.

[27] S. Corovic, I. Lackovic, P. Sustaric, T. Sustar, T. Rodic, and D. Miklavcic, "Modeling of electric field distribution in tissues during electroporation," *Biomed Eng Online*, vol. 12, p. 16, 2013.

[28] A. Paganin-Gioanni, E. Bellard, J. M. Escoffre, M. P. Rols, J. Teissié, and M. Golzio, "Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells," *Proc Natl Acad Sci U S A*, vol. 108, no. 26, pp. 10443–10447, 2011.

[29] J.-M. Escoffre, A. Debin, J.-P. Reynes, D. Drocourt, G. Tiraby, L. Hellaudais, J. Teissie, and M. Golzio, "Long-lasting in vivo gene silencing by electrotransfer of shRNA expressing plasmid.," *Technol. Cancer Res. Treat.*, vol. 7, no. 2, pp. 109–16, Apr. 2008.

[30] K. E. Broderick, A. Chan, F. Lin, X. Shen, G. Kichaev, A. S. Khan, J. Aubin, T. S. Zimmermann, and N. Y. Sardesai, "Optimized in vivo transfer of small interfering RNA targeting dermal tissue using in vivo surface electroporation," *Mol Ther Nucleic Acids*, vol. 1, p. e11, 2012.

[31] C. Marie, G. Vandermeulen, M. Quiviger, M. Richard, V. Préat, and D. Scherman, "pFARs, plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells.," *J. Gene Med.*, vol. 12, no. 4, pp. 323–32, Apr. 2010.

[32] G. Vandermeulen, H. Richiardi, V. Escriou, J. Ni, P. Fournier, V. Schirrmacher, D. Scherman, and V. Préat, "Skin-specific promoters for genetic immunisation by DNA electroporation.," *Vaccine*, vol. 27, no. 32, pp. 4272–7, Jul. 2009.

[33] N. Tesic and M. Cemazar, "In vitro targeted gene electrotransfer to endothelial cells with plasmid DNA containing human endothelin-1 promoter," in *Journal of Membrane Biology*, 2013, vol. 246, no. 10, pp. 783–791.

[34] S. Chabot, J. Orio, M. Schmeer, M. Schleef, M. Golzio, and J. Teissié, "Minicircle DNA electrotransfer for efficient tissue-targeted gene delivery.," *Gene Ther.*, vol. 20, no. 1, pp. 62–8, Jan. 2013.

[35] G. Tevz, S. Kranjc, M. Cemazar, U. Kamensek, A. Coer, M. Krzan, S. Vidic, D. Pavlin, and G. Sersa, "Controlled systemic release of interleukin-12 after gene electrotransfer to muscle for cancer gene therapy alone or in combination with ionizing radiation in murine sarcomas," *J Gene Med*, vol. 11, no. 12, pp. 1125–1137, 2009.

[36] M. Cemazar, D. Pavlin, S. Kranjc, A. Grosel, S. Mesojednik, and G. Sersa, "Sequence and time dependence of transfection efficiency of electrically-assisted gene delivery to tumors in

mice," *Curr. Drug Deliv.*, vol. 3, no. 1, 2006.

[37] S. Mesojednik, D. Pavlin, G. Sersa, A. Coer, S. Kranjc, A. Grosel, G. Tevz, and M. Cemazar, "The effect of the histological properties of tumors on transfection efficiency of electrically assisted gene delivery to solid tumors in mice," *Gene Ther.*, vol. 14, no. 17, 2007.

[38] J. M. McMahon, E. Signori, K. E. Wells, V. M. Fazio, and D. J. Wells, "Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase -- increased expression with reduced muscle damage.," *Gene Ther*, vol. 8, no. 16, pp. 1264–1270, 2001.

[39] M. Cemazar, M. Golzio, G. Sersa, P. Hojman, S. Kranjc, S. Mesojednik, M. P. Rols, and J. Teissie, "Control by pulse parameters of DNA electrotransfer into solid tumors in mice," *Gene Ther.*, vol. 16, no. 5, pp. 635–644, 2009.

[40] S. Mesojednik, D. Pavlin, G. Sersa, A. Coer, S. Kranjc, A. Grosel, G. Tevz, and M. Cemazar, "The effect of the histological properties of tumors on transfection efficiency of electrically assisted gene delivery to solid tumors in mice," *Gene Ther.*, vol. 14, no. 17, pp. 1261–1269, 2007.

[41] M. Cemazar, G. Sersa, J. Wilson, G. M. Tozer, S. L. Hart, A. Grosel, and G. U. Dachs, "Effective gene transfer to solid tumors using different nonviral gene delivery techniques: Electroporation, liposomes, and integrin-targeted vector," *Cancer Gene Ther.*, vol. 9, no. 4, 2002.

[42] K. Znidar, M. Bosnjak, M. Cemazar, and L. C. Heller, "Cytosolic DNA Sensor Upregulation Accompanies DNA Electrotransfer in B16.F10 Melanoma Cells," *Mol. Ther. - Nucleic Acids*, vol. 5, no. 6, 2016.

[43] P. Chiarella, E. Massi, M. De Robertis, A. Sibilio, P. Parrella, V. M. Fazio, and E. Signori, "Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration.," *Expert Opin. Biol. Ther.*, vol. 8, no. 11, pp. 1645–57, Nov. 2008.

[44] J. J. Drabick, J. Glasspool-Malone, A. King, and R. W. Malone, "Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization.," *Mol. Ther.*, vol. 3, no. 2, pp. 249–55, Feb. 2001.

[45] M. L. Yarmush, A. Golberg, G. Serša, T. Kotnik, and D. Miklavčič, "Electroporation-based technologies for medicine: principles, applications, and challenges.," *Annu. Rev. Biomed. Eng.*, vol. 16, pp. 295–320, Jul. 2014.

[46] M. Nomura, Y. Nakata, T. Inoue, A. Uzawa, S. Itamura, K. Nerome, M. Akashi, and G. Suzuki, "In vivo induction of cytotoxic T lymphocytes specific for a single epitope introduced into an unrelated molecule.," *J. Immunol. Methods*, vol. 193, no. 1, pp. 41–9, Jul. 1996.

[47] G. L. Niu, R. Heller, R. Catlett-Falcone, D. Coppola, M. Jaroszeski, W. Dalton, R. Jove, and H. Yu, "Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor in vivo," *Cancer Res.*, vol. 59, no. 20, pp. 5059–5063, 1999.

[48] F. M. Andre and L. M. Mir, "Nucleic acids electrotransfer in vivo: mechanisms and practical aspects," *Curr Gene Ther*, vol. 10, no. 4, pp. 267–280, 2010.

[49] T. Dolinsek, B. Markelc, G. Sersa, A. Coer, M. Stimac, J. Lavrencak, A. Brozic, S. Kranjc, and M. Cemazar, "Multiple Delivery of siRNA against Endoglin into Murine Mammary Adenocarcinoma Prevents Angiogenesis and Delays Tumor Growth," *PLoS One*, vol. 8, no. 3, 2013.

[50] L. Heller, C. Pottinger, M. J. Jaroszeski, R. Gilbert, and R. Heller, "In vivo electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces long-term antitumour immunity," *Melanoma Res*, vol. 10, no. 6, pp. 577–583, 2000.

[51] S. L. Li, X. J. Zhang, and X. Q. Xia, "Regression of tumor growth and induction of long-term antitumor memory by interleukin 12 electro-gene therapy," *J. Natl. Cancer Inst.*, vol. 94, no. 10, pp. 762–768, 2002.

[52] L. Heller, V. Todorovic, and M. Cemazar, "Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas," *Cancer Gene Ther.*, vol. 20, no. 12, pp. 695–700, 2013.

[53] M. Cemazar, T. Jarm, and G. Sersa, "Cancer electrogene therapy with interleukin-12.," *Curr. Gene Ther.*, vol. 10, no. 4, pp. 300–311, 2010.

[54] G. Trinchieri, "Interleukin-12 and the regulation of innate resistance and adaptive immunity," *Nat. Rev. Immunol.*, vol. 3, no. 2, pp. 133–146, 2003.

[55] L. C. Heller and R. Heller, "In vivo electroporation for gene therapy," *Hum. Gene Ther.*, vol. 17, no. 9, pp. 890–897, 2006.

[56] J. M. Escoffre, J. Teissié, and M. P. Rols, "Gene transfer: how can the biological barriers be overcome?," *J Membr Biol*, vol. 236, no. 1, pp. 61–74, 2010.

[57] M. Cemazar and G. Sersa, "Electrotransfer of therapeutic molecules into tissues," *Curr Opin Mol Ther*, vol. 9, no. 6. pp. 554–562, 2007.

[58] T. Cichoń, L. Jamrozy, J. Glogowska, E. Missol-Kolka, and S. Szala, "Electrotransfer of gene encoding endostatin into normal and neoplastic mouse tissues: inhibition of primary tumor growth and metastatic spread.," *Cancer Gene Ther.*, vol. 9, no. 9, pp. 771–7, Oct. 2002.

[59] M. Uesato, Y. Gunji, T. Tomonaga, S. Miyazaki, T. Shiratori, H. Matsubara, T. Kouzu, H. Shimada, F. Nomura, and T. Ochiai, "Synergistic antitumor effect of antiangiogenic factor genes on colon 26 produced by low-voltage electroporation.," *Cancer Gene Ther.*, vol. 11, no. 9, pp. 625–32, Sep. 2004.

[60] J. M. Weiss, R. Shivakumar, S. Feller, L.-H. Li, A. Hanson, W. E. Fogler, J. C. Fratantoni, and L. N. Liu, "Rapid, in vivo, evaluation of antiangiogenic and antineoplastic gene products by nonviral transfection of tumor cells.," *Cancer Gene Ther.*, vol. 11, no. 5, pp. 346–53, May 2004.

[61] N. Tesic, U. Kamensek, G. Sersa, S. Kranjc, M. Stimac, U. Lampreht, V. Preat, G. Vandermeulen, M. Butinar, B. Turk, and M. Cemazar, "Endoglin (CD105) Silencing Mediated by shRNA Under the Control of Endothelin-1 Promoter for Targeted Gene Therapy of Melanoma," *Mol. Ther. Acids*, vol. 4, 2015.

[62] T. Dolinsek, G. Sersa, L. Prosen, M. Bosnjak, M. Stimac, U. Razborsek, and M. Cemazar, "Electrotransfer of plasmid DNA encoding an anti-mouse endoglin (CD105) shRNA to B16 melanoma tumors with low and high metastatic potential results in pronounced anti-tumor effects," *Cancers (Basel).*, vol. 8, no. 1, 2015.

[63] J. M. McMahon and D. J. Wells, "Electroporation for gene transfer to skeletal muscles: current status.," *BioDrugs*, vol. 18, no. 3, pp. 155–65, 2004.

[64] P. Lefesvre, J. Attema, and D. van Bekkum, "A comparison of efficacy and toxicity between electroporation and adenoviral gene transfer.," *BMC Mol. Biol.*, vol. 3, p. 12, Aug. 2002.

[65] N. Perez, P. Bigey, D. Scherman, O. Danos, M. Piechaczyk, and M. Pelegrin, "Regulatable systemic production of

monoclonal antibodies by in vivo muscle electroporation.," *Genet. Vaccines Ther.*, vol. 2, no. 1, p. 2, Mar. 2004.

[66] A. I. Daud, R. C. DeConti, S. Andrews, P. Urbas, A. I. Riker, V. K. Sondak, P. N. Munster, D. M. Sullivan, K. E. Ugen, J. L. Messina, and R. Heller, "Phase I Trial of Interleukin-12 Plasmid Electroporation in Patients With Metastatic Melanoma," *J. Clin. Oncol.*, vol. 26, no. 36, pp. 5896–5903, 2008.

[67] D. Pavlin, M. Cemazar, A. Cor, G. Sersa, A. Pogacnik, and N. Tozon, "Electrogene therapy with interleukin-12 in canine mast cell tumors," *Radiol Oncol*, vol. 45, no. 1, pp. 31–39, 2011.

[68] D. Pavlin, M. Cemazar, G. Sersa, and N. Tozon, "IL-12 based gene therapy in veterinary medicine.," *J. Transl. Med.*, vol. 10, p. 234, 2012.

[69] G. Sersa, J. Teissie, M. Cemazar, E. Signori, U. Kamensek, G. Marshall, and D. Miklavcic, "Electrochemotherapy of tumors as in situ vaccination boosted by immunogene electrotransfer.," *Cancer Immunol. Immunother.*, vol. 64, no. 10, pp. 1315–27, Oct. 2015.

[70] C. Y. Calvet and L. M. Mir, "The promising alliance of anti-cancer electrochemotherapy with immunotherapy.," *Cancer Metastasis Rev.*, vol. 35, no. 2, pp. 165–77, Jun. 2016.

[71] A. Sedlar, T. Dolinsek, B. Markelc, L. Prosen, S. Kranjc, M. Bosnjak, T. Blagus, M. Cemazar, and G. Sersa, "Potentiation of electrochemotherapy by intramuscular IL-12 gene electrotransfer in murine sarcoma and carcinoma with different immunogenicity," *Radiol. Oncol.*, vol. 46, no. 4, 2012.

[72] S. Kranjc, G. Tevz, U. Kamensek, S. Vidic, M. Cemazar, and G. Sersa, "Radiosensitizing effect of electrochemotherapy in a fractionated radiation regimen in radiosensitive murine sarcoma and radioresistant adenocarcinoma tumor model," *Radiat Res*, vol. 172, no. 6, pp. 677–685, 2009.

[73] A. Sedlar, S. Kranjc, T. Dolinsek, M. Cemazar, A. Coer, and G. Sersa, "Radiosensitizing effect of intratumoral interleukin-12 gene electrotransfer in murine sarcoma," *BMC Cancer*, vol. 13, 2013.

[74] J. Cutrera, M. Torrero, K. Shiomitsu, N. Mauldin, and S. Li, "Intratumoral bleomycin and IL-12 electrochemogenetherapy for treating head and neck tumors in dogs.," *Methods Mol. Biol.*, vol. 423, pp. 319–25, Jan. 2008.

[75] J. Cutrera, G. King, P. Jones, K. Kicenuik, E. Gumpel, X. Xia, and S. Li, "Safety and efficacy of tumor-targeted interleukin 12 gene therapy in treated and non-treated, metastatic lesions.," *Curr. Gene Ther.*, vol. 15, no. 1, pp. 44–54, Jan. 2015.

[76] S. D. Reed, A. Fulmer, J. Buckholz, B. Zhang, J. Cutrera, K. Shiomitsu, and S. Li, "Bleomycin/interleukin-12 electrochemogenetherapy for treating naturally occurring spontaneous neoplasms in dogs," *Cancer Gene Ther*, vol. 17, no. 8, pp. 571–578, 2010.

[77] M. Cemazar, J. Ambrozic Avgustin, D. Pavlin, G. Sersa, A. Poli, A. Krhac Levacic, N. Tesic, U. Lampreht Tratar, M. Rak, and N. Tozon, "Efficacy and safety of electrochemotherapy combined with peritumoral IL-12 gene electrotransfer of canine mast cell tumours," *Vet Comp Oncol.* vol. 15, no. 2, pp. 641-654, Jun 2017.

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Electrotransfer of DNA vaccine

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DNA VACCINES

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

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

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

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

ELECTROPORATION-MEDIATED DELIVERY OF DNA VACCINES

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

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

Consequently, electroporation-mediated delivery of DNA vaccines enhances up to100-fold the immune responses elicited compared to simple injection. It is a useful strategy to increase both humoral and cellular responses in small and large animals including

primates. A survey of the preclinical studies indicates that electroporation-mediated DNA vaccination induces long-lasting and robust cellular responses characterised by the induction of CTL, interferon γ and interleukin-2 by CD4+ and CD8+ T cells. Antibodies are usually detected. Combination with adjuvant (e.g. TLR-9 stimulation by CpG or interleukin-12) enhances the potency of DNA vaccination.

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

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

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

RECOMMENDED PAPERS

DNA vaccines

[1] Liu MA. DNA vaccines: an historical perspective and view to the future. Immunol Rev. 2011;239(1):62-84. Review

[2] Vandermeulen G, Marie C, Scherman D, Préat V. New Generation of Plasmid Backbones Devoid of Antibiotic Resistance Marker for Gene Therapy Trials. Mol Ther. 2011;19(11):1942-9. Review

Electroporation of DNA vaccines

[3] van Drunen Littel-van den Hurk S, Hannaman D. Electroporation for DNA immunization: clinical application. Expert Rev Vaccines. 2010;9(5):503-17. Review

[4] Sardesai NY, Weiner DB. Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol. 2011;23(3):421-9. Review.

[5] Frelin L, Brass A, Ahlén G, Brenndörfer ED, Chen M, Sällberg M. Electroporation: a promising method for the nonviral delivery of DNA vaccines in humans? Drug News Perspect. 2010 ;23(10):647-53. Review.

[6] Vandermeulen G, Staes E, Vanderhaeghen ML, Bureau MF, Scherman D, Préat V. Optimisation of intradermal DNA electrotransfer for immunisation. J Control Release. 2007;124(1-2):81-7.

Clinical trials with DNA vaccines and electroporation

[7] El-Kamary SS, Billington M, Deitz S, Colby E, Rhinehart H, Wu Y, Blackwelder W, Edelman R, Lee A, King A. Safety and tolerability of the Easy Vax™ clinical epidermal electroporation system in healthy adults. Mol Ther. 2012;20(1):214-20.

[8] Yang FQ, Yu YY, Wang GQ, Chen J, Li JH, Li YQ, Rao GR, Mo GY, Luo XR, Chen GM. A pilot randomized controlled trial of dual-plasmid HBV DNA vaccine mediated by in vivo electroporation in chronic hepatitis B patients under lamivudine chemotherapy. J Viral Hepat. 2012;19(8):581-93.

[9] Vasan S, Hurley A, Schlesinger SJ, Hannaman D, Gardiner DF, Dugin DP, Boente-Carrera M, Vittorino R, Caskey M, Andersen J, Huang Y, Cox JH, Tarragona-Fiol T, Gill DK, Cheeseman H, Clark L, Dally L, Smith C, Schmidt C, Park HH, Kopycinski JT, Gilmour J, Fast P, Bernard R, Ho DD. In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. PLoS One. 2011;6(5):e19252.

[10] Chudley L, McCann K, Mander A, Tjelle T, Campos-Perez J, Godeseth R, Creak A, Dobbyn J, Johnson B, Bass P, Heath C, Kerr P, Mathiesen I, Dearnaley D, Stevenson F, Ottensmeier C. DNA fusion-gene vaccination in patients with prostate cancer induces high-frequency CD8(+) T-cell responses and increases PSA doubling time. Cancer Immunol Immunother. 2012 May 22

Optimisation of delivery methods

[11] Lin F, Shen X, Kichaev G, Mendoza JM, Yang M, Armendi P, Yan J, Kobinger GP, Bello A, Khan AS, Broderick KE, Sardesai NY. Optimization of electroporation-enhanced intradermal delivery of DNA vaccine using a minimally invasive surface device. Hum Gene Ther Methods. 2012;23(3):157-68.

[12] Hallengard D, Bråve A, Isaguliants M, Blomberg P, Enger J, Stout R, King A, Wahren B.A combination of intradermal jet-injection and electroporation overcomes in vivo dose restriction of DNA vaccines. Genet Vaccines Ther. 2012;10(1):5.

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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.

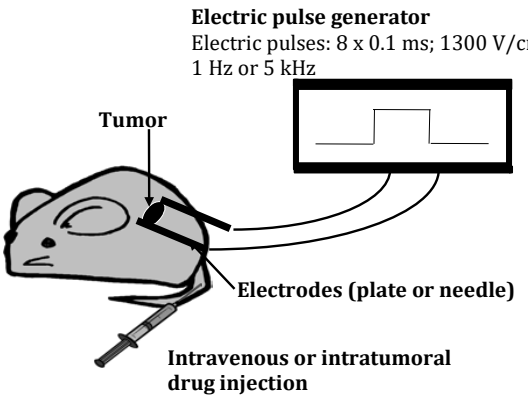

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumourally at doses which do not usually exert an antitumor effect. After an interval which allows sufficient drug accumulation in the tumors, electric pulses are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

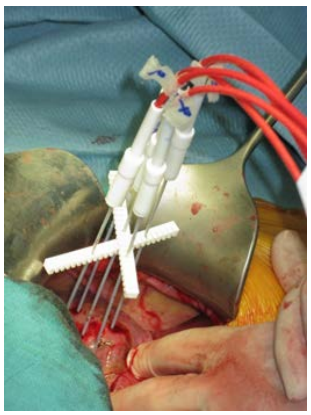

Figure 2: Electrochemotherapy of liver metastasis. Electrodes were inserted into the tumor and around the tumor in healthy liver tissue and connected to electric pulse generator. Electric pulses were delivered between the pairs of electrodes according to the treatment plan.

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.

REFERENCES

[1] Yarmush ML, Goldberg A, Sersa G, Kotnik T, Miklavcic D. Electroporation-based technologies for medicine: principles, applications, and challenges. Ann Rev Biomed Eng 2014; 16: 295-320.

[2] Miklavcic D, Mali B, Kos B, Heller R, Sersa G. Electrochemotherapy: from the drawing board into medical practice. *BioMedical OnLine* 2014; **13:** 29.

[3] Howell SB, Safaei R, Larson CA, Sailor MJ. Sopper transporters and the cellular pharmacology of the Platinum-containing cancer drugs. *Mol Pharmacol* 2010; **77**:887-94.

[4] Sersa G. Electrochemotherapy: animal work review. In. Jaroszeski MJ, Heller R, Gilbert R, editors. *Electrochemotherapy, electrogenetherapy, and transdermal drug delivery. Electrically mediated delivery of molecules to cells.* Totowa, New Jersey: Humana Press, 2000. p. 119-36.

[5] Mir LM. Therapeutic perspectives of in vivo cell electropermeabilization. *Bioelectrochem* 2001; 53: 1-10.

[6] Gehl J. Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol Scan* 2003; **177**: 437-47.

[7] Mir LM. Bases and rationale of the electrochemotherapy. *EJC Suppl* 2006; **4**: 38-44.

[8] Agerholm-larsen B, Iversen HK, Ibsen P, Moller JM, Mahmood F, Jansen KS, Gehl J. Preclinical validation of electrochemotherapy as an effective treatment for brain tumors. *Cancer Res* 2011; **71**:3753-62.

[9] Vásquez JL, Ibsen P, Lindberg H, Gehl J. In vitro and in vivo experiments on electrochemotherapy for bladder cancer. *J Urol.* 2015; **193**: 1009-15.

[10] Miklavcic D, Beravs K, Semrov D, Cemazar M, Demsar F, Sersa G. The importance of electric field distribution for effective in vivo electroporation of tissues. *Biophys J* 1998; **74**: 2152-8.

[11] Miklavcic D, Corovic S, Pucihar G, Pavselj N. Importance of tumor coverage by sufficiently high local electric field for effective electrochemotherapy. *EJC Suppl* 2006; **4**: 45-51.

[12] Corovic S, Al Hakere B, Haddad V, Miklavcic D, Mir LM. Importance of the contact surface between electrodes and treated tissue in electrochemotherapy. *Tech Cancer Res Treat* 2008; **7**: 292—99.

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

[14] Miklavcic D, Pucihar G, Pavlovec M, Ribaric S, Mali M, Macek-Lebar A, Petkovsek M, Nastran J, Kranjc S, Cemazar M, Sersa G. The effect of high frequency electric pulses on muscle contractions and antitumor efficiency in vivo for a potential use in clinical electrochemotherapy. *Bioelectrochemistry* 2005; **65**: 121-8.

[15] Sersa G, Kranjc S, Cemazar M, Scancar J, Krzan M, Neumann E. Comparison of antitumor effectiveness of electrochemotherapy using different electric pulse repetition frequencies. *J membrane biology* 2010; **236**: 155-162.

[16] Sersa G, Cemazar M, Parkins CS, Chaplin DJ. Tumour blood flow changes induced by application of electric pulses. *Eur J Cancer* 1999; **35:** 672-7.

[17] Bellard E, Markelc B, Pelofy S, Le Guerroué F, Sersa G, Teissié J, Cemazar M, Golzio M. Intravital microscopy at the single vessel level brings new insights of vascular modification mechanisms induced by electropermeabilization. *J Control Release* 2012; **163**: 396-403.

[18] Gehl J, Skovsgaard T and Mir LM. Vascular reactions to in vivo electroporation: characterization and consequences for drug and gene delivery. *Biochim Biophys Acta* 2002; **1569**: 51-8.

[19] Cemazar M, Parkins CS, Holder AL, Chaplin DJ, Tozer GM and Sersa G. Electroporation of human microvascular endothelial cells: evidence for anti-vascular mechanism of electrochemotherapy. *Br J Cancer* 2001; **84**: 556-70

[20] Jarm T, Cemazar M, Miklavcic D, Sersa G. Antivascular effects of electrochemotherapy: implications in treatment of bleeding metastases. *Exp Rev Anticancer Ther* 2010; **10**: 729-746.

[21] Sersa G, Jarm T, Kotnik T, Coer A, Podkrajsek M, Sentjurc M, Miklavcic D, Kadivec M, Kranjc S, Secerov A, Cemazar M. Vascular disrupting action of electroporation and electrochemotherapy with bleomycin in murine sarcoma. *Brit J Cancer*, 2008, **98**: 388-98

[22] Markelc B, Bellard E, Sersa G, Pelofy S, Teissie J, Coer A, Golzio M, Cemazar. In vivo molecular imaging and histological analysis of changes induced by electric pulses used for plasmid DNA electrotransfer to the skin: a study in a dorsal window chamber in mice. *J Membrane Biol* 2012; **245**: 545-554.

[23] Gehl J, Geertsen PF. Palliation of haemorrhaging and ulcerated cutaneous tumours using electrochemotherapy. EJC Suppl 2006; **4**: 35-37.

[24] Sersa G, Miklavcic D, Cemazar M, Belehradek JJr, Jarm T, Mir LM. Electrochemotherapy with CDDP on LPB sarcoma: comparison of the anti-tumor effectiveness in immunocompetent and immunodeficient mice. *Bioelectroch Bioener* 1997; **43**: 279-283.

[25] Sersa G, Cemazar M, Menart V, Gaberc-Porekar V, Miklavčič D. Antitumor effectiveness of electrochemotherapy is increased by TNF-α on SA-1 tumors in mice. *Cancer Letters* 1997; **116**: 85-92.

[26] Mir LM, Roth C, Orlowski S, Quintin-Colona F, Fradelizi D, Belahradek J, Kourilsky P. Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin 2. *J Immunother* 1995; **17:** 30-8.

[27] Heller L, Pottinger C, Jaroszeski MJ, Gilbert R, Heller R. In vivo electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanoma combined with electrochemotherapy inducing long-term antitumour immunity. *Melanoma Res* 2000; **10**: 577-83.

[28] Cemazar M, Todorovic V, Scancar, J Lampreht U, Stimac M, Kamensek U, Kranjc S, Coer A, Sersa G. Adjuvant INF-a therapy to electrochemotherapy with intravenous cisplatin in murine sarcoma exerts synergistic antitumor effectiveness *Radiol Oncol* 2015; **49**:32-40

[29] Mir LM, Belehradek M, Domenge C, Orlowski S, Poddevin B, Belehradek J Jr, Schwaab G, Luboinski B, Paoletti C. Electrochemotherapy, a new antitumor treatment: first clinical trial. *C R Acad Sci III* 1991; **313:** 613-8.

[30] Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M, et al. Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. *EJC Suppl* 2006; **4**: 3-13.

[31] Mir LM, Gehl J, Sersa G, Collins CG, Garbay JR, Billard V, et al. Standard operating procedures of the electrochemotherapy:

Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by Cliniporator ™ by means of invasive or non-invasive electrodes. *EJC Supp*l 2006; **4**: 14-25.

[32] Campana LG, Mocellin S, et al. Bleomycin-based electrochemotherapy: clinical outcome from a single institution's experience with 52 patients. *Ann Surg Oncol* 2008; **16**: 191-9.

[33] Quaglino P, Mortera C, et al. Electrochemotherapy with intravenous bleomycin in the local treatment of skin melanoma metastases. *Ann Surg Oncol* 2008; **15**: 2215-22.

[34] Testori A, Rutkowski P, Marsden J, Bastholt L, Chiarion-Sileni V, Hauschild A, Eggermont AM, Surgery and radiotherapy in the treatment of cutaneous melanoma, *Ann Oncol* 2009; **20 Suppl 6**: 22-9.

[35] Mali B, Jarm T, Snoj M, Sersa G, Miklavcic D. Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. *Eur J Surg Oncol* 2013; **39:** 4-16.

[36] Mali B, Miklavcic D, Campana L, Cemazar M, Sersa G, Snoj M, Jarm T. Tumor size and effectiveness of electro-chemotherapy. *Radiol Oncol* 2013; **47:** 32-41.

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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.

REFERENCES

[1] Kinosita K, Tsong TY. Formation and resealing of pores of controlled sizes in human erythrocyte membrane. Nature 1977;268:438-41.

[2] Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1982;1(7):841-45.

[3] Skovsgaard T, Nissen NI. Membrane transport of anthracyclines. Pharmacol Ther 1982;18(3):293-311.

[4] Okino M, Mohri H. Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. JpnJCancer Res 1987;78(0910-5050 SB - M SB - X):1319-21.

[5] Orlowski S, Belehradek Jr J, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. Biochemical Pharmacology 1988;37(24):4727-33.

[6] Mir LM, Orlowski S, Belehradek J, Jr., Paoletti C. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. Eur J Cancer 1991;27(1):68-72.

[7] Belehradek M, Domenge C, Luboinski B, Orlowski S, Belehradek Jr J, Mir LM. Electrochemotherapy, a new antitumor treatment. First clinical phase I-II trial. Cancer 1993;72(12):3694-700.

[8] Heller R. Treatment of cutaneous nodules using electrochemotherapy. [Review] [32 refs]. Journal of the Florida Medical Association 1995;82(2):147-50.

[9] Sersa G, Stabuc B, Cemazar M, Jancar B, Miklavcic D, Rudolf Z. Electrochemotherapy with cisplatin: Potentiation of local cisplatin antitumor effectiveness by application of electric pulses in cancer patients. European Journal of Cancer 1998;34(8):1213-18.

[10] Gehl J, Geertsen PF. Efficient palliation of haemorrhaging malignant melanoma skin metastases by electrochemotherapy. Melanoma Res 2000;10(6):585-9.

[11] Andre FM, Gehl J, Sersa G, Preat V, Hojman P, Eriksen J, et al. Efficiency of High- and Low-Voltage Pulse Combinations for Gene Electrotransfer in Muscle, Liver, Tumor, and Skin. Human Gene Therapy 2008;19(11):1261-71.

[12] Hojman P, Gissel H, Andre F, Cournil-Henrionnet C, Eriksen J, Gehl J, et al. Physiological effect of high and low voltage pulse combinations for gene electrotransfer in muscle. HumGene Ther 2008(1557-7422 (Electronic)).

[13] Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M, et al. Electrochemotherapy - An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of

Electrochemotherapy) study. Ejc Supplements 2006;4(11):3-13.

[14] Mir LM, Gehl J, Sersa G, Collins CG, Garbay JR, Billard V, et al. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. European Journal of Cancer Supplements 2006;4(11):14-25.

[15] National Institute for H, Care E. Electrochemotherapy for metastases in the skin from tumours of non-skin origin and melanoma. http://publicationsniceorguk/electrochemotherapy-for-metastases-in-the-skin-from-tumours-of-non-skin-origin-and-melanoma-ipg446 2013.

[16] (NICE) NIfHaCE. Electrochemotherapy for primary basal cell carcinoma and primary squamous cell carcinoma. www.nice.org.uk2014.

[17] Stratigos A, Garbe C, Lebbe C, Malvehy J, Del Marmol V, Pehamberger H, et al. Diagnosis and treatment of invasive squamous cell carcinoma of the skin: European consensus-based interdisciplinary guideline. Eur J Cancer 2015;51(14):1989-2007.

[18] Matthiessen LW, Chalmers RL, Sainsbury DC, Veeramani S, Kessell G, Humphreys AC, et al. Management of cutaneous metastases using electrochemotherapy. Acta Oncol 2011;50:621-29.

[19] Matthiessen LW, Johannesen HH, Hendel HW, Moss T, Kamby C, Gehl J. Electrochemotherapy for large cutaneous recurrence of breast cancer: A phase II clinical trial. Acta Oncologica 2012;51(6):713-21.

[20] Campana LG, Valpione S, Falci C, Mocellin S, Basso M, Corti L, et al. The activity and safety of electrochemotherapy in persistent chest wall recurrence from breast cancer after mastectomy: a phase-II study. Breast Cancer ResTreat 2012;134:1169-78.

[21] Campana LG, Bianchi G, Mocellin S, Valpione S, Campanacci L, Brunello A, et al. Electrochemotherapy treatment of locally advanced and metastatic soft tissue sarcomas: results of a non-comparative phase II study. World JSurg 2014;38:813-22.

[22] Campana LG, Mali B, Sersa G, Valpione S, Giorgi CA, Strojan P, et al. Electrochemotherapy in non-melanoma head and neck cancers: a retrospective analysis of the treated cases. BrJOral MaxillofacSurg 2014.

[23] Curatolo P, Mancini M, Clerico R, Ruggiero A, Frascione P, Di Marco P, et al. Remission of extensive merkel cell carcinoma after electrochemotherapy. Arch Dermatol 2009;145(4):494-5.

[24] Curatolo P, Quaglino P, Marenco F, Mancini M, Nardo T, Mortera C, et al. Electrochemotherapy in the treatment of Kaposi sarcoma cutaneous lesions: a two-center prospective phase II trial. Ann Surg Oncol 2012;19(1):192-8.

[25] Quaglino P, Mortera C, Osella-Abate S, Barberis M, Illengo M, Rissone M, et al. Electrochemotherapy with intravenous bleomycin in the local treatment of skin melanoma metastases. AnnSurgOncol 2008;15:2215-22.

[26] Quaglino P, Matthiessen LW, Curatolo P, Muir T, Bertino G, Kunte C, et al. Predicting patients at risk for pain associated with electrochemotherapy. Acta Oncol 2015;54(3):298-306.

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NOTES

NOTES

Development of devices and electrodes

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Abstract: Since first reports on electroporation, numerous electroporation based biotechnological and biomedical applications have emerged. The necessary pulse generators are characterized by the shape of the pulses and their characteristics: pulse amplitude and duration. In addition, the electrodes are the important "connection" between the cells/tissue and pulse generator. The geometry of the electrodes together with the cell/tissue sample properties determine the necessary output power and energy that the electroporators need to provide. The choice of electroporator – the pulse generator depends on biotechnological and biomedical application but is inherently linked also to the electrodes choice.

INTRODUCTION

Since first reports on electroporation (both irreversible and reversible), a number of applications has been developed and list of applications which are based on electroporation is 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.

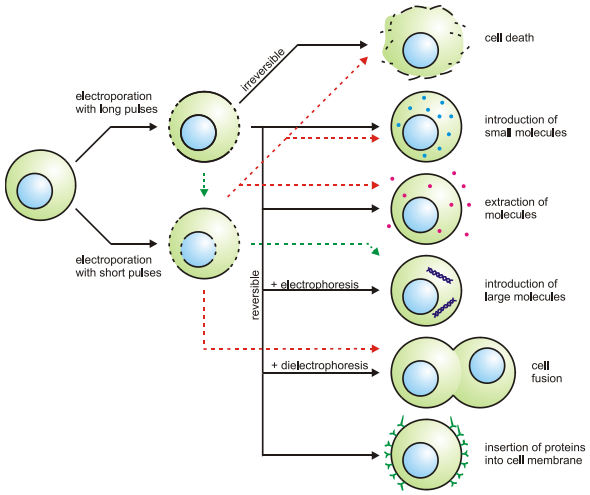

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications 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 [20–23].

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, 24-26]. Smilarly as in electrochemotherapy pulses of 50 or 100 µs with amplitudes up to 3000 V are used [27]. 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 [28].

GENE ELECTROTRANSFER

Exogenous genetic material can be delivered to cells by using non-viral methods such as electropermeabilization [29]. 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 [30]. 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 [31]. 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) [32]. 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 [33].

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 [34]. 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 [35, 36]. Electrofusion of cells of different sizes can be achieved by nanosecond pulsed electric fields [37].

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) [38–42]. An economic assessment of microalgae-based bioenergy production was recently made [43]. Recommendations guidelines on the key information to be reported in biotechnological studies because of variability in results obtained in different laboratories [44].

ELECTRO- PASTEURIZATION AND STERILIZATION

Irreversible electroporation can be used in applications where destruction of microorganisms is required, i.e. food processing and water treatment [45]. 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], [46].

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 [47]. 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 [48] with models of electric field distribution [49–53]. 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 [54, 55].

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 [47]. 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 [56–60]. 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 [61–63]. 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 [64], which further leads to increased sample conductivity [65].

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 [66]. The cell membrane damage and uptake of ions can be significantly reduced when using bipolar ns pulses instead of monopolar [67]. Shapes other than "rectangular" have been investigated with respect to electroporation efficiency [68]. 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 [32].

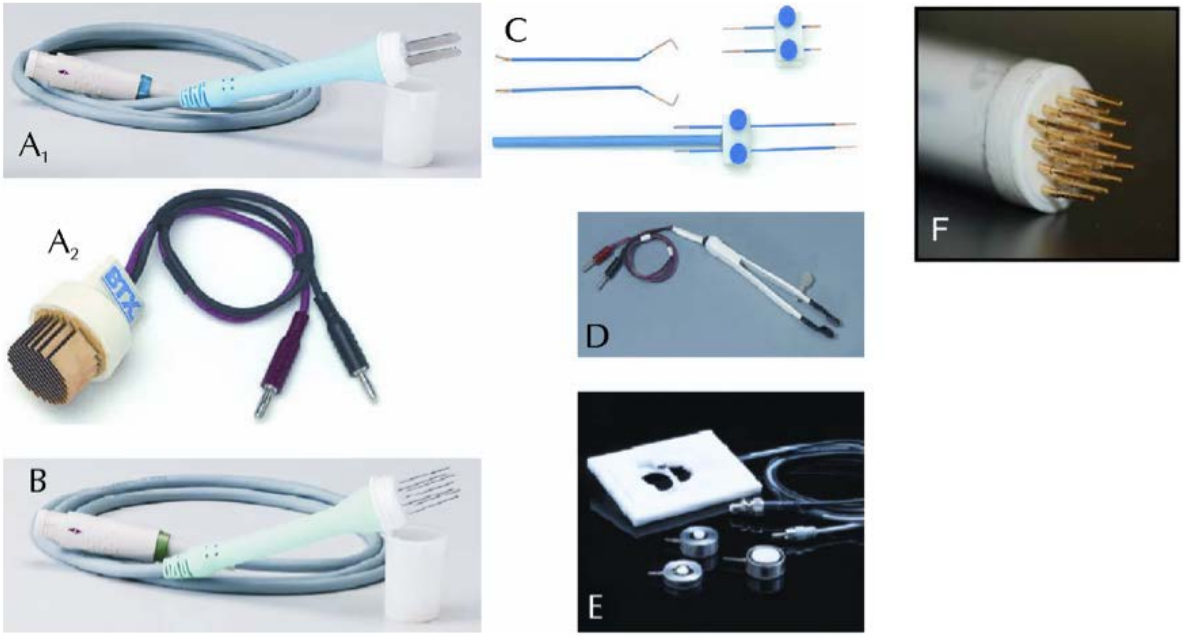

Figure 2: Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: A_1 and A_2 –parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes, E – coaxial electrodes and F – multiple electrodes array. Electrodes A_1 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 [31].

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 [69].

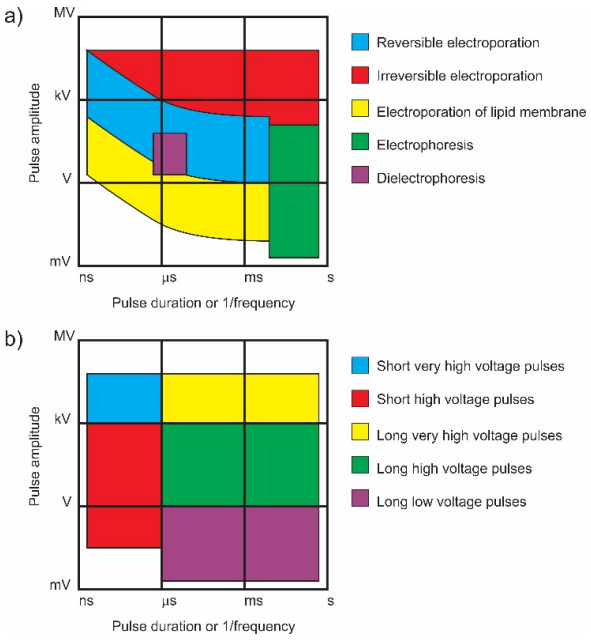

Figure 3: Areas of amplitude and duration of electrical pulses which are used in the research of electroporation and related effects (a). Five different areas of electroporation pulse generation (b). To amplify or to generate very-high-voltage electroporation pulses (over a few kV) spark gaps and similar 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 [70], 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 [56, 58, 71]. Short bipolar pulses were also investigated as they mitigate nerve/muscle stimulation [72–74], but these pulses may already fall into vicinity of "cancellation effect" [74].

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. 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.

REFERENCES

[1] M. Reberšek, D. Miklavčič, C. Bertacchini, and M. Sack, "Cell membrane electroporation-Part 3: the equipment," Electr. Insul. Mag. IEEE, vol. 30, no. 3, pp. 8–18, 2014.

[2] K. Flisar, M. Puc, T. Kotnik, and D. Miklavcic, "Cell membrane electropermeabilization with arbitrary pulse waveforms," IEEE Eng. Med. Biol. Mag. Q. Mag. Eng. Med. Biol. Soc., vol. 22, no. 1, pp. 77–81, Feb. 2003.

[3] M. Reberšek and D. Miklavčič, "Concepts of Electroporation Pulse Generation and Overview of Electric Pulse Generators for Cell and Tissue Electroporation," in Advanced Electroporation Techniques in Biology and Medicine, A. G. Pakhomov, D. Miklavčič, and M. S. Markov, Eds. Boca Raton: CRC Press, 2010, pp. 323–339.

[4] L. G. Staal and R. Gilbert, "Generators and Applicators: Equipment for Electroporation," in Clinical Aspects of Electroporation, S. T. Kee, J. Gehl, and E. W. Lee, Eds. New York: Springer, 2011, pp. 45–65.

[5] G. A. Hofmann, "Instrumentation and electrodes for in vivo electroporation," in Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery, M. J. Jaroszeski, R. Heller, and R. Gilbert, Eds. Totowa: Humana Press, 2000, pp. 37–61.

[6] E. Pirc, M. Reberšek, and D. Miklavčič, "Dosimetry in Electroporation-Based Technologies and Treatments," in Dosimetry in Bioelectromagnetics, M. Markov, Ed. 6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487–2742: CRC Press, 2017, pp. 233–268.

[7] M. Reberšek and D. Miklavčič, "Advantages and Disadvantages of Different Concepts of Electroporation Pulse Generation," Automatika, vol. 52, no. 1, pp. 12–19, Mar. 2011.

[8] M. Reberšek et al., "Electroporator with automatic change of electric field direction improves gene electrotransfer in-vitro," Biomed. Eng. Online, vol. 6, p. 25, 2007.

[9] P. Kramar, D. Miklavcic, and A. M. Lebar, "A system for the determination of planar lipid bilayer breakdown voltage and its applications," NanoBioscience IEEE Trans. On, vol. 8, no. 2, pp. 132–138, 2009.

[10] J. M. Sanders, A. Kuthi, Yu-Hsuan Wu, P. T. Vernier, and M. A. Gundersen, "A linear, single-stage, nanosecond pulse generator for delivering intense electric fields to biological loads," IEEE Trans. Dielectr. Electr. Insul., vol. 16, no. 4, pp. 1048–1054, Aug. 2009.

[11] S. Haberl, J. Teissié, W. Frey, and D. Miklavčič, "Cell Membrane Electroporation – Part 2: The Applications," IEEE Electr. Insul. Mag., vol. 29, no. 1, pp. 19–27, Feb. 2013.

[12] C. Jiang, R. V. Davalos, and J. C. Bischof, "A Review of Basic to Clinical Studies of Irreversible Electroporation Therapy," IEEE Trans. Biomed. Eng., vol. 62, no. 1, pp. 4–20, Jan. 2015.

[13] S. Mahnič-Kalamiza, E. Vorobiev, and D. Miklavčič, "Electroporation in Food Processing and Biorefinery," J. Membr. Biol., vol. 247, no. 12, pp. 1279–1304, Dec. 2014.

[14] M. L. Yarmush, A. Golberg, G. Serša, T. Kotnik, and D. Miklavčič, "Electroporation-Based Technologies for

Medicine: Principles, Applications, and Challenges," Annu. Rev. Biomed. Eng., vol. 16, no. 1, pp. 295–320, Jul. 2014.
[15] T. Kotnik, W. Frey, M. Sack, S. Haberl Meglič, M. Peterka, and D. Miklavčič, "Electroporation-based applications in biotechnology," Trends Biotechnol., vol. 33, no. 8, pp. 480–488, Aug. 2015.
[16] A. Golberg et al., "Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development," Biotechnol. Biofuels, vol. 9, no. 1, Dec. 2016.
[17] M. Morales-de la Peña, P. Elez-Martínez, and O. Martín-Belloso, "Food Preservation by Pulsed Electric Fields: An Engineering Perspective," Food Eng. Rev., vol. 3, no. 2, pp. 94–107, Mar. 2011.
[18] D. Miklavčič, B. Mali, B. Kos, R. Heller, and G. Serša, "Electrochemotherapy: from the drawing board into medical practice," Biomed. Eng. Online, vol. 13, no. 1, p. 29, 2014.
[19] B. Mali, T. Jarm, M. Snoj, G. Sersa, and D. Miklavcic, "Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis," Eur. J. Surg. Oncol. EJSO, vol. 39, no. 1, pp. 4–16, Jan. 2013.
[20] Edhemovic et al., "Intraoperative electrochemotherapy of colorectal liver metastases: Electrochemotherapy of Liver Metastases," J. Surg. Oncol., vol. 110, no. 3, pp. 320–327, Sep. 2014.
[21] D. Miklavčič et al., "Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors," Med. Biol. Eng. Comput., vol. 50, no. 12, pp. 1213–1225, Dec. 2012.
[22] A. Gasbarrini, W. K. Campos, L. Campanacci, and S. Boriani, "Electrochemotherapy to Metastatic Spinal Melanoma: A Novel Treatment of Spinal Metastasis?," Spine, vol. 40, no. 24, pp. E1340-1346, Dec. 2015.
[23] M. Djokić, M. Čemažar, P. Popovič, B. Kos, R. Dežman, M. Bošnjak, M. Nikšić Žakelj, D. Miklavčič, S. Potrč, B. Štabuc, A. Tomažič, G. Serša, B. Trotovšek, "Electrochemotherapy as treatment option for hepatocellular carcinoma, a prospective pilot study." Eur. J. Surg. Oncol. 44: 651-657, 2018.
[24] J. Lavee, G. Onik, P. Mikus, and B. Rubinsky, "A novel nonthermal energy source for surgical epicardial atrial ablation: irreversible electroporation," Heart Surg. Forum, vol. 10, no. 2, pp. E162-167, 2007.
[25] P. G. Wagstaff et al., "Irreversible electroporation: state of the art," OncoTargets Ther., vol. 9, pp. 2437–2446, Apr. 2016.
[26] V.Y. Reddy, J. Koruth, P. Jais, et al. "Ablation and atrial fibrillation with pulsed electric fields: an ultra-rapid, tissue-selective modality for cardiac ablation." J Am Coll Cardiol EP 2018: 4: 987–95.
[27] C. Bertacchini, P. M. Margotti, E. Bergamini, A. Lodi, M. Ronchetti, and R. Cadossi, "Design of an irreversible electroporation system for clinical use," Technol. Cancer Res. Treat., vol. 6, no. 4, pp. 313–320, Aug. 2007.
[28] R. E. Neal, P. A. Garcia, J. L. Robertson, and R. V. Davalos, "Experimental Characterization and Numerical Modeling of Tissue Electrical Conductivity during Pulsed Electric Fields for Irreversible Electroporation Treatment Planning," IEEE Trans. Biomed. Eng., vol. 59, no. 4, pp. 1076–1085, Apr. 2012.
[29] L. Lambricht, A. Lopes, S. Kos, G. Sersa, V. Préat, and G. Vandermeulen, "Clinical potential of electroporation for gene therapy and DNA vaccine delivery," Expert Opin. Drug Deliv., vol. 13, no. 2, pp. 295–310, 2016.
[30] A. Gothelf and J. Gehl, "What you always needed to know about electroporation based DNA vaccines," Hum. Vaccines Immunother., vol. 8, no. 11, pp. 1694–1702, Nov. 2012.
[31] R. Heller, Y. Cruz, L. C. Heller, R. A. Gilbert, and M. J. Jaroszeski, "Electrically mediated delivery of plasmid DNA to the skin, using a multielectrode array," Hum. Gene Ther., vol. 21, no. 3, pp. 357–362, Mar. 2010.
[32] S. Satkauskas et al., "Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis," Mol. Ther. J. Am. Soc. Gene Ther., vol. 5, no. 2, pp. 133–140, Feb. 2002.
[33] M. Kandušer, D. Miklavčič, and M. Pavlin, "Mechanisms involved in gene electrotransfer using high- and low-voltage pulses — An in vitro study," Bioelectrochemistry, vol. 74, no. 2, pp. 265–271, Feb. 2009.
[34] M. Usaj, K. Flisar, D. Miklavcic, and M. Kanduser, "Electrofusion of B16-F1 and CHO cells: The comparison of the pulse first and contact first protocols," Bioelectrochemistry, vol. 89, pp. 34–41, Feb. 2013.
[35] H. Mekid and L. M. Mir, "In vivo cell electrofusion," Biochim. Biophys. Acta BBA - Gen. Subj., vol. 1524, no. 2–3, pp. 118–130, Dec. 2000.
[36] R. Heller and R. J. Grasso, "Reproducible layering of tissue culture cells onto electrostatically charged membranes," J. Tissue Cult. Methods, vol. 13, no. 1, pp. 25–29.
[37] L. Rems, M. Ušaj, M. Kandušer, M. Reberšek, D. Miklavčič, and G. Pucihar, "Cell electrofusion using nanosecond electric pulses," Sci. Rep., vol. 3, Nov. 2013.
[38] M. Zakhartsev, C. Momeu, and V. Ganeva, "High-Throughput Liberation of Water-Soluble Yeast Content by Irreversible Electropermeation (HT-irEP)," J. Biomol. Screen., vol. 12, no. 2, pp. 267–275, Jan. 2007.
[39] M. Sack et al., "Electroporation-Assisted Dewatering as an Alternative Method for Drying Plants," IEEE Trans. Plasma Sci., vol. 36, no. 5, pp. 2577–2585, Oct. 2008.
[40] M. Sack et al., "Research on Industrial-Scale Electroporation Devices Fostering the Extraction of Substances from Biological Tissue," Food Eng. Rev., vol. 2, pp. 147–156, Mar. 2010.
[41] E. Puértolas, G. Saldaña, S. Condón, I. Álvarez, and J. Raso, "Evolution of polyphenolic compounds in red wine from Cabernet Sauvignon grapes processed by pulsed electric fields during aging in bottle," Food Chem., vol. 119, no. 3, pp. 1063–1070, Apr. 2010.
[42] S. Haberl, M. Jarc, A. Štrancar, M. Peterka, D. Hodžić, and D. Miklavčič, "Comparison of Alkaline Lysis with Electroextraction and Optimization of Electric Pulses to Extract Plasmid DNA from Escherichia coli," J. Membr. Biol., Jul. 2013.
[43] A. L. Gonçalves, M. C. M. Alvim-Ferraz, F. G. Martins, M. Simões, and J. C. M. Pires, "Integration of Microalgae-Based Bioenergy Production into a Petrochemical Complex: Techno-Economic Assessment," Energies, vol. 9, no. 4, p. 224, Mar. 2016.
[44] J. Raso et al., "Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes," Innov. Food Sci. Emerg. Technol., Aug. 2016.
[45] J. R. Beveridge, S. J. MacGregor, L. Marsili, J. G. Anderson, N. J. Rowan, and O. Farish, "Comparison of the effectiveness of biphase and monophase rectangular pulses for the inactivation of micro-organisms using pulsed electric fields," IEEE Trans. Plasma Sci., vol. 30, no. 4, pp. 1525–1531, Aug. 2002.

[46] S. Toepfl, "Pulsed electric field food processing industrial equipment design and commercial applications," Stewart Postharvest Rev., vol. 8, no. 2, pp. 1–7, 2012.
[47] T. Kotnik, P. Kramar, G. Pucihar, D. Miklavcic, and M. Tarek, "Cell membrane electroporation- Part 1: The phenomenon," IEEE Electr. Insul. Mag., vol. 28, no. 5, pp. 14–23, Oct. 2012.
[48] M. Kranjc et al., "In Situ Monitoring of Electric Field Distribution in Mouse Tumor during Electroporation," Radiology, vol. 274, no. 1, pp. 115–123, Jan. 2015.
[49] D. Miklavcic, K. Beravs, D. Semrov, M. Cemazar, F. Demsar, and G. Sersa, "The importance of electric field distribution for effective in vivo electroporation of tissues.," Biophys. J., vol. 74, no. 5, pp. 2152–2158, May 1998.
[50] N. Pavselj, Z. Bregar, D. Cukjati, D. Batiuskaite, L. M. Mir, and D. Miklavcic, "The Course of Tissue Permeabilization Studied on a Mathematical Model of a Subcutaneous Tumor in Small Animals," IEEE Trans. Biomed. Eng., vol. 52, no. 8, pp. 1373–1381, Aug. 2005.
[51] D. Sel, D. Cukjati, D. Batiuskaite, T. Slivnik, L. M. Mir, and D. Miklavcic, "Sequential Finite Element Model of Tissue Electropermeabilization," IEEE Trans. Biomed. Eng., vol. 52, no. 5, pp. 816–827, May 2005.
[52] D. Miklavcic, S. Corovic, G. Pucihar, and N. Pavselj, "Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy," Eur. J. Cancer Suppl., vol. 4, no. 11, pp. 45–51, Nov. 2006.
[53] D. Miklavcic et al., "Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy," Biomed Eng Online, vol. 9, no. 10, pp. 1–12, 2010.
[54] S. Corovic, I. Lackovic, P. Sustaric, T. Sustar, T. Rodic, and D. Miklavcic, "Modeling of electric field distribution in tissues during electroporation," Biomed. Eng. Online, vol. 12, no. 1, p. 16, 2013.
[55] J. Langus, M. Kranjc, B. Kos, T. Šuštar, and D. Miklavčič, "Dynamic finite-element model for efficient modelling of electric currents in electroporated tissue," Sci. Rep., vol. 6, p. 26409, May 2016.
[56] R. A. Gilbert, M. J. Jaroszeski, and R. Heller, "Novel electrode designs for electrochemotherapy," Biochim. Biophys. Acta, vol. 1334, no. 1, pp. 9–14, Feb. 1997.
[57] S. Mazères et al., "Non invasive contact electrodes for in vivo localized cutaneous electropulsation and associated drug and nucleic acid delivery," J. Control. Release Off. J. Control. Release Soc., vol. 134, no. 2, pp. 125–131, Mar. 2009.
[58] M. Reberšek, S. Čorović, G. Serša, and D. Miklavčič, "Electrode commutation sequence for honeycomb arrangement of electrodes in electrochemotherapy and corresponding electric field distribution," Bioelectrochemistry, vol. 74, no. 1, pp. 26–31, Nov. 2008.
[59] J. Čemažar, D. Miklavčič, and T. Kotnik, "Microfluidic devices for manipulation, modification and characterization of biological cells in electric fields - a review," Inf. MIDEM, vol. 43, no. 3, pp. 143–161, Sep. 2013.
[60] P. F. Forde et al., "Preclinical evaluation of an endoscopic electroporation system," Endoscopy, vol. 48, no. 5, pp. 477–483, May 2016.
[61] M. Pavlin et al., "Effect of Cell Electroporation on the Conductivity of a Cell Suspension," Biophys. J., vol. 88, no. 6, pp. 4378–4390, Jun. 2005.
[62] D. Cukjati, D. Batiuskaite, F. André, D. Miklavčič, and L. M. Mir, "Real time electroporation control for accurate and safe in vivo non-viral gene therapy," Bioelectrochemistry, vol. 70, no. 2, pp. 501–507, May 2007.
[63] M. Kranjc, F. Bajd, I. Serša, and D. Miklavčič, "Magnetic resonance electrical impedance tomography for measuring electrical conductivity during electroporation," Physiol. Meas., vol. 35, no. 6, pp. 985–996, Jun. 2014.
[64] M. Phillips, L. Rubinsky, A. Meir, N. Raju, and B. Rubinsky, "Combining Electrolysis and Electroporation for Tissue Ablation," Technol. Cancer Res. Treat., vol. 14, no. 4, pp. 395–410, Aug. 2015.
[65] I. Lackovic, R. Magjarevic, and D. Miklavcic, "Three-dimensional finite-element analysis of joule heating in electrochemotherapy and in vivo gene electrotransfer," Dielectr. Electr. Insul. IEEE Trans. On, vol. 16, no. 5, pp. 1338–1347, 2009.
[66] K. Mitsutake, A. Satoh, S. Mine, K. Abe, S. Katsuki, and H. Akiyama, "Effect of pulsing sequence of nanosecond pulsed electric fields on viability of HeLa S3 cells," Dielectr. Electr. Insul. IEEE Trans. On, vol. 19, no. 1, pp. 337–342, 2012.
[67] B. L. Ibey et al., "Bipolar nanosecond electric pulses are less efficient at electropermeabilization and killing cells than monopolar pulses," Biochem. Biophys. Res. Commun., vol. 443, no. 2, pp. 568–573, Jan. 2014.
[68] T. Kotnik, D. Miklavčič, and L. M. Mir, "Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses: Part II. Reduced electrolytic contamination," Bioelectrochemistry, vol. 54, no. 1, pp. 91–95, Aug. 2001.
[69] B. Mali et al., "Electrochemotherapy of colorectal liver metastases-an observational study of its effects on the electrocardiogram," Biomed. Eng. Online, vol. 14, no. Suppl 3, p. S5, 2015.
[70] M. Puc, S. Čorović, K. Flisar, M. Petkovšek, J. Nastran, and D. Miklavčič, "Techniques of signal generation required for electropermeabilization: Survey of electropermeabilization devices," Bioelectrochemistry, vol. 64, no. 2, pp. 113–124, Sep. 2004.
[71] M. Reberšek, M. Kandušer, and D. Miklavčič, "Pipette tip with integrated electrodes for gene electrotransfer of cells in suspension: a feasibility study in CHO cells," Radiol. Oncol., vol. 45, no. 3, pp. 204–208, 2011.
[72] C. B. Arena et al., "High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction," Biomed. Eng. OnLine, vol. 10, p. 102, Nov. 2011.
[73] M. B. Sano et al., "Bursts of Bipolar Microsecond Pulses Inhibit Tumor Growth," Sci. Rep., vol. 5, p. 14999, Oct. 2015.
[74] D. C. Sweeney, M. Reberšek, J. Dermol, L. Rems, D. Miklavčič, and R. V. Davalos, "Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses," Biochim. Biophys. Acta BBA - Biomembr., vol. 1858, no. 11, pp. 2689–2698, Nov. 2016

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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.

Recent references (former references can be found in these papers):

[1] A. Azan, F. Gailliègue, L.M. Mir and M. Breton. Cell Membrane Electropulsation: Chemical Analysis of Cell Membrane Modifications and Associated Transport Mechanisms. In: Advs Anatomy, Vol. 227, Transport Across Natural and Modified Biological Membranes and its Implications in Physiology and Therapy, eds. J. Kulbaka and S. Satkauskas. ISBN : 978-3-319-56894-2

[2] H. Hanna, A. Denzi, M. Liberti, F.M. Andre and L.M. Mir. Electropermeabilization of inner and outer membranes of cells with microsecond pulsed electric fields: Quantitative study with calcium ions. Scientific Reports in press 2017

[3] H. Hanna, F.M. Andre and L.M. Mir. Electrical control of calcium oscillations in mesenchymal stem cells using microsecond pulsed electric fields. Stem cell research and therapy, vol 8, art 91, 2017, DOI: 10.1186/s13287-017-0536-z

[4] M. Breton and L. M. Mir. Investigation of the Chemical Mechanisms Involved in the Electropulsation of Membranes at the Molecular Level. Bioelectrochemistry 119 (2018) e7966; doi:10.1016/j.bioelechem.2017.09.005

[5] A. Azan, V. Untereiner, C. Gobinet, G. D. Sockalingum, M. Breton, O. Piot and L. M. Mir. Demonstration of Protein Involvement in Living Cell Electropulsation using Confocal Raman Microspectroscopy. Scientific Reports 7. 297–306, 2017. doi:10.1038/srep40448.

[6] A. Azan, V. Untereiner, L. Descamps, C. Merla, C. Gobinet, M. Breton, O. Piot and L. M. Mir. Comprehensive Characterization of the Interaction between Pulsed Electric Fields and Live Cells by Confocal Raman Microspectroscopy. Analytical Chemistry in press 2017.

[7] A. Denzi, E. della Valle, G. Esposito, L. M. Mir, F. Apollonio and Micaela Liberti. Technological and Theoretical Aspects for Testing Electroporation on Liposomes. BioMed Research International, vol. 2017, Article ID 5092704, 10 pages, 2017. doi:10.1155/2017/5092704.

[8] D.Voyer, A. Silve, L. M. Mir, R. Scorretti and C. Poignard. Dynamic modeling of tissue electroporation. Bioelectrochemistry in press 2017

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INVITED LECTURERS

PEF treatment for cooking meal components

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Abstract: Pulsed Electrical Field treatment of raw tissues such as meats and vegetables creates unique properties in micro-structure. These properties can not be explained on a single cell level by the presence of interconnected tissue. In case that meal components are prepared using PEF technology, the occurrence of a temperature increase by Ohmic heating may be considered as an essential part for the final stage preparation. The combined effect of electroporation and temperature increment has been studied in vegetable tissue by monitoring the release of cellular KCl and was detected by bulk conductivity measurement during treatment. A two state conductivity model was formulated with temperature and ion concentration as independent variables. Cell damage of plant tissues was observed in the temperature range of 40-100ºC. Non-thermal assisted cell damage by PEF was demonstrated in Brussel sprouts and potato at temperatures between 45-55ºC at an electrical field strength of 175V/cm. The electroporation mechanism observed for cells in tissue is considered to be an important factor in the formation of specific micro-structure for vegetables.

INTRODUCTION

Quality of meal components much relies on the intensity of cooking. The temperature-time combination for final stage preparation of meal components such as meat, fish and vegetables is a critical factor in achieving an acceptable quality. Electrical field assisted cooking is investigated by the authors as a technology to optimise the cooking process. In the food industry Pulsed Electric Fields (PEF) processing has been proved a useful non-thermal processing method for mild preservation of juices [1] and mild extraction of intercellular components [2]. An established industrial application is the PEF (pre-) treatment of potato in fries processing lines to enhance the cutting efficiency and quality of cuts [3].

In this paper the role of electroporation is investigated in vegetable tissues when the temperature is increased by the ohmic heating effect [4]. It is well documented that exposing plant tissue to a pulsed electric field increases the permeability of cell membranes and induces structural changes by a local membrane breakdown [2,5]. The significance of the electrical field strength has been mentioned for both pulsed and non-pulsed conditions [5-7].

At higher temperatures the role of electrical induced damage in plant tissue is more pronounced. A high tissue disintegration degree may be achieved at moderate electrical field with minor loss in product characteristics [4].

In this work we consider PEF treatment as alternative method for cooking i.e. a method to prepare raw produce such as meat, vegetables and potatoes. More particular, the direct electrical effect on the tissue degradation in presence of ohmic heating is previously studied in more detail.

The objective of this experimental work is the in-situ measurement and modelling of electrical conductivity changes in the tissue as a function of temperature and ion concentration.

Electrical conductivity measurements are a versatile method to determine the concentration of dissolved minerals in liquids and to quantify the degree of cell damage in membrane structures [5]. From the generalized model of conductivity of ion solutions [8], it is well known that a change in ion concentration, temperature, viscosity and ion strength, all affect the overall conductivity but in a characteristic fashion.

In this study a thermodynamic model for conductivity is presented to disentangle effects of concentration, temperature and viscosity of free ions in the tissue after membrane permeabilization of the cell. The aim of the work is to establish a method to isolate the effect of viscosity in vegetable and meat like tissues [7] by bulk conductivity measurement to characterize the tenderness of foods when exposed to Pulsed Electrical Field as terminal preparation step of raw meal components.

MATERIALS AND METHODS

Potatoes (variety Solist) and Brussel sprouts of uniform quality were purchased at the local supermarket (Culemborg, The Netherlands). Whole potatoes with skin and whole Brussel sprouts were washed in tap water at ambient temperature 18-20°C. The first outer layer of leaves of the Brussel sprouts were removed in advance.

The treatment device is basically an oversized electroporator cuvette of approx. 500 mL volume. It consists of two vertically placed Titanium electrode plates spaced by an electrical insulating piece of polymer. The distance between the electrodes is 40 mm. In each run the pan was filled with 300 g tap water and 300 g product.

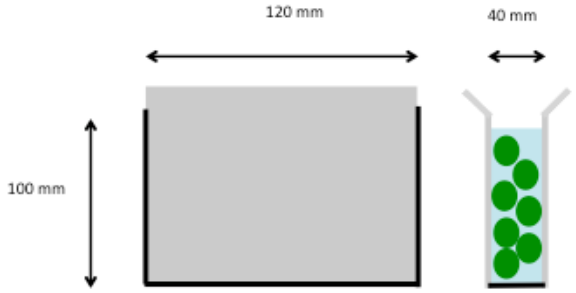

Figure 1**:** *Schematic representation and dimensions of the Titanium electrode configuration (side view and cross section). The 500 mL pan is fully stacked with potatoes or Brussel sprouts that are immersed in tap water.*

A temperature probe, that is inserted 10-20 mm into the tissue, is periodically read by software that allows for cooking control. The temperature sensor is a medical grade NTC thermoelement mounted in a sealed PP tube with a sharp point that is stuck into the tissue under investigation. Temperatures were registered as momentary core temperatures while pulsing.

The electrodes were connected to a pulsed power source that delivers 700V peak voltage and a maximum of 1kA peak current (IXL Netherlands, Schalkwijk, The Netherlands). The power unit provides bipolar pulses of near-rectangular shape at a voltage of 700 V. The pulse duration was fixed to 40 microseconds. The total number of pulses that are delivered was fixed to 5000. The repetition rate was programmed at 2kHz. After each series of a number of pulses the conductivity of an immersed potato or sprout was measured using an LCR meter (Keysight U1733C). The test frequency for impedance measurement was 1kHz. The electrode configuration for conductivity measurement is a two pin set-up using two standard multi meter probes with 3 mm diameter, 15 mm long tips at 15 mm distance (effective cell constant 2.2 cm^{-1}). Probes were inserted to its full length into the tissue under investigation. The impedance measurements, read as a true Ohmic resistance, were converted to electrical conductivity after calibration to a standard KCl solution. The impedance and temperature of the sprout were recorded after finalising each series of pulses. Recordings were taken at an increasing number of pulses such that after each series of 5000 pulses the temperature has increased by a few degrees to obtain a heating curve. After reaching 100°C, just below the boiling point, the pulses were terminated and the pan tissue were left to cool. The recordings of temperature and conductivity were continued to obtain a cooling curve. The heating and cooling curves that were obtained define the tissue state before and after the treatment. A heating curve of the rinsing water of the raw tissue was obtained as a control that defines the reference levels.

Model parameters were estimated by the method of Maximum Likelihood Estimation (MLE) using 'R' language and environment for statistical computing [12]. Models were coded as a single primary model to include all experimental variables and model parameters. The (global) optimisation and parameter estimates were performed according to the method of Simulated Annealing using the GenSA package [13]. Global optimisation over the entire, relevant domain of parameters was carried out using box constraints, to avoid guessing of starting values and rendering a sub optimal model.

MODELING CONDUCTIVITY σ(C,T)

The electrical conductivity depends on the ion mobility, which is a function of temperature, concentration and viscosity [8,10,11]. Accurate calibration data for KCl solutions are provided by Wu et. al. [10] for temperatures in the range of 0-50°C and concentrations of 0.01-1.0 M KCl.

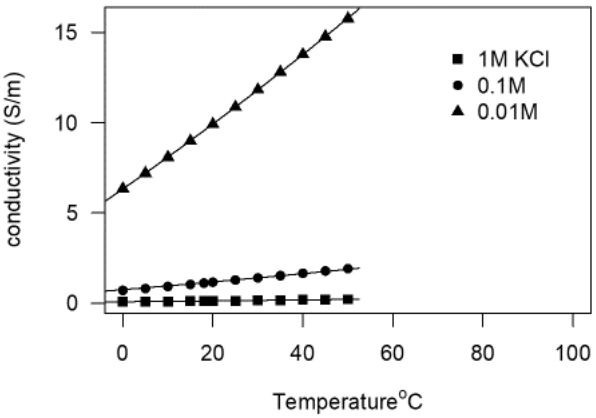

Figure 2: *Fit of the virial model to the KCl reference data set [10]. The conductivity depends on concentration and temperature.*

Model	Number of parameters	Bayesian Information Criterium
Debye-Huckel	3	-8
Robertson	4	-18
Chen-Onsager	6	-53
Virial expansion (this work)	5	-160

Table 1: *Performance of the bivariate model for electrical conductivity by the Bayesian Information (BIC) using the NIST data set for conductivity as a function of KCl concentration and temperature.*

Several line shapes (table 1) were evaluated to model the reference data on its domain. The predicted line shape of the exact analytical expression provided by Chen and Onsager [8] is a good description of the data. However, upon comparison using the Bayesian Information Criterium (BIC), it was found that the best model for conductivity (σ) is the product of the concentration (C) dependence, multiplied by a virial expansion in the temperature (T) given by:

$$\sigma(C,T) = \left[L - A\sqrt{C} + \epsilon_1 C\right] \cdot C \cdot (1 + \epsilon_2 T + \epsilon_2 T^2)$$

This five parameter model was fitted to the calibration data provided by Wu *et. al*. [10] to obtain parameter estimates and confidence intervals. For an estimate of the free ion concentration in a PEF experiment, the conductivity of both the heating and cooling curve were fitted using these parameter estimates. In this way the (unknown) ion concentration in the tissue and the confidence interval were obtained before and after electroporation.

RESULTS

In figure 3 the conductivity of Brussel sprouts is shown when treated at 175 V/cm . After preparation at 18°C, the sprouts were treated by applying series of 5000 pulses. After each series the temperature and the conductivity of the tissue was measured. For temperatures from 20-55°C the conductivity increases linear and the conductivity coincides with the surrounding water. At 55°C a strong onset in conductivity is found, which cannot be explained by the conductivity of ions in the surrounding water. The treatment was stopped just below the boiling point of water. After the boiling point was reached, the system was left to cool down and the conductivity and temperatures were recorded until a temperature of 25°C was reached. Is was observed that the conductivity at the cooling curve approaches a straight line. It was hypnotised that the observed conductivity increase is by intracellular release of ions (mainly KCl) after cell membrane damage had occurred. This membrane permeabilisation is assumed to be the sum of the heating and electroporation effect.
The corresponding KCl concentration in the tissue was retrieved by fitting the linear part of the cooling curve to the reference data for KCl in solution as a function of temperature. A corresponding total ion concentration of 129 mg per 100 gr is found after treatment. The confidence interval (CI) of this concentration is [126-132]. Taking into account the initial added water with a ion concentration of 0.13 [0.09-0.17] mg per 100 gr water and the approximate concentration of 258 mg per 100 gr raw sprouts approximately half the value of the typical total mineral concentration of 548 mg per 100 gr for Brussel sprouts.

A similar series of experiments were carried out for potato for indirect heating in immersed water and for PEF treatment at 100 V/cm and 175 V/cm. The result of this series is shown in figure 4. A sharp increase in conductivity is observed at the onset of low temperature for field strengths at 175 V/cm. Up to 100 V/cm the increase is not distinguishable from ions dissolved in the surrounding water until a temperature is reached of more than 40°C. This is dramatically different at 175 V/cm where an immediate incline in conductivity is observed, indicating an immediate concentration increase of intracellular ions that are released by electroporation.

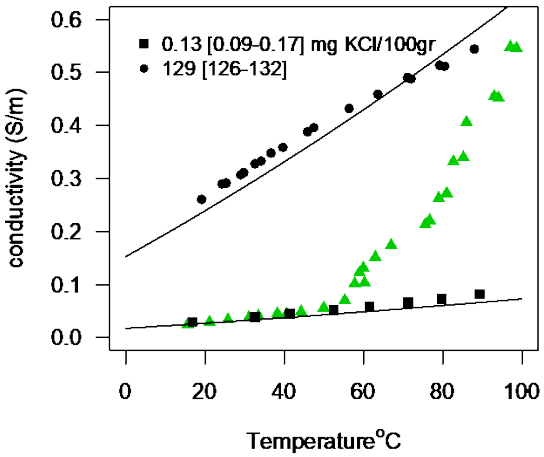

Figure 3: *Tissue conductivity of PEF treated Brussels sprouts at 175 V/cm while heating (▲) and during cooling (●). The conductivity of the surrounding water is the reference level for tissue conductivity (■). The onset of the observed conductivity increase of the tissue and decrease at cooling is by temperature change only. These two tissue states are well approximated by the bivariate linear model for conductivity. A transit from low to high KCl concentration could be resolved which is attributed to electroporation.*

Remarkably, the final level of free ions that was determined by a fit of the cooling curves to the reference data, was the highest for indirect heated potato: 362 [357-367] mg per 100 gr. This yields a mineral content of 714 mg per 100 gr for the raw potato. This is in fair agreement with a tabulated characteristic total mineral content of 512 mg per 100 gr.

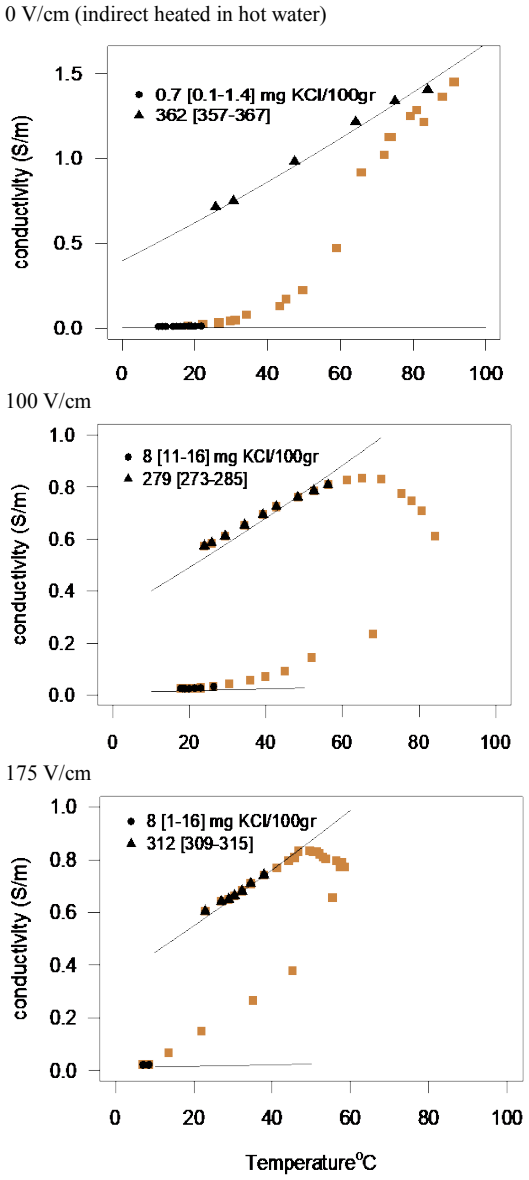

Figure 4: *Conductivity of PEF treated potato tissue at different applied field strengths. At 0 V/cm the temperature of the potato was controlled by heating the surrounding water. At an electrical field strength of 100 and 175 V/cm the temperature of the tissue is determined by Ohmic heating, inevitable present in a PEF treatment.*

DISCUSSION AND CONCLUSIONS

The release of intercellular ions after electroporation of membranes of plant cells and the increase in conductivity of the tissue is well established [2,4-6]. The ion mobility is dependent on the concentration, temperature and viscosity of the surrounding medium in which the ions are present [8]. For tissues with a dense cellular structure, one would expect that the ion motion is hindered by the tissue and cellular fragments. If so, this may yield a detectable change in mobility leading to deviations in the conductivity as a function of concentration and temperature. In this work an accurate method was presented to accurately retrieve the concentration of dissolved KCl in water by bulk conductivity measurements. This method relies on the bivariate model for conductivity of dissolved KCl at different temperature and concentration.

The conductivity of conventional cooked Brussel sprouts and potato are compared with PEF treated specimens. Profound differences are observed in the heating curve for at an electrical field strength of 175 V/cm and beyond. The conductivity data for the cooling curve, and the comparison to the reference data using the bivariate model, indicates that the ions that are released in the tissue are dissolved as in water. That is the mobility is not noticeable affected by the presence of interconnected tissue or cellular materials. A change in conductivity by hindered ion motion seems to be a good indicator for the local viscosity of degraded plant cells in a tissue and a precursor of the level of tenderness of tissue.

The observation of (thermal assisted) tissue degradation in presence of ohmic heating at electric field strengths less than 100 V/cm [6,7] suggests that a combination of longer pulse duration in combination with at a lower electric field strength should be able to reproduce tissue and cell damage in the same fashion. However, when vegetables (sprouts, carrots, potato) were exposed to conditions less then 100 V/cm a totally different sensorial quality was obtained even at pulse durations in excess of 1 millisecond (data not shown).

The aim of this work was to find a method to monitor and characterise the culinary doneness of Brussel sprouts and potato by bulk conductivity measurement. Conductivity measurement is a versatile method as it can be implemented in-situ. The study on the particular response curves that are observed, combined with the particular sensory quality (texture, color, taste) of the vegetables that were prepared (data not shown), is a challenging but rewarding subject which requires more efforts and certainly deserves more attention in future research.

REFERENCES

[1] Lelieveld, H.L.M., Notermans, S. & de Haan, S.W.H. (Eds.). (2007) Food preservation by pulsed electric fields. From research to application. Woodhead publishing

[2] Vorobiev, E, Lebovka, N.I. (2006). Pulsed power systems for application of pulsed electric fields in the food industry. In: Raso,J. & Heinz, V. (Eds.). Pulsed Electric Fields Technology for the Food Industry. Fundamentals and application. Springer, New York.

[3] Oey, Indrawati & Faridnia, Farnaz & Ying Leong, Sze & Burritt, David & Liu, Tingting. (2016). Determination of Pulsed Electric Fields Effects on the Structure of Potato Tubers. 10.1007/978-3-319-26779-1_151-1.

[4] Lebovka, N.I., Praporscic I, Ghnimi, Vorobiev E., (2005), *J. of Food Science, 70(5) 308-311*

[5] Angersbach, A., Heinz, V., Knorr, D. (1999) *Biotechnology Progress, 15*(4), 753-762

[6] Lebovka, N.I., Praporscic I, Ghnimi, Vorobiev E., (2005), *J. of Food Eng, 69(5) 177-184*

[7] K Halden, AAP de Alwis, PJ Freyer, Int J of Food Science and Technology (1990) 25, 9-25

[8] Chen M.S., Onsager L., (1997) The Journal of Physical Chemistry 81 (21)

[9] Gudmundsson M., Hafsteinsson H, (2001), *Trends in Food Science and Technology 12, 122-128*

[10] Wu, Y. C., Koch, W. F., and Pratt, K. W., J. Res. Natl. Inst. Stand.Technol. 96, 191, (1991)

[11] Wright, M.R. (2007). An Introduction to Aqueous Electrolyte Solutions.

[12] R Development Core Team. (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0

[13] Xiang, Y., Gubian, S., Suomela, B. & Hoeng, J. (2013). Generalized Simulated Annealing for Efficient Global Optimization: the GenSA Package for R. The R Journal, 5

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NOTES

NOTES

Imaging electroporation in artificial bilayers

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Abstract: Much of our understanding of electroporation stems from direct measurements of ionic flux across the membrane. However, such measurements inherently average signals from the whole bilayer. Here we explore the advantages and limitations of applying optical imaging to help overcome these restrictions and improve our picture of pore-formation in lipid membranes. In these brief proceedings we focus on the methods pioneered in our lab, which exploit a shift from direct measurements of ionic flux into equivalent optical readouts from Droplet Interface Bilayers.

TOROIDAL PORES

Membrane disruption via electroporation can be thought of in terms of increased electrical conductance, transient permeability to small molecules, or total breakdown of the membrane. Overall, the physical phenomena underlying this membrane disruption is typically described in terms of the voltage-dependent formation of toroidal defects in the lipid bilayer forming transient aqueous pores. How and why such defects occur is an important question for improving our understanding of gene transfection, food sterilisation, drug encapsulation, drug delivery, and tumour ablation. Here we present a brief overview of the mechanism of defect formation, methods to visualise such defects, and our contribution to this problem. This is a brief introduction and is unashamedly non-exhaustive.

Toroidal pores are *hydrophilic pores*, membrane-spanning defects in which the lipid headgroups line the edge of a water-filled inclusion [1]. The mechanism of the voltage-dependent toroidal pore formation, sometimes called the transient aqueous pore hypothesis, remains the foundation of much of our understanding of electroporation. In this model, first *hydrophobic pores* expose the bilayer interior to the surrounding electrolyte, followed by structural rearrangement of the lipids to link the two lipid monolayers and remove this unfavourable configuration.

Let us take a moment to consider this two-step process in more detail: So why not just a hydrophobic pore? The energy cost of placing a monovalent ion within the hydrophobic bilayer core is estimated at ≥40–70 k_BT, (although this barrier is reduced to around 10 k_BT if the ion crosses the bilayer within a 5 Å water-lined hydrophobic pore) [2,3]. This barrier is clearly significant, and a hydrophobic pore also is therefore unlikely to allow significant passage of ions.

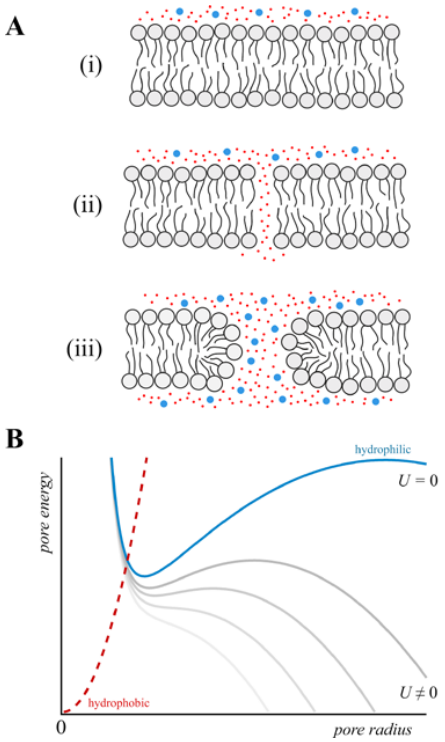

Figure 1: (A) Toroidal pore formation: (i) Unperturbed bilayer. (ii) Lateral fluctuations result in a non-conducting, hydrophobic pore that may pass water (*red*) but not ions (*blue*). (iii) Inversion of the hydrophobic pore leads to a hydrophilic pore, conductive to ions. (B) Influence of a transmembrane potential U on the energy of a membrane pore. The energy barrier to expansion of a hydrophilic pore (*solid lines*) is high in the absence of a potential (*blue line*), but decreases with increasing transmembrane voltage (*grey lines*).

In contrast, the headgroup-lined lumen of a hydrophilic pore readily permits flow of electrolytes across the membrane, with essential no barrier to ionic flux. However, without an external driving force, the barrier to conversion from hydrophobic to a hydrophilic pore is too large to be overcome spontaneously by the thermal energy [4,5], and membranes (thankfully) remain essentially impermeant to ions. However, with an external input of

energy from an electric field, the barrier to hydrophobic pore inversion may be surmounted, producing membrane-spanning, conductive defects characteristic of electroporation.

This sequence of events, proposed in 1979 and subsequently (mathematically) refined in the following decades [1,6-8], has been borne out in both experiment [9-12], and simulations [5,13-15], which have demonstrated the formation of hydrophilic channels from membrane-spanning hydrophobic pores.

BEYOND ELECTROPORATION

It is worthwhile taking pause to recognise the scope of toroidal pore formation in lipid membranes; it is a likely actor in a wide range of important biological processes [16], well beyond the confines of just electroporation. Mounting evidence suggests that many 'proteolipidic' pores form via toroidal structures. For example, the folding machinery of gram negative outer membrane Bam, catalyses outer membrane porin insertion by lowering the kinetic barrier imposed by phospholipid head groups [17], presumably by lateral opening [18, 19]. It seems likely that if such a mechanism is correct, it would require the rearrangement of lipids to form a partial toroid.

Toroidal pore formation is also a well-established mechanism of action for many antimicrobial peptides [20,21], here in essence the energy barrier to defect formation is lowered by the interaction of peptides with lipids at the mouth of the pore, stabilizing its structure [22].

One of the most often observed features of the membrane attack complex perforin / cholesterol dependent cytolysin superfamily is the presence of both ring-shaped and arc-shaped oligomers [16]. Arcs of subunits would have to form pores at a protein–lipid interface with a toroidal lipidic structure. Functional assays suggest that both rings and arcs are capable of forming pores [23].

If we are to understand these processes, a firm understanding of pore-formation in pure lipid bilayers is a first step. In understanding toroidal pore formation in the context of electroporation, we also help lay the foundations for understanding this broad range of other important biological processes.

IMAGING PORES, WHY BOTHER?

Although electrical recording provides us with a clear measurement of the result of membrane permeabilization, it cannot extend our understanding beyond this output. Alternative techniques are required to expand our understanding. A compelling first step would be to 'see' electropores in action to allow us to characterise their behaviour directly. Although modern molecular dynamics simulations have helped us model the existence of hydrophilic pores under conditions representative of bilayer membranes exposed to strong electric fields, the experimental evidence to bolster this work is lacking.

Historically experiments have been conducted that attempt to image the proposed pores by inducing permeabilization, and immediately freezing the membrane, followed by visualisation with using electron microscopy [10]. More recent experiments of note include the high-speed imaging of macropore formation in giant unilamellar vesicles [11,12].

If we relinquish the pursuit of direct dynamic visualisation of nanoscopic toroidal pores, the indirect detection of ionic flux might also benefit us. Using a fluorogenic dye, sensitive to the transport of ions across the membrane, the flux associated with a single pore can be visualised [9], even when many permeabilization events might be present in a single membrane.

Although other fluorogenic indicators might be considered [24, 25], it is calcium indicator dyes that have the dynamic range, and temporal responsivity required to act as a proxy measurement for ionic flux. Such optical Single Channel Recording (oSCR) methods are well established in order to visualize calcium flux in cells and oocytes [26]. For example, in the membrane disruption by small amyloidogenic oligomers [27].

To achieve efficient imaging of individual electroporation events we require both an intact bilayer, control of the membrane potential, and single-molecule sensitive optical imaging. Our lab has achieved this through the development of Droplet Interface Bilayers (DIBs) [28,29]. We recently reported the optical imaging of electroporation events in this system [9, 30].

DIBs can be formed by bringing into contact an aqueous droplet and a planar hydrogel substrate in the presence of a solution consisting of lipid dissolved in an alkane oil. The lipid self-assembles as a monolayer on both surfaces; when they come into contact a bilayer is formed. Electrical access may then be gained by insertion of electrodes into the droplet and the hydrogel. Total Internal Reflection Fluorescence (TIRF) microscopy can then be used to visualise permeabilization events in the bilayer.

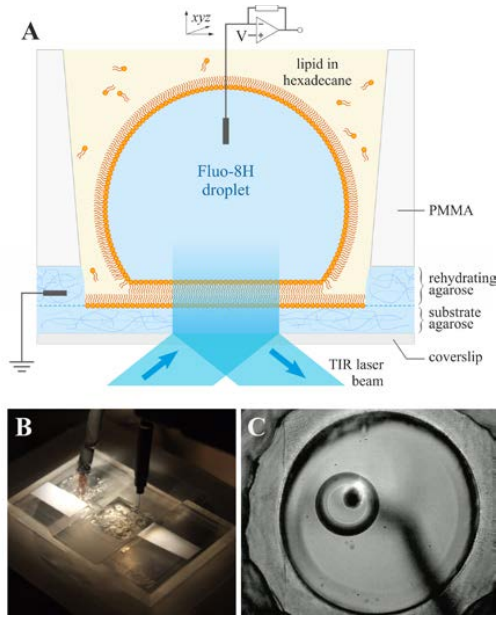

Figure 2: (A) Simultaneous electrical and optical recording from a Droplet Interface Bilayer. Electrodes in the droplet and the hydrating agarose monitor the membrane current. Total Internal Reflection illuminates the bilayer and allows the monitoring of Ca^{2+} flux through membrane pores by imaging fluorescence signal from Fluo-8H dye present in the droplet. (B) Device and electrodes ready for experiment. (C) View of a droplet with inserted electrode.

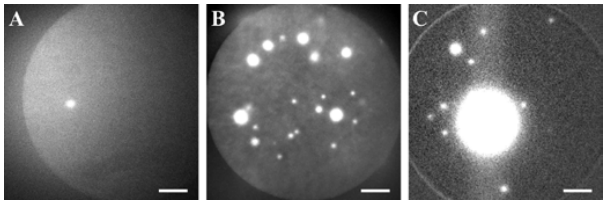

Figure 3: Visualising electropores in DIBs. (A) A single electropore in a bilayer held at 200 mV. Image is the raw data, recorded at 9.9 Hz. (B) Multiple electropores at 485 mV. Image is the maximum pixel intensity of 40 frames recorded at 61.7 Hz. (C) A very large pore at 260 mV. Image is a single, background-corrected frame, recorded at 61.7 Hz. All scale bars: 20 µm.

ELECTROPORES IN DROPLET INTERFACE BILAYERS

Under an applied DC potential electroporation occurs in the DIB, calcium flows from the agarose into the droplet where the indicator dye, Fluo-8, is present. When a pore opens this can be detected as a bright spot in the bilayer. Figure 3 shows typical data acquired during electropore visualisation.

This approach enables us to assign components of the overall ionic flux to an individual pore. Although stepwise gating events can be observed in these single-component lipid bilayers, overall it is interesting to note that even single isolated pores exhibit the 'noisy' ionic currents associated with a toroidal defect that fluctuates in size.

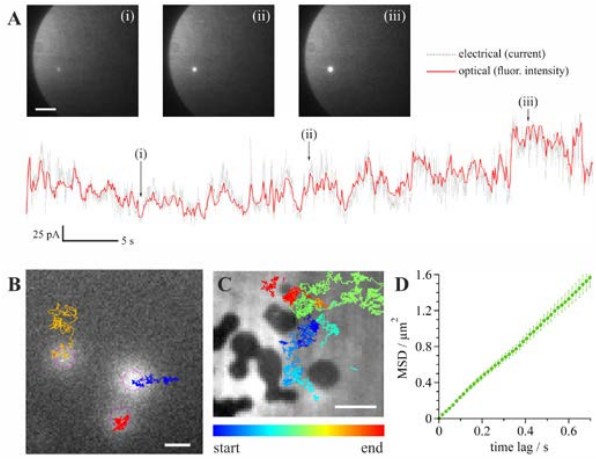

Figure 4: (A) Correlating fluorescence and electrical recordings during electroporation of a bilayer at -195 mV. Overlaying traces of the total membrane current and fluorescence intensity of an isolated pore in the membrane reveal clear correlation between the two signals. Frames are 100 ms exposures at the indicated points in the trace. Scale bar: 25 µm. (B) Tracking the diffusion of individual electropores in a DIB. Scale bar: 5 µm. (C) Electropore diffusion a in DPhPC/DPPG/Chol phase-separated membrane. The disordered region was labelled with 1 mol% of the lipophilic dye $DiIC_{18}(3)$. Coloured trajectories show motion of electropores; their colour indicates the time at which the trajectory begins with respect to the start of the recording. Scale bar: 5 µm. (D) Mean-squared displacement analysis of individual pores shows Brownian diffusivity.

Figure 4 shows a simultaneous electrical and fluorescence experiment at -195 mV where only a single pore is present in the membrane; the correspondence between these two measurements is apparent. We have previously exploited this method to characterise the the kinetics [9] (Fig. 5B) and thermodynamics [30] of electropore formation in detail. We can also use these methods to examine the spatial distribution of pore formation, tracking pore diffusion and location relative to the composition of the lipid membrane. For example, Figure 4C shows pore formation is confirmed to liquid disordered phases on the bilayer.

Examining multiple individual electroporation events in parallel also provides new information, for example Figure 5A shows anti-correlation between signals from two closely spaced electropores. This opens discussion as to the mechanisms underlying this behaviour.

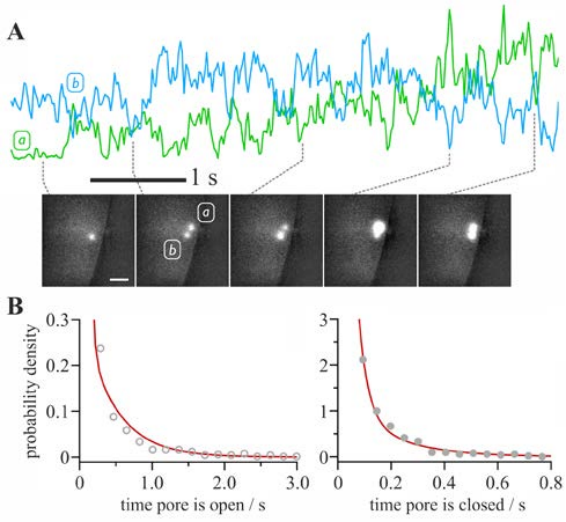

Figure 5: (A) Anticorrelated fluorescence signals of two adjacent electropores, suggesting that dynamic local changes in the membrane environment from nearby pores can influence pore behaviour. Scale bar: 10 μm. (B) Probability density histograms for electropore open and closed times at 260 mV (open) and 160 mV (closed). The double exponential fits (*red*) indicate that gating is complex.

SUMMARY & LIMITATIONS

oSCR is an optical complement to electrophysiological measurements of ion flux. However, whereas electrical recording alone can only report on the ensemble of pores, yielding information about the total bilayer conductivity but revealing little about the contribution from individual defects, oSCR relays a signal from each component pore.

Although powerful, this method comes with some significant limitations: (i) Although the positions of individual permeabilization events are super-resolved (~40 nm) using this method, the signal is a diffraction-limited cloud of fluorescence around the location of the defect. We do not image the pore directly; (ii) Given the limitation of current fluorogenic probes, these methods are limited to imaging calcium flux; (iii) The temporal resolution of these methods are lower than the corresponding direct electrical measurement, here typically we are limited to millisecond resolution optical recording.

By switching from electrical to a proxy, optical, measurement of ionic current these methods provide new insights into pore formation in lipid membranes.

REFERENCES

[1] I. G. Abidor *et al*. “Electric breakdown of bilayer lipid membranes: 1. The main experimental facts and their qualitative discussion”. Journal of Electroanalytical Chemistry and Interfacial Electrochemistry 104 (1979), pp. 37–52.

[2] A. Parsegian. “Energy of an Ion crossing a Low Dielectric Membrane: Solutions to Four Relevant Electrostatic Problems”. Nature 221.5183 (1969), pp. 844–846.

[3] I. V. Khavrutskii *et al*. “Free Energy for the Permeation of Na+ and Cl- Ions and Their Ion-Pair through a Zwitterionic Dimyristoyl Phosphatidylcholine Lipid Bilayer by Umbrella Integration with Harmonic Fourier Beads”. Journal of the American Chemical Society 131.5 (2009), pp. 1706–1716.

[4] R. W. Glaser *et al*. “Reversible electrical breakdown of lipid bilayers: formation and evolution of pores”. Biochimica et Biophysica Acta (BBA) - Biomembranes 940.2 (1988), pp. 275–287.

[5] W. F. D. Bennett, N. Sapay, and D. P. Tieleman. “Atomistic Simulations of Pore Formation and Closure in Lipid Bilayers”. Biophysical Journal 106.1 (2014), pp. 210–219.

[6] J. C. Neu and W. Krassowska. “Asymptotic model of electroporation”. Physical Review E 59.3 (1999), pp. 3471–3482.

[7] G. Saulis, M. S. Venslauskas, and J. Naktinis. “Kinetics of pore resealing in cell membranes after electroporation”. Journal of Electroanalytical Chemistry and Interfacial Electrochemistry 321.1 (1991), pp. 1–13.

[8] R. P. Joshi and K. H. Schoenbach. “Electroporation dynamics in biological cells subjected to ultrafast electrical pulses: a numerical simulation study”. Physical Review. E, Statistical Physics, Plasmas, Fluids, and Related Interdisciplinary Topics 62.1 Pt B (2000), pp. 1025–1033.

[9] J. T. Sengel and M. I. Wallace “Imaging the dynamics of individual electropores” Proceedings of the National Academy of Sciences 113.19 (2016), pp. 5281-5286.

[10] D. C. Chang and T. S. Reese. “Changes in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy.” Biophysical Journal 58.1 (1990), pp. 1–12.

[11] N. Rodriguez, S. Cribier, and F. Pincet. “Transition from long- to short-lived transient pores in giant vesicles in an aqueous medium”. Physical Review E 74.6 (2006), p. 061902.

[12] T. Portet and R. Dimova. “A new method for measuring edge tensions and stability of lipid bilayers: effect of membrane composition”. Biophysical Journal 99.10 (2010), pp. 3264–3273.

[13] D. P. Tieleman. “The molecular basis of electroporation”. BMC Biochemistry 5.1 (2004), pp. 1–12.

[14] M. Tarek. “Membrane electroporation: a molecular dynamics simulation”. Biophysical Journal 88.6 (2005), pp. 4045–4053.

[15] S. A. Kirsch and R. A. Böckmann. “Membrane pore formation in atomistic and coarse-grained simulations”. Biochimica et Biophysica Acta (BBA) - Biomembranes 1858.10 (2016), pp. 2266–2277.

[16] R. J. C. Gilbert, M. D. Serra, C. J. Froelich, M. I. Wallace and G. Angerluh. "Membrane pore formation at protein-lipid interfaces". Trends in Biochemical Sciences 39.11 (2014), pp. 510–516.

[17] D. Gessmann, Y. H. Chung, E. J. Danoff, A. M. Plummer, C. W. Sandlin, N. R. Zaccai, K. G. Fleming. “BamA-catalyzed OMP folding”. Proceedings of the National Academy of Sciences 111.16 (2014), pp. 5878-5883.

[18] L. Han, J. Zheng, Y. Wang *et al*. “Structure of the BAM complex and its implications for biogenesis of outer-

membrane proteins". Nature Structural & Molecular Biology 23 (2016), pp. 192–196.
[19] N. Noinaj, A. J. Kuszak, J. C. Gumbart, P. Lukacik, H. Chang, N. C. Easley, T. Lithgow and S. K. Buchanan. "Structural insight into the biogenesis of β-barrel membrane proteins". Nature 501 (2013), pp. 385–390.
[20] K. V. R. Reddy, R. D. Yedery, and C. Aranha. "Antimicrobial peptides: premises and promises". International Journal of Antimicrobial Agents 24.6 (2004), pp. 536–547.
[21] K. A. Brogden. "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" Nature Reviews Microbiology 3.3 (2005), pp. 238–250.
[22] D. Sengupta, H. Leontiadou, A. E. Mark, and S.-J. Marrink. "Toroidal pores formed by antimicrobial peptides show significant disorder". Biochimica et Biophysica Acta (BBA) - Biomembranes 1778.10 (2008), pp. 2308-2317.
[23] M. Palmer, R. Harris, C. Freytag, M. Kehoe, J. Tranum-Jensen and S. Bhakdi. "Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization". The EMBO Journal 17 (1998), pp. 1598-1605.
[24] M. Szabo and M. I. Wallace. "Imaging potassium-flux through individual electropores in droplet interface bilayers". Biochimica et Biophysica Acta 1858 (2016), pp. 613–617.
[25] G. C. Kemmer, S. A. Bogh, M. Urban, M. G. Palmgren, T. Vosch, J. Schiller and T. G. Pomorski. "Lipid-conjugated fluorescent pH sensors for monitoring pH changes in reconstituted membrane systems". Analyst 140 (2015), pp. 6313-6320
[26] A. Demuro and I. Parker. "Optical single-channel recording: imaging Ca2+ flux through individual N-type voltage-gated channels expressed in Xenopus oocytes". Cell Calcium 34.6 (2003), pp. 499-509.
[27] A. Demuro, E. Mina, R. Kayed, S. C. Milton, I. Parker and C. G. Glabe. "Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers". The Journal of Biological Chemistry 280.17 (2005), pp. 17294-17300.
[28] H. Bayley, B. Cronin, A. Heron, M. A. Holden, W. L. Hwang, R. Syeda, J. R. Thompson and M. I. Wallace. "Droplet interface bilayers". Molecular BioSystems 4(2008), pp. 1191-1208.
[29] S. Leptihn, O. K. Castell, B. Cronin, E.-H. Lee, L. C. M. Gross, D. P. Marshall, J. R. Thompson, M. Holden and M. I. Wallace. "Constructing droplet interface bilayers from the contact of aqueous droplets in oil". Nature Protocols 8.6 (2013), pp. 1048-1057.
[30] J. T. Sengel and M. I. Wallace. "Measuring the potential energy barrier to lipid bilayer electroporation". Philosophical Transactions of the Royal Society B 372.1726 (2017).

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Developing plastic bioelectronic devices for measuring and manipulating cells in vitro and in vivo

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Abstract: Organic conductive polymers are highly versatile materials that have been widely adopted in commercial electronics as display technology, transistors, LEDs and solar cells. Our research is exploring their use for interfacing biology with electronics. We have developed microelectrode array devices using the conductive polymer PEDOT:PSS for electrophysiology, electropulsation, neurostimulation and electroporation investigations in vitro and in vivo. Here I will present our ongoing work using plastic bioelectronics to develop devices for in vitro studies using live cell imaging, and flexible, implantable electrodes that are suitable for studying electropulsation effects with in vivo imaging and electrophysiology.

PLASTIC BIOELECTRONICS

Plastic bioelectronics are a now a burgeoning area of research that use conductive organic polymer materials to interface biology with electronics [1]. Although we are typically taught that plastics are insulators, and metals conductors, pioneering work in the 1970s by Heeger, MacDiarmid and Shirakawa demonstrated that polymers could be altered to make them conductive. This seminal discovery was so significant it was awarded the Nobel prize for Chemistry in 2000[1]. Now, conductive organic polymers are widely used in consumer electronics, while their application in bioelectronics has just begun.

PEDOT:PSS is an example of a conductive polymer routinely used in bioelectronics, and also known as poly (3,4-ethylenedioxythiophene) polystyrene sulfonate. The properties of this organic polymer that make it useful in biology are its biocompatibility, mechanical flexibility, transparency to visible light, and mixed conductivity. PEDOT:PSS is conductive as it forms a degenerately doped p-type semiconductor, in which holes in the conjugated polymer PEDOT are compensated by the PSS anions. This type of material has significant advantages over inorganic electrodes made of either metal or p-type silicon, at the level of surface interactions with the electrolyte and biological tissue.

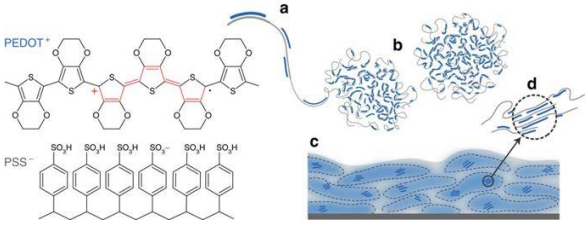

Figure 1: The chemical structure of PEDOT:PSS and commonly described microstructure of the conductive polymer system [2].

A further benefit of organic electronic materials for bioelectronics is that they are soft, stretchable and conformable, which allows them to better match the mechanical properties of soft biological tissues. Another feature is that conductive polymers can uptake and interact with ions in biological solutions, giving PEDOT:PSS both ionic and electronic conductivity [2]. The ingress of biological ions into such materials allows a 3D coupling of the electrical behaviour of the tissue, as shown by the cartoon in Figure 2. Finally, low temperature processability and the low cost of these polymer materials allows for large scale manufacturing of devices for single use and disposability, if required.

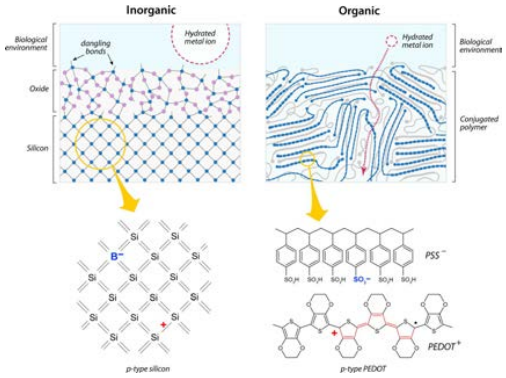

Figure 2: Inorganic vs. Organic PEDOT:PSS electrodes

The microstructure of PEDOT:PSS forms a spongy thin layer as shown in Figures 1 and 2. PEDOT:PSS is commercially available in a highly conductive aquaeous form (1000 S/cm) (Clevios™, Heraeus) known to have good chemical stability. Microelectronic devices can be fabricated with this colloidal gel dispersion of PEDOT:PSS by using

an adapted photolithographic technique suitable for organic layers [3].

The coating of metal electrodes with PEDOT:PSS, by either spin casting or electrodeposition, modifies their surface properties improving their coupling to tissue by reducing interfacial impedance. For example, Figure 3 compares the electrical impedance spectra of bare gold electrodes versus the same sized electrodes coated with 350 nm of PEDOT:PSS [5]. These modifications increases the effective surface area and decrease impedance observed on electrodes, enhancing the signal-to-noise ratio for signals at lower frequencies, where biological signals primarily occur (< 1 kHz).

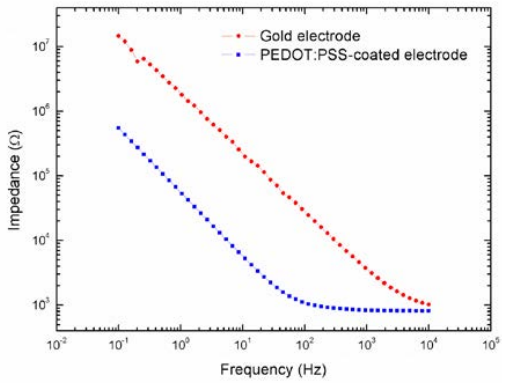

Figure 3: Impedance spectroscopy of spun-cast PEDOT:PSS film, 350 nm on a 500 µm x 500 µm square electrode (blue squares), in comparison with bare gold electrode (red circles) [5].

MICROELECTRODE ARRAYS (MEA)

Extracellular recordings of electrogenic cells like neurons can be performed in vitro [6] and in vivo [7] through the use of microelectrode arrays made from inorganic or organic materials. MEAs are now essential tools routinely used to measure the network behaviour of neurons to understand their functions in the brain and to see how this differs in neurological diseases like epilepsy and Parkinson's disease. As shown in Figure 4, MEAs come in a variety of designs, depending on the target tissue type and location of the recording site.

Inorganic, silicon or metal MEAs (Figure 4 a-h) have been widely successful in obtaining important neurophysiological datasets, however, the stiffness of these materials is known to lead to tissue inflammation, increasing electrode impedance, and eventually causing the loss of signals [7]. It is here where soft, flexible organic electronic materials have risen to the task of mechanically matching biological tissue.

Thin layers of conductors and/or conductive polymers like PEDOT:PSS can be patterned on extremely thin (2 µm) layers of biocompatible materials like parylene C, which produces highly conformable and soft MEAs (Figure 5). The resulting devices are mechanically more similar to the properties of brain or nerve tissue and cause significantly less inflammation responses, giving superior signal-to-noise recordings for longer recording periods [7,8].

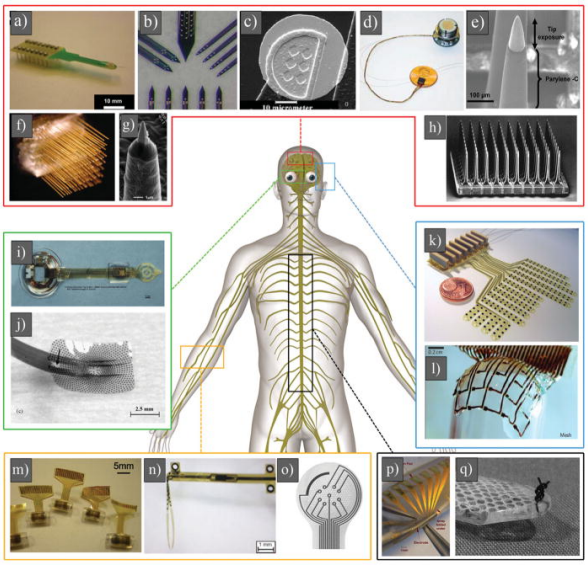

Figure 4: The many designs of inorganic and organic microelectrode arrays used for recording electrophysiological signals [7].

Several strategies have been applied to temporarily increase the stiffness of MEAs for insertion, including the use of delaminating removable shuttles [9] and bioresorbable materials [10]. This leaves behind only the very thin and flexible parylene support with its conductive polymer recording pads and interconnects.

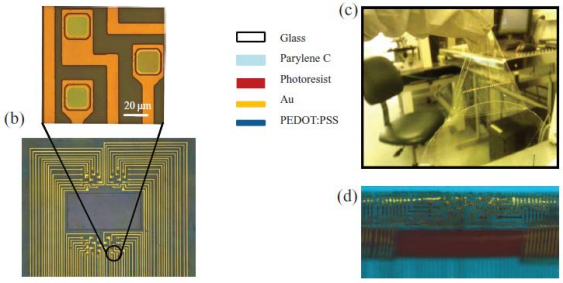

Figure 5: Highly conformable microelectrode arrays fabricated at the Microelctronic Center of Provence by BEL from PEDOT:PSS coated gold electrodes patterned on parylene C [8].

This very short review covers the current state-of-the-art in flexible recording MEAs that use conductive polymers like PEDOT:PSS. Other promising approaches using conductive materials like graphene or carbon nanotubes have not been discussed, however, they are comprehensively reviewed by Fattahi et al, [7].

ACTIVE DEVICES FOR ELECTRICAL RECORDING

Organic electrochemical transistors (OECTs) are an active microelectronic device made with an organic semiconductor film that is in contact with an electrolyte in which a gate electrode is immersed. They make use of ion injection from the electrolyte to modulate the bulk conductivity of an organic semiconductor channel made from PEDOT:PSS [4]. The coupling between ionic and electronic charges within the entire volume of the channel gives OECTs a high transconductance compared with that of field-effect transistors, but it also limits their response time [11]. Figure 6b illustrates a schematic of an OECT, while 6c shows an image of an actual OECT device made at BEL.

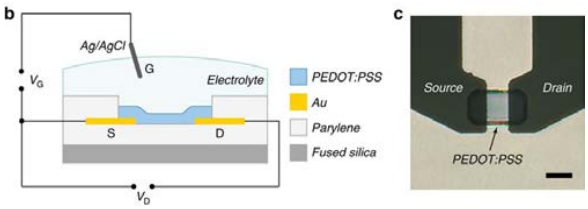

Figure 6: Organic electrochemical transistors. b, Schematic of an OECT cross-section and the wiring diagram for device operation. (c) Optical micrograph of an individual transistor. Scale bar, 10 µm [4].

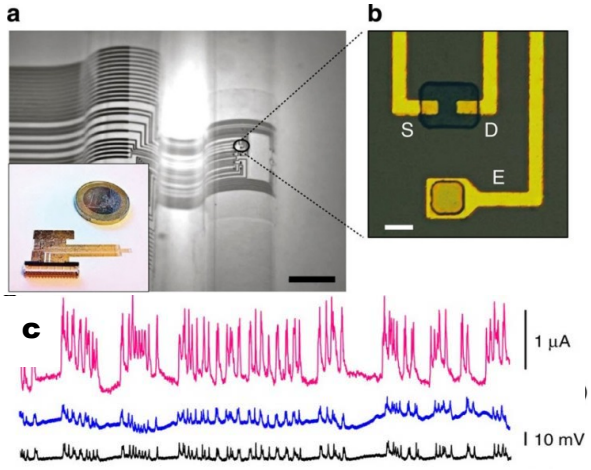

Figure 7: In vivo recordings of brain activity.

OECTs can be used for sensing electrical activity from excitable cells like neurons [12], sensing impedance across tissue barriers [4], or detecting analytes. In the case of electrical recordings from excitable cells, OECTs provide amplification of the biological signal as ions ingress into the device and dedope the PEDOT:PSS, a p-type semiconductor operating in depletion mode. Figure 7a shows the flexible thin OECT array used to record action potentials from the surface of the brain. Electrical signals recorded with OECTs are thus amplified (Fig 7c pink trace), in comparison to those registered on passive electrodes (Fig 7c blue and black traces).

ORGANIC POLYMER ELECTRODES FOR ELECTROPULSATION / ELECTROPORATION

Organic electronic materials have not yet been thoroughly evaluated for their use in electropulsation or electroporation. However, cyclic voltammetry (CV) measurement of the charge injection capacity of PEDOT electrodes versus iridium oxide (IrOx) and platinum iridium (PtIr) electrodes of similar size has been compared, as shown in Figure 8 below, from [14].

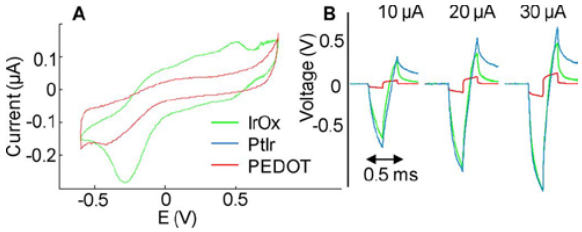

Figure 8: Cyclic voltammetry measurements on PEDOT and IrOx coated electrodes show that IrOx exhibits larger charge storage capacity for the particular parameters used. (b) When subjected to biphasic constant current pulses (10 µA, 200 µs), the same PEDOT coated electrodes exhibit a much smaller voltage transient (48 mV) compared to IrOx (616 mV) and bare PtIr (713 mV) [14].

Whilst IrOx and PtIr have a larger charge storage capacity than PEDOT during these type of slow measurements (typically 10–1000 mV/s), only a fraction of this charge capacity would be available when using sub-millisecond current pulses. When the charge-injection limit was considered (the quantity of charge which polarizes the electrode interface to the potential for water reduction or oxidation), PEDOT-coated electrodes were found to deliver 15 times more charge compared to IrOx and PtIr electrodes without causing electrolysis of water [14].

Our laboratory are now developing PEDOT:PSS microelectrode arrays for studying the performance of this organic electronic material for electropulsation and electroporation. We have recently presented our first devices, designed as interdigitated microelectrode arrays, for in vitro and in vivo studies of electropulsation and electroporation (Figure 9) [15]. Our recent in vitro results using these devices confirm

that PEDOT:PSS microelectrodes can indeed be used for electropulsation and electroporation and there are benefits to conductive polymers; particularly at the µ-scale and when transparency is desirable for imaging.

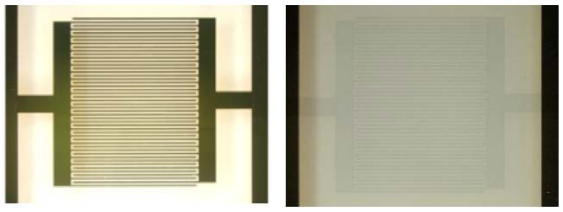

Figure 9: Interdigitated microelectrode arrays made at BEL for electropulsation. Left: Gold electrode coated with PEDOT:PSS, Right: Transparent PEDOT:PSS electrode designed for microscopy.

REFERENCES

[1] Someya T, Bao Z, Malliaras GG. The rise of plastic bioelectronics. *Nature.* 540: 379-385, 2016.

[2] Rivnay J, Inal S, Collins BA, Sessolo M, Stavrinidou E, Strakosas X, Tassone C, Delongchamp DM &. Malliaras GG Structural control of mixed ionic and electronic transport in conducting polymers. Nature Communications. 7: 11287, 2016.

[3] J. A. DeFranco, B. S. Schmidt, M. Lipson, and G. G. Malliaras, Photolithographic patterning of organic electronic materials. Organic Electronics, vol. 7, pp. 22-28, 2006.

[4] Khodagholy D, Rivnay J, Sessolo M, Gurfinkel M, Leleux P, Jimison LH, Stavrinidou E, Herve T, Sanaur S, Owens RM & Malliaras GG. High transconductance organic electrochemical transistors. Nature Communications 4: 2133, 2013.

[5] Koutsouras DA, Gkoupidenis P, Stolz C, Subramanian V, Malliaras GG and Martin DC. Impedance spectroscopy of spun cast and electrochemically deposited PEDOT:PSS films on microfabricated electrodes with various areas. ChemElectroChem, 4(9): 2321-2327, 2017.

[6] Nam Y, Wheeler BC. In vitro microelectrode array technology and neural recordings. Crit Rev Biomed Eng. 39:45-61, 2011.

[7] Fattahi P, Yang G, Kim G, and Abidian MR. A Review of Organic and Inorganic Biomaterials for Neural Interfaces. Advanced Materials. 2014 Mar 26; 26(12): 1846–1885.

[8] Khodagholy D, Doublet T, Gurfinkel M, Quilichini P, Ismailova E, Leleux P, Herve T, Sanaur S, Bernard C, and Malliaras GG. Highly Conformable Conducting Polymer Electrodes for In Vivo Recordings. Advanced Materials, 23(36), H268–H272, 2011.

[9] Williamson A, Ferro M, Leleux P, Ismailova E, Kaszas A, Doublet T, Quilichini P, Rivnay J, Rõzsa B, Katona G, Bernard C, Malliaras GG. Localized Neuron Stimulation with Organic Electrochemical Transistors on Delaminating Depth Probes. Advanced Materials, 27, 4405-4410, 2015.

[10] Pas J, Rutz AL, Quilichini PP, Slézia A, Ghestem A, Kaszas A, Donahue MJ, Curto VF, O'Connor RP, Bernard C, Williamson A, Malliaras GG. A bilayered PVA/PLGA-bioresorbable shuttle to improve the implantation of flexible neural probes. Journal of Neural Engineering. 15(6): 065001, 2018.

[11] Rivnay J, Inal S, Salleo A, Owens RM, Berggren M, Malliaras, GG. Organic electrochemical transistors. Nature Reviews Materials 3: 17086, 2018.

[12] Khodagholy D, Gelinas, JN, Thesen T, Doyle W, Devinsky O, Malliaras GG, Buzsáki G. NeuroGrid: recording action potentials from the surface of the brain. Nat Neurosci. 18(2): 310–315, 2015.

[13] Khodagholy D, Doublet T, Quilichini P, Gurfinkel M, Leleux P, Ghestem A, Ismailova E, Hervé T, Sanaur S, Bernard C, Malliaras GG. In vivo recordings of brain activity using organic transistors. Nat Commun. Mar 12; 4: 1575, 2013.

[14] Venkatraman S, Hendricks J, King ZA, Sereno AJ, Richardson-Burns S, Martin D, Carmena JM. In vitro and in vivo evaluation of PEDOT microelectrodes for neural stimulation and recording. IEEE Trans Neural Syst Rehabil Eng. 2011 Jun;19(3):307-16.

[15] Dijk G, Ruigrok H, O'Connor RP. Flexible Conductive Polymer Microelectrode Arrays for Electropulsation, Neurostimulation and Electroporation In Vitro and In Vivo. BioEM June 29, Portoroz, Slovenia, 2018.

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He is currently Professor of Neurotechnology and Bioelectronics and Head of the Department of Bioelectronics at École des Mines de Saint-Étienne, located at the Centre Microelectronics of Provence campus in Gardanne, France (in the south of France between Aix-en-Provence and Marseille). His current research at BEL focuses on the application of flexible organic electronic materials for interfacing with tissue for sensing and treating disease, including cancer. He is interested the bioelectrical basis of disease and the fusion of Bioelectronics and Bioelectrics approaches to create implantable or wearable electroceutical devices for therapeutics. He is an author of over 40 publications and two book chapters, with an h-index of 17, according to Google scholar (09/2018).

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Treating cancer with electrochemotherapy: cure, palliation, or treatment consolidation?

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Abstract: Treatment of superficial tumors with electrochemotherapy (ECT) has shown a steep rise over the past decade. The Standard Operating Procedures for clinical application were established in 2006, and then updated in 2018. Ease of administration, patient tolerability, efficacy across tumor histotypes, and repeatability are peculiar advantages, which make *standard* ECT (i.e., ECT using fixed-geometry electrodes) an emerging therapy in the field of surgical oncology. Consolidated indications include superficially metastatic melanoma (complete response [CR] rate, 20-50%), breast cancer (CR rate, 40-75%), head and neck skin tumors (CR rate, 48-97%), and Kaposi sarcoma (CR rate, 60-100%). Recently, ECT has been investigated in well-selected patients with oropharyngeal cancers (CR rate, ~19%), in whom it ensured appreciable symptom control. Repeatability and integration with other oncological therapies allow for consolidation of response and sustained tumor control. Beyond the consolidation of standard ECT, the last decade witnessed a rapid incremental development of the technique, and the emergence of new clinical data. First, technical developments have improved ECT equipment, with custom electrode probes and dedicated tools supporting individual treatment planning in anatomically challenging tumors. Second, standard ECT has been explored in new groups of cancer patients, and in some non-cancerous skin lesions. Third, the feasibility and short-term efficacy of ECT has been established in deep-seated tumors, including bone metastases, liver malignancies, and pancreatic and prostate cancers (*long-needle variable electrode-geometry* ECT), and gastrointestinal tumors (*endoscopic* ECT). Pioneering studies indicate lung and brain tumors as suitable future targets. A further advance relates to new combination strategies with immunotherapy, gene electro transfer (GET), calcium EP, and radiotherapy. Finally, cross-institutional collaborative groups such as the International Network for Sharing Practices of ECT (InspECT) have been established to promote high-quality research, refine procedural guidelines, and explore new indications.

INTRODUCTION

Solid tumors present several barriers towards targeted delivery of drugs [1]. If anticancer drugs are unable to access tumor cells, their effectiveness will be hindered. Reversible electroporation (EP) has been developed to achieve transient permeabilization of the cell membrane by means of short electric pulses, thus increasing intracellular uptake of chemotherapy [2]. Using reversible EP, electrochemotherapy (ECT) has been introduced in the field of surgical oncology with the aim to treat superficial tumors not amenable to resection or radiotherapy [3]. After extensive development, the procedure entered the clinic in 2006, when the European Standard Operating Procedures of ECT (ESOPE) were released [4]. Based on ESOPE, the application of ECT has shown a steep rise, mainly in Europe, and large multicenter studies have demonstrated its efficacy, tolerability, and high levels of patient satisfaction. Key aspects favoring its broad acceptance are the simplicity and versatility of the procedure. In fact, *standard* ECT (i.e. ECT applied by means of fixed-geometry electrodes) is based on a flexible technology, suitable for targeting different types of cancers. Interestingly, recent technological advances are opening new avenues for expanding treatment indications. In particular, the development of long, freely placeable needle electrodes has enabled targeting also deep-seated malignancies (*long needle variable electrode-geometry* ECT), while *endoscopic* ECT is gleaming into the clinic with the aim to palliate gastrointestinal tumors. Herein, we provide an overview on shared clinical indications of *standard* ECT, as well as an update on the emerging ECT indications.

ISTANDARDIZATION OF THE TECHNIQUE

The standardization of the procedure represented a landmark in clinical ECT and allowed confirming the results of the ESOPE study on a larger scale. The standard operating procedures have been recently updated and offer guidelines concerning patient selection, chemotherapy, anesthesia, patient preparation, electrode selection, and post-treatment care [5]. Briefly, ECT can be administered according to four treatment modalities, based on type of anaesthesia (local/general) and route of chemotherapy administration (intratumoral/intravenous).

CONSOLIDATED INDICATIONS

ECT is now included in several cancer treatment guidelines. However, timing of application, and positioning within cancer-specific algorithms remain open to debate, due to the heterogeneity of published studies and lack of randomized trials. Nonetheless, the most recent clinical experiences are focusing on more homogeneous populations, are investigating ECT in the real-world context (i.e., as a complementary therapy), and, importantly, are evaluating also patient-reported outcomes. An effort to improve the quality of ECT clinical studies and their reporting has been made through specific recommendations and a dedicated checklist [6].

Melanoma

Due to its propensity to recur on the skin, melanoma represents a leading indication to ECT. With the advent of the new targeted and immune therapies, prolonged survival is being achieved and new patterns of disease have emerged [7]. Thus, ECT is increasingly applied not only as a palliative, but also as a complementary therapy, to enhance the care of patients with superficial metastases. Since 2006 ESOPE guidelines, nine case series have been published. Complete response (CR) rate ranged between 20% and 50% [8]. In well-selected cases, ECT allowed to managing metastases in challenging anatomical locations such as the face, oral cavity, and perianal region. Interestingly, ECT can be combined with surgery, with either a neoadjuvant or adjuvant intent. Moreover, it can represent an effective rescue option to consolidate the response achieved with other treatments (**Fig. 1**). Patient-reported outcomes were investigated in 36 patients by means of a dedicated questionnaire. In the short-term, 34 of them reported a positive impact on wound healing, bleeding, aesthetic impairment, activities of daily living, social relations, or pain [9]. These findings were confirmed in 211 melanoma patients by the EORTC QLQ-C30 questionnaire [10].

Breast cancer

Skin involvement has an overall 24% incidence in the progression of breast cancer and may cause relevant physical and psychologic distress. This condition represents a therapeutic dilemma, particularly in heavily pretreated women. Skin tumor involvement can be the hallmark of either metastatic or locoregional disease (i.e., chest wall recurrence after mastectomy). In both cases, ECT may represent an opportunity to control tumor growth locally and palliate the associated symptoms. Under careful anesthesiological management, the procedure is tolerable by elderly patients; the chance to obtain an effective chest wall control inversely correlates with the spread of skin metastases [11]; ECT is active in previously irradiated skin, although retreatment may be associated with increased pain and local toxicity; finally, ECT can be safely combined with systemic chemotherapy.

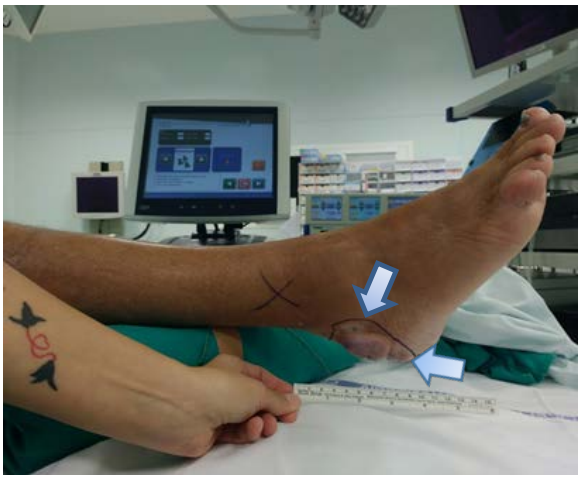

Figure 1: Locoregional metastases from a melanoma of the heel. The patient underwent previous hyperthermic isolated limb perfusion (ILP) with tumor necrosis factor-α and melphalan. Nine months after ILP, he developed a 5-cm subcutaneous recurrence (arrows) and was treated with electrochemotherapy by intravenous bleomycin and a hexagonal needle electrode, under mild general sedation. The procedure was well tolerated and the patient was discharged the same day with the prescription for minor analgesics and basic instructions concerning wound care.

Head and neck cancer

Despite optimal treatment, local recurrence develops in 10-30% of head and neck cancer patients, and second primary tumors develop at a rate of 2-3% per annum. The treatment of these tumors is challenging, especially after previous extensive surgery or radiotherapy, and ECT can represent a low-invasive option with unique advantages in terms of hospitalization, tissue preservation, and costs. From a practical standpoint, treatment of these tumors is technically challenging, due to relevant anatomic constraints, and should be performed at referral centers. A European multicenter study on 43 patients with recurrent mucosal cancers, indicated a 56% overall response rate (CR rate, 19%), with transient mild pain and tissue swelling [12]. Another European study based on 105 patients with skin tumors of various histotypes indicated 62% CR rate, with significant improvement of patient quality of life according to the EQ-5D, EORTC-QLQC30, and EORTC QLQ-H&N35 questionnaires [13].

Non-melanoma skin cancers

Results of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) treatment have been reported in several case series, while clinical

experience in Merkel cell carcinoma, sebaceous carcinoma, and keratoacanthoma is still patchy. Multifocal BCC (e.g. patients with Gorlin-Goltz syndrome or Xeroderma Pigmentosum) may be particularly suited for treatment to reduce the morbidity of repetitive surgical excisions [14], and an increasing number of reports highlight the value of ECT as an alternative option in well-selected patients (**Fig. 2**).

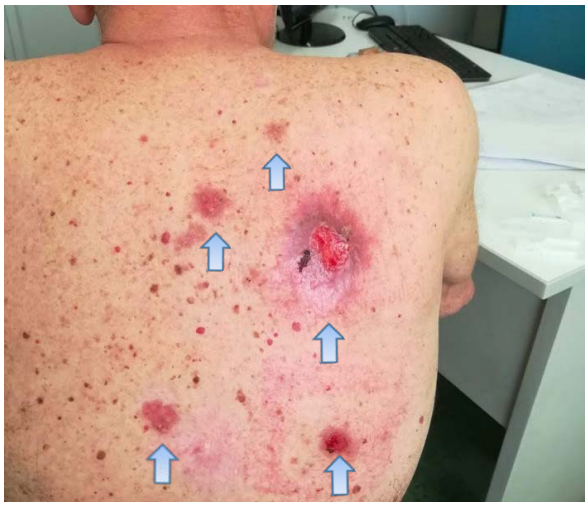

Figure 2: Multifocal basal cell carcinoma (arrows) in an elderly patient with relevant medical comorbidities. Treatment options include extensive surgical resection, Mohs surgery, superficial radiotherapy, targeted therapy, topical immunotherapy, photodynamic therapy. Electrochemotherapy can be offered as an effective tissue-sparing alternative. Treatment parameters (anesthesia, electrode type, route of drug administration) should be selected in order to ensure optimal tolerability of the procedure and provide best treatment outcome.

Superficial soft tissue sarcomas

Clinical experience is still sparse. Small tumor size and superficial location make Kaposi's sarcoma an ideal target for ECT, and its prominent vascularization favors the exposure to chemotherapy, thus allowing exploiting ECT peculiar anti-vascular effect. A 60%-100% CR rate has been reported following a single course of treatment, with possibility to administer further cycles. Mild side effects and prolonged local control are invariably reported across studies. These findings prompted researchers to investigate ECT also in more aggressive histotypes such as angiosarcoma.

NEW INVESTIGATIONAL INDICATIONS

Metastases from visceral or hematologic cancers

Several solid organ and hematologic neoplasms can metastasize to the skin, and the occurrence of superficial metastases is 0.7-10%. Standard ECT has been investigated in patients with head and neck, lung, gastric, bladder, kidney, thyroid cancer, and cutaneous lymphomas. However, since the evidence relies on isolated reports, the clinical benefit should be carefully evaluated on an individual basis, considering patient prognosis, expected results, and eventual side effects.

Gynecologic cancers

Vulvar cancer comprises 5% of gynecologic malignancies, and one-third of patients presents with locally advanced disease and debilitating symptoms. In the first clinical experience, nine elderly women with recurrent disease were treated with no relevant side effects. The CR rate was 62.5%, with a significant reduction of pain, bleeding, odor, and urinary discomfort compared with baseline assessment. In a phase-II study ECT was investigated in 25 women (median tumor size 4.5 cm^2). Only minimal blood loss and transient tissue edema were noted, and all patients were discharged within 24 hours. After 1 month, 13 patients (52%) achieved CR and 7 (28%) achieved partial response. The 6-month local progression-free survival and symptom-free survival were 53% and 40%, respectively [15].

Non-cancerous skin lesions

Keloid scars are benign fibroproliferative lesions with a propensity for the earlobe, and the pre-sternal and deltoid regions. Because intratumoral (i.t.) BLM is a well-documented agent in keloid treatment, its association with EP represents a rational approach. Capillary vascular malformations, instead, consist of abnormal blood vessels, which may vary greatly in size and EP is currently investigated to promote bleomycin uptake and achieve their minimally-invasive clearance.

Liver tumors

The introduction of long freely placeable needle electrodes and a new pulse generator, which allow customizing the applied voltages according to the distance between electrodes (*variable electrode-geometry* ECT) has allowed to investigate ECT in patients with liver malignancies (metastases from colorectal cancer, hepatocellular carcinoma, colangiocarcinoma) [16]. Overall, these experiences support the feasibility of ECT in patients with liver malignancies, with no major safety concerns. On these bases, ECT can be envisioned a promising tool in interventional oncology for treating hepatic tumors that are unresectable or in proximity to vessels.

Bone tumors

In the first clinical experience, a patient with metastatic spinal melanoma was treated using four long needle probes inserted at L5 level, through a mini-open surgery. Their placement was monitored through fluoroscopy and neuronavigation control, under

general anesthesia. Positron-emission tomography-computed tomography (PET-CT) assessment indicated a near CR, which lasted 6 months and was associated with significant improvement of pain. Between July 2009 and June 2017, 55 patients underwent ECT, with the first 29 in the frame of a phase-II study. The patients had metastases of the pelvis or appendicular skeleton smaller than 6 cm, and no associated fracture. The procedure was tolerable, and no intra- or early post-operative complications were reported. After a mean 7-month follow-up, 20 (84%) of the 24 evaluable patients indicated a 50% or greater decrease in bone pain [17].

Other investigational indications

The introduction of *variable electrode-geometry* ECT has allowed targeting large and deep-seated soft tissue tumors, prostate and pancreatic cancer and, in theory, also lung and brain tumors.

Gastrointestinal cancers

The newly available customized electrodes provide excellent maneuverability in confined anatomical spaces and make gastrointestinal cancers a suitable target for ECT. In particular, the patients with inoperable esophageal or rectal cancer may benefit from treatment with *endoscopic* ECT. The first in-human phase-I study has been conducted in Denmark in six patients with advanced esophageal carcinoma [18]. Treatment was performed under general anesthesia, and an electrocardiogram triggering monitor was used to prevent cardiac arrhythmias. The duration of the procedure varied from 24 to 59 minutes. There were no major safety issues, and an endoscopic visual response was reported in all cases (confirmed by PET-CT in four patients). Ongoing studies will clarify the safety and feasibility of this approach.

Combined approaches

Immunotherapy, gene electro transfer (GET), calcium EP, and radiotherapy provide exciting opportunities for innovative therapeutic strategies. A further approach combining irreversible EP (IRE) on the target tumor and simultaneous administration of chemotherapy, which can take advantage of the reversible EP effect around the tumor, has also been postulated. At present, and following the advent of new immunotherapies, research efforts are focused on converting the local effect of ECT into a systemic one. Previous studies reported that ECT-mediated tumor regression is dramatically impaired in immunodeficient mice. Moreover, preclinical and clinical experiences, particularly in melanoma patients, have characterized the local immune infiltrate at the ECT site. In addition, EP can induce an immunogenic cell death through the liberation of molecules, which act as damage-associated molecular patterns (DAMP) signals towards the immune system. Preliminary experiences in melanoma patients support the feasibility and safety of this combined approach [19] and prompt for further investigation.

CONCLUSIONS

ECT is a flexible skin-directed therapy, which relies on a multi-faceted mechanism of action. Consolidated indications include a range of superficial cancers, which can be effectively controlled, thus ensuring preservation of patients' quality of life. Since low tumor burden and limited disease spread are invariably associated with better outcomes, moving ECT application earlier in the course of the disease will be a crucial. Noteworthy, the number of cancers potentially amenable to ECT has expanded. The widespread use of *standard* ECT and the introduction of *variable electrode-geometry* and *endoscopic* ECT, together with the new combination strategies, has enlarged the number of patients who might benefit from this therapy. Based on available evidence, it is conceivable that thoughtful incorporation of ECT into standard health-care setting will improve the treatment of several patients with cancer. For ECT to be consolidated at the level of care, specific training of clinical staff (**Fig. 3**) and continuous improvement of research evidence are warranted [6].

Figure 3. European Society of Surgical Oncology (ESSO) Course on Electrochemotherapy of Cutaneous and Deep Seated Tumors (Ljubljana, 22-23 October 2018). Live session on electrochemotherapy in liver metastases.

REFERENCES

[1] Dewhirst M.W, Secomb T.W. Transport of drugs from blood vessels to tumour tissue. *Nat Rev Cancer,* 2017. 17(12):738-750.

[2] Rems L., Miklavcic D. Tutorial: Electroporation of cells in complex materials and tissue. *Journal of Applied Physics*, 2016. 119 (20).

[3] Sersa, G., et al., Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol*, 2008. 34(2):232-40.
[4] Mir, L.M., et al., Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator by means of invasive or non-invasive electrodes. EJC Supplements, 2006. 4(11):14-25.
[5] Gehl, J., et al., Updated standard operating procedures for electrochemotherapy of cutaneous tumours and skin metastases. Acta Oncol, 2018:1-9.
[6] Campana, L.G., et al., Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. *Radiol Oncol*, 2016. 50(1):1-13.
[7] Valpione, S., et al., Consolidation electrochemotherapy with bleomycin in metastatic melanoma during treatment with dabrafenib. *Radiol Oncol,* 2015. 49(1):71-4.
[8] Campana L.G., et al., Electrochemotherapy for disseminated superficial metastases from malignant melanoma. *Br J Surg*, 2012. 99(6):821-30.
[9] Campana L.G., et al., Bleomycin-based electrochemotherapy: clinical outcome from a single institution's experience with 52 patients. *Ann Surg Oncol*, 2009. 16(1):191-9.
[10] Campana L.G., et al., Treatment efficacy with electrochemotherapy: A multi-institutional prospective observational study on 376 patients with superficial tumors. *Eur J Surg Oncol*, 2016. 42(12):1914-1923.
[11] Campana L.G., et al., The activity and safety of electrochemotherapy in persistent chest wall recurrence from breast cancer after mastectomy: a phase-II study. *Breast Cancer Res Treat*, 2012. 134(3): 1169-78
[12] Plaschke C.C., et al., European Research on Electrochemotherapy in Head and Neck Cancer (EURECA) project: Results from the treatment of mucosal cancers. *Eur J Cancer,* 2017. 87:172-181.
[13] Bertino G., et al., European Research on Electrochemotherapy in Head and Neck Cancer (EURECA) project: Results of the treatment of skin cancer. *Eur J Cancer*, 2016. 63:41-52.
[14] Campana L.G., et al., Basal cell carcinoma: 10-year experience with electrochemotherapy. *J Transl Med,* 2017. 15(1): 122.
[15] Perrone A.M., et al., Palliative electro-chemotherapy in elderly patients with vulvar cancer: A phase II trial. *J Surg Oncol,* 2015. 112(5):529-32.
[16] Edhemovic, I., et al., Intraoperative electrochemotherapy of colorectal liver metastases. *J Surg Oncol*, 2014. 110(3):320-7.
[17] Bianchi G., et al., Electrochemotherapy in the Treatment of Bone Metastases: A Phase II Trial. *World J Surg,* 2016. 40(12):3088-3094.
[18] Egeland C., et al., Endoscopic electrochemotherapy for esophageal cancer: a phase I clinical study. *Endosc Int Open*, 2018. 6(6): E727-E734.
[19] Heppt M.V., et al., Immune checkpoint blockade with concurrent electrochemotherapy in advanced melanoma: a retrospective multicenter analysis. *Cancer Immunol Immunother*, 2016. 65(8):951-9.

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NOTES

NOTES

SHORT PRESENTATIONS

Pulsed Electric Field Treatment of *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, Bacteria with Probiotic Potential

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INTRODUCTION

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host, as accepted by FAO/WHO. Some of probiotic bacteria are also high lactic acid producing strains and used in functional food production and biorefinery processes [1]. Application of pulsed electric field (PEF) treatment in food industry is mainly related to the inactivation of undesired microorganisms in foodstuff and partially for improvement of other processes like drying, cutting, extraction etc. [2]. In this work, the possibility to change probiotic profile of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* by PEF treatment while preserving viability was examined.

METHODS

Continuous PEF treatment experiments were performed in flow through chamber with build-in electrodes (d=2 mm) [2]. *Lactobacillus rhamnosus* ATCC 7469 and *Lactobacillus paracasei* NRRL B-4564 were collected in exponential growth phase in MRS media by centrifugation (4248×g, 30min, 4 °C) and suspended in sterile distilled water to attain viable cell number of around 5×10^7 CFU/ml. After the treatments, viability was determined using pour plate counting method.

Membrane permeabilization was evaluated by means of propidium iodide after the PEF treatment of bacterial cells [3]. Effect of PEF treatment on lactic acid production and susceptibility of these bacteria to antibiotics was also studied [1].

RESULTS

The permeabilization and viability of studied probiotics after different PEF treatments are presented in Fig.1. *L. rhamnosus* is more susceptible to PEF treatment, being permeabilized and inactivated at lower field strengths than *L. paracasei*. In terms of shape and size, both bacteria are rod-shaped but on average *L. rhamnosus* cells are slightly larger which is in accordance to general observation that larger cells are more easily permeabilized by PEF [4]. Kinetics of lactic acid fermentation is the same or even slightly increased for *L. rhamnosus* in comparison to control after 24h. Only susceptibility to chloramphenicol was significantly increased for treated *L. rhamnosus*, while treated *L. paracasei* was more susceptible to chloramphenicol, penicillin, erythromycin and tetracycline.

CONCLUSIONS

L. rhamnosus has shown higher susceptibility to PEF treatment with slight increase in overall lactic acid production in comparison to control. Susceptibility to antibiotics of *L. paracasei* was more affected by PEF treatment than *L. rhamnosus*, suggesting other mechanism not directly linked to permeabilization.

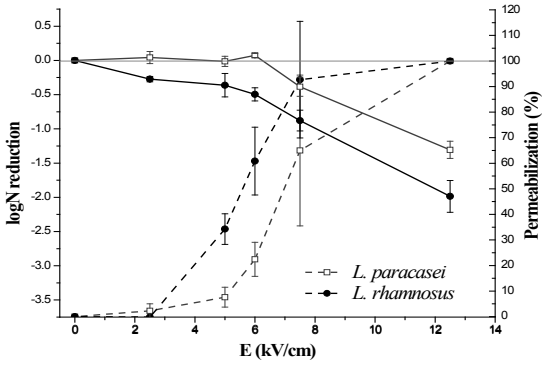

Figure 1: Permeabilization and inactivation curve for *L. rhamnosus* and *L. paracasei* by PEF treatment.

ACKNOWLEDGEMENTS

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REFERENCES

[1] A. P. Djukić-Vuković, L. V. Mojović, V. V. Semenčenko, M. M. Radosavljević, J. D. Pejin, and S. D. Kocić-Tanackov, "Effective valorisation of distillery stillage by integrated production of lactic acid and high quality feed," *Food Res. Int.*, vol. 73, pp. 75–80, 2015.

[2] K. Flisar, S. H. Meglic, J. Morelj, J. Golob, and D. Miklavcic, "Testing a prototype pulse generator for a continuous flow system and its use for E. coli inactivation and microalgae lipid extraction," *Bioelectrochemistry*, vol. 100, pp. 44–51, Dec. 2014.

[3] S. Haberl-Meglič, E. Levičnik, E. Luengo, J. Raso, and D. Miklavčič, "The effect of temperature and bacterial growth phase on protein extraction by means of electroporation," 2016.

[4] M. Coustets, V. Ganeva, B. Galutzov, and J. Teissie, "Millisecond duration pulses for flow-through electro-induced protein extraction from E. coli and associated eradication," *Bioelectrochemistry*, vol. 103, pp. 82–91, 2015.

Optimization of pulsed electric field assisted extraction of bioactive compounds from blackcurrant

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INTRODUCTION

In the last decades an increasing interest in natural foods with high antioxidant activity has been observed worldwide. Blackcurrant is a berry grown in Argentinian Patagonia which is recognized for being a very rich source of bioactive compounds, such as anthocyanins and other polyphenols, with inmunomodulatory, antiinflamatory and anticancer properties [1]. However, these components are very difficult to extract from the fruit matrix. For this purpose, pulsed electric field technology offers a "green", economically viable, and nonthermal solution to assist the extraction process without destroying the bioactive compounds.

The objective of this work was to optimize pulsed electric field (PEF) pre-treatment to enhance the extraction of bioactive compounds from blackcurrant by cold pressing.

MATERIAL AND METHODS

IQF blackcurrants (*Ribes nigrum*, Titania cultivar) were thawed under controlled conditions. For PEF treatment a custom electroporation chamber was constructed, consisting of an acrylic cuvette with two confronted rows of 22 needles each, separated by 80mm.

Response surface methodology was used to determine the optimal PEF conditions using the desirability approach. The independent variables analysed were the electric field intensity (0 to 1950 V/cm, determined by a high-voltage probe (100x)) and the number of pulses (of constant duration; 50 to 500 pulses). The effect of these parameters over the total polyphenolic content (TPC) (Folin–Ciocalteu method [2]) and the antioxidant activity (AA) (bleaching method of radical cation (ABTS+.) [3]) was studied with the objective of maximizing both responses. A factorial design of 4x3 levels was used and a complete replicate of the whole design was conducted. Statgraphics Centurion XVI.I software was used for response optimization.

RESULTS AND DISCUSSION

Both the electric field intensity and the number of pulses showed a positive effect over the response variables though, the first one was more influencing than the latter one. The AA increased when the electric field increased up to 1300 V/cm but a further increase to 1950 V/cm caused a decrease of the response (Figure 1). Other authors have reported that high levels of energy may cause degradation of bioactive compounds in food [4].

The response surface is shown in Figure 2. The desirability function reached a maximum value of 0.804, indicating that a good maximization for both responses was achieved. The optimal treatment conditions determined by the system were an electric field of 1256 V/cm and 319 pulses which would result in TPC and AA values of 3.8 and 1.8 mg GA/g. The predicting model was tested applying the optimal treatment to a new set of samples reaching average values of 3.8 ± 0.2 and 1.88 ± 0.06 mg GA/g for TPC y AA respectively.

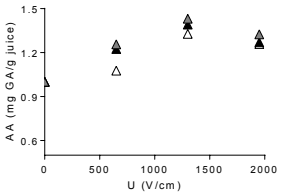

Figure 1: AA as a function of the electric field for: 50 (white), 275 (grey) and 500 (black) pulses.

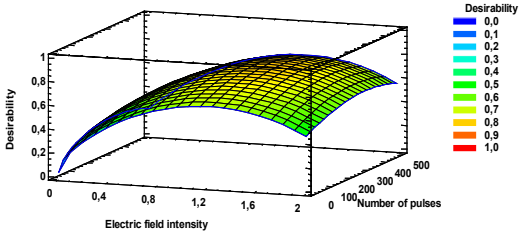

Figure 2: Desirability response surface as a function of electric field values and number of pulses.

ACKNOLEDGEMENTS

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REFERENCES

[1] Gopalan, A., Reuben, S. C., Ahmed, S., Darvesh, A. S., Hohmann, J. & Bishayee, A. (2012). "The health benefits of blackcurrants". *Food & Function*, 3, 795.

[2] Archaina, D., Leiva, G., Salvatori, D. & Schebor, C. (2018). "Physical and functional properties of spray-dried powders from blackcurrant juice and extracts obtained from the waste of juice processing". *Food Science and Technology International*, 24(1).

[3] Cayupán C., Ochoa Y. S. & Nazareno M. J. (2011). "Health-promoting substances and antioxidant properties of Opuntia sp. fruits. Changes in bioactive-compound contents during ripening process". *Food Chemstry*, 126(2), 514–519.

[4] Bobinaitė, R., Pataro, G., Lamanauskas, N., Šatkauskas, S., Viškelis, P., & Ferrari, G. (2015). "Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products". *Journal of Food Science and Technology*, 52(9), 5898–5905.

Clinical Electroporators – Standards and Certification within EU

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INTRODUCTION

For successful implementation of electroporation we need an electronic device – electroporator and electrodes. Electroporators are able to generate high voltage pulses of specific shape, different amplitudes, number of pulses, duration and repetition frequency. Generated pulses create an electric field around the electrodes which intensity is controlled by the pulse amplitude. Depending on the biotechnological or biomedical applications, electroporators are divided in several groups of which clinical electroporators are medical devices used for medical treatment in clinics [1]. Currently there is a lack of suitable clinical electroporators on the European market due to the limited approval and strict undergo procedure for their certification.

In this work we present the most significant requirements and standards that have to be considered as well as the necessary documentation which has to be prepared for the certification of a clinical electroporator.

MEDICAL DEVICE REGULATION 2017/745

Medical Device Regulation 2017/745 (MDR) [2] is composed of 17 Annexes and it is intended to harmonize the laws related to medical devices within the European Union. Each manufacturer of medical devices must submit technical documentation providing evidence of conformity with the Regulation in order to obtain certification mark (CE) for EU. During the development and manufacturing phase of the clinical electroporator it is important to be acquainted with the MDR by studying the technical documentation needed to obtain a CE mark, setting the general requirements regarding design and construction, providing the necessary standards and presenting relevant evidence that all the requirements are met by standards. For all medical devices, MDR provides detailed instruction on the minimum content and the necessary elements to be included in the technical documentation which is clearly stated in Annexes II and III of the MDR (Table 1).

STANDARDS

Within technical documentation, manufactures must present suitable evidence to show that the device fulfills the requirements detailed in Annex I in consideration with the related standards. On the beginning of the process, quality management system has to be implemented in compliance with the standard EN ISO 13485:2016. Emphasis is put on the safety of the patients due to the high voltage pulse generator that can be potentially hazardous for patients as well as for operators. In that purpose, clinical electroporators are obligated to meet the standard for risk analysis EN ISO 14971:2012 and prove that the benefits for the patient always outweigh the risks as well as the general standard for basic safety and essential performance of medical devices EN 60601-1:2006. Clinical electroporators must also meet collateral standards such as EN 60601- 1- 2:2015 for electromagnetic disturbances and EN 60601-1-6:2010 for usability, which has to be followed by EN ISO 62366:2008. Since clinical electroporators are programmable medical devices, the standard for medical device software, EN ISO 62304:2006 should be considered.

Table 1: Content of the Technical Documentation.

	(a) Annex II – Technical Documentation:
1.	Device description and specification, including variants and accessories
	1.1 Device description and specification
	1.2 Reference to previous and similar generations of the device
2.	Information to be supplied by the manufacturer
3.	Design and manufacturing information
4.	General safety and performance requirements
5.	Benefit – Risk analysis and risk management
6.	Product verification and validation
	6.1 Pre-clinical and clinical data
	6.2 Additional information required in specific cases
	(b) Annex III – Technical Documentation on Post-Market Surveillance:
1.	The Post-Market Surveillance Plan
2.	Periodic Safety Update Report
3.	Post-Market Surveillance Report

CONCLUSIONS

It is a manufacturer's responsibility to carry out the conformity assessment, prepare the required technical documentation to include the elements set out in Annexes II and III, continuously ensure that the technical documentation is always up to date, take into consideration all the above listed standards and issue the EU declaration of conformity. Only then the CE mark can be affixed.

ACKNOWLEGEMENTS

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REFERENCES

[1] M. Rebersek, D. Miklavcic, C. Bertacchini, and M. Sack, "Cell membrane electroporation - Part 3: the equipment," *IEEE Electr. Insul. Mag.*, 2014.

[2] "REGULATION (EU) 2017/745 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL - of 5 April 2017 - on medical devices, amending Directive 2001/ 83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC".

Effects of Interphase Delay and Delay between Bipolar Pulses on Permeabilization and Survival of CHO Cells After Electroporation

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INTRODUCTION

Numerous studies have focused on optimization of electroporation conditions. Of the various parameters, the impact of the pulse repetition rate (PRR) is one of the least understood. Previous studies reported electroporation-induced cell sensitization [1].

Recently, bursts of bipolar square wave pulses have attracted attention since they have shown reduced muscle contractions compared to monopolar treatments [2]. However, it was also reported (for nanosecond electric pulses) that the opposite polarity phase cancels the effects of the first if the delay between them is short enough [3].

The scope of this work was to investigate the effect of interphase delay and delay between bipolar pulses of bipolar square pulse treatments on the permeability and survival of CHO-K1 cells.

METHODS

We used two types of bipolar square pulse treatments. For both types, the length was 1 µs for the positive and 1 µs for the negative phase. For pulse type 1, the interphase delay was fixed at 1 µs and the delay between bipolar pulses was set to 0.5, 10, 100, 1000 or 10 000 µs. For pulse type 2, the interphase delay and delay between bipolar pulses were of equal duration: 0.5, 10, 100, 1000 or 10 000 µs. We treated the cells with 1 burst of 400 pulses with both type 1 and type 2 pulses.

Permeability was determined by flow cytometry as percentage of cells that fluoresce propidium iodide 3 min after pulse treatment at 1500 V/cm.

Cell survival was evaluated 24 h after pulse treatment at 2500 V/cm with MTS assay.

RESULTS

The survival of CHO cells decreased when increasing the interphase and/or delay between bipolar pulses. We noticed significantly lower survival when cells were treated with pulses of type 2 of longer delays compared to type 1.

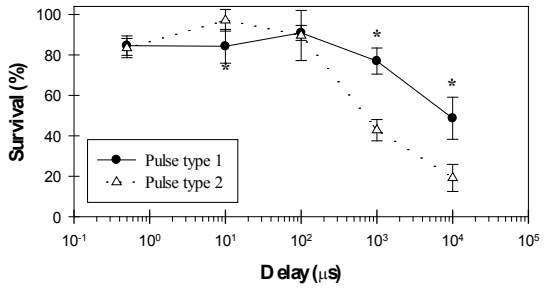

Figure 1: Survival of CHO cells 24 h after treatment with bipolar pulses (E = 2500 V/cm) with different delays.

The permeability increased with increasing the delay between bipolar pulses in the case of pulses of type 1. For type 2, it increased until a certain point and then started decreasing.

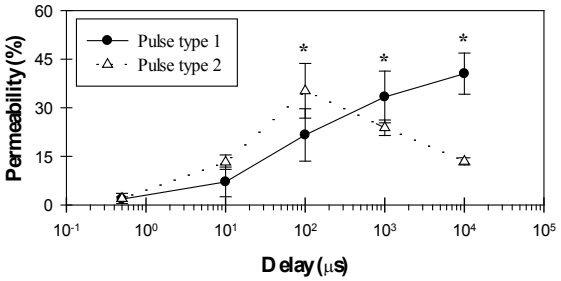

Figure 2: Permeability of CHO cells 3 min after treatment with bipolar pulses (E = 1500 V/cm) with different delays.

CONCLUSIONS

Higher survival in the case of type 1 pulses is in line with the previously reported cancellation effect of the first phase by the second. The survival decreases for both pulse types with increasing the delay between bipolar pulses, which is in accordance with observations from literature that lower PRR are more effective than high ones.

To explain the bell-shaped trend of permeability values of cells treated with pulse type 2, further studies are needed.

ACKNOWLEDGEMENTS

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REFERENCES

[1] O.N. Pakhomova, B.W. Gregory, V.A. Khorokhorina, A.M. Bowman, S. Xiao, and A.G. Pakhomov, "Electroporation-induced electrosensitization," *PLoS One*, vol. 6, pp. 36–38, 2011.

[2] C.B. Arena, M.B. Sano, J.H.J. Rossmeisl, J.L. Caldwell, P.A. Garcia, M.N. Rylander and R.F. Davalos, "High-frequency irreversible electroporation (H-FIRE) for non-thermal ablation without muscle contraction," *Biomed. Eng. Online*, vol. 10, pp. 102, 2011.

[3] A.G. Pakhomov, I. Semenov, S. Xiao, O.N. Pakhomova, B.W. Gregory, K.H. Schoenbach, J.S. Ullery, H.T. Beier, S.R. Rajulapati and B.L. Ibey, "Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity," *Cell. Mol. Life Sci.*, vol. 71, pp. 4431–4441, 2014.

Preliminary results on the *in vitro* comparison between Irreversible Electroporation and the combination of Electroporation and Electrolysis (E2)

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INTRODUCTION

The combination of electroporation with electrolysis (E2) is a novel, non-thermal tissue ablation modality. Compared to Irreversible Electroporation (IRE), E2 affords tissue ablation with lower voltages, lower energy and shorter procedure times. The E2 technology was developed from basic concept in small animal studies to large animal studies [1-5]. This study was performed to obtain initial results comparing the two ablation techniques *in vitro* and to test the set-up for future experiments to quantify the ablation efficiency of E2.

MATERIALS AND METHODS

HEK-293 cells were cultivated in DMEM + 10% FBS. For cell suspension preparation, cells were washed once with DPBS, removed from cell culture plates using 0.25% trypsin-EDTA solution and centrifuged at 1,500rpm for 5min at room temperature. The cell pellet was resuspended in DPBS to a final concentration of 2.5 million cells per ml.

150µL of cell suspension was pipetted into a custom made cuvette (polycarbonate) with stainless steel electrodes and treated with either E2 or IRE. Treatment parameters are summarized in Table 1. Experiments were carried out with a custom-made generator that is able to apply both types of treatments. Immediately after treatment application, the electrodes were gently pulled out and cell solution was left in the cuvette for 30min. The cells were seeded on 24-well plates with 700µL of media and incubated for 24h before vitality measurements the following day.

The applied currents and voltages were measured and both the total charge as well as the energy delivered was calculated retrospectively.

Table 1: Applied treatment parameters. E2: exponential decay waveform with additional 60V pre-pulse, mean time constant 9.3 ± 0.3ms. Indicated E-fields are peak values. IRE: 100µs pulse length, 1kHz, delivered in trains of 10p.

Modality	E-field /V/cm	# Pulses	Charge /C/cm
E2	625	2	0,0153
E2	750	2	0,0164
E2	800	2	0,0205
IRE	625	220	0,0165
IRE	750	300	0,0240
IRE	800	320	0,0288

RESULTS

The total energy applied for IRE applications was between 8-18J, while for the E2 applications it ranged from 2-3.4J.

Cell viability showed comparable results between the two treatment modalities at 625V/cm, with a viability of 2% for E2 and 6.6% for IRE relative to sham-treated cells. Application at 800V/cm shows similar results, with cell viability of 8.6% for E2 versus 4.7% for IRE. Figure 1 shows the results for the application at 750V/cm: Cell count appears similar between the treatment modalities, and both show significant decrease of cell concentration compared to control, though statistical analysis reveals that cell vitalities are significantly different (t-test p=0.0313).

Figure 1: Trypan-blue stained cell solutions, which were treated at 750 V/cm and measured the following day. **A.** E2 treatment with Q(E2) = 0.0164C/cm **B.** IRE treatment with Q(IRE) = 0.0240C/cm **C.** sham-treated cells which underwent the same procedure but were not exposed to electroporation-based therapies.

CONCLUSION

These preliminary results indicate that E2 and IRE kill cells comparably in the range of 625-800V/cm with the given parameters. Further studies at different applied voltages are necessary to investigate treatment thresholds and ablation efficiency of E2 *in vitro*.

REFERENCES

[1] N. Klein, E. Guenther, P. Mikus, M. Stehling & B. Rubinsky. „Single exponential decay waveform; a synergistic combination of electroporation and electrolysis (E2) for tissue ablation.” *PeerJ* 5, e3190, 2017.

[2] L. Rubinsky, E. Guenther, P. Mikus, M. Stehling & B. Rubinsky. „Electrolytic Effects During Tissue Ablation by Electroporation.” *Technol. Cancer Res. Treat.* 15, NP95-NP103, 2016.

[3] M. Stehling, E. Guenther, P. Mikus, N. Klein, L. Rubinsky & B. Rubinsky. „Synergistic combination of electrolysis and electroporation for tissue ablation.” *Plos one*, 11(2), e0148317, 2016.

[4] M. Phillips, R. Krishnan & B. Rubinsky. “Tissue Ablation by a Synergistic Combination of Electroporation and Electrolysis Delivered by a Single Pulse.” *Ann. Biomed. Eng.* 44, 3144–3154, 2016.

PEDOT:PSS : A Conductive Polymer for Electropulsation, Neurostimulation and Electroporation

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INTRODUCTION

Organic conductive polymers are highly versatile materials that have been widely adopted in commercial electronics as display technology, transistors, LEDs and solar cells. Our research is exploring their use for interfacing biology with electronics [1]. We have developed microelectrode array devices using PEDOT:PSS for electropulsation, neurostimulation and electroporation investigations.

METHODS

Stimulation electrodes are fabricated using standard photolithography processes [2]. The electrodes consist of arrays of interdigitated fingers that act as electrodes to generate an electric field.

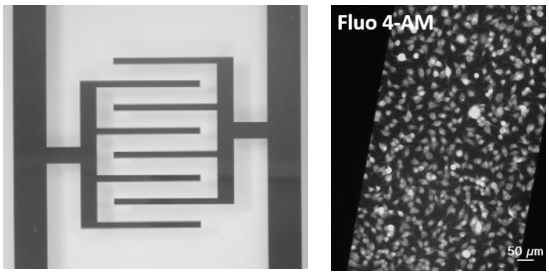

Figure 1: Interdigitated stimulation electrodes (left) and the calcium response of U-87 cells (right).

The difference between gold only electrodes and gold electrodes coated with PEDOT:PSS is verified in vitro with human glioblastoma cells U-87. Cells were loaded with fluo4-AM and propidium iodide for real-time live-dead staining that reveals cellular calcium responses (Figure 1). Monophasic pulses of 100 µs at 30 V were applied with a distance of 500 µm between the electrode fingers.

RESULTS AND FIGURES

Key aspect of this work is the demonstration of improved stimulation capabilities when metallic electrodes are coated with PEDOT:PSS. Figure 2 shows the temporal cytosolic calcium responses for a gold electrode and a gold electrode coated with PEDOT:PSS. 8x100 µs pulses were applied at 1Hz and 30V. By tuning the voltage it is possible to target for different stimulation effects; low voltages stimulate the voltage-gated ion channels whereas higher voltage will results in electroporation of the cell membrane. The stimulation response of the coated electrodes is about 3 times higher than the gold only electrodes which shows that PEDOT:PSS enhances stimulation.

This is explained by the unique coupling of the material with the biological environment. When a coated electrode is placed in an aqueous solution, ions are allowed to enter the polymer which effectively increases the capacitance of the electrode. A larger capacitance means a higher charge injection capacity which results in a higher response.

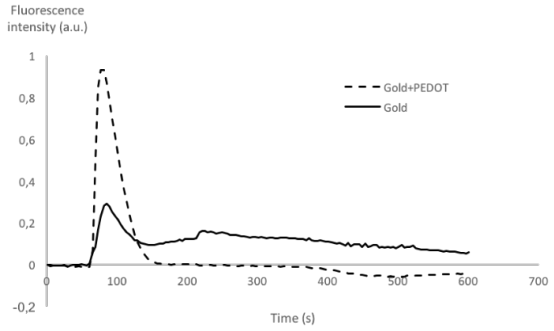

Figure 2: Calcium response of U-87 cells for gold and gold coated with PEDOT:PSS electrodes.

CONCLUSION AND PERSPECTIVES

Coating metallic electrodes with the conductive polymer PEDOT:PSS enhances their stimulation performance. Moreover, PEDOT:PSS is soft, flexible and biocompatible which makes this material suitable for applications in implantable medical devices.

At the moment we're studying PEDOT:PSS electrodes only. These electrodes are optically transparent and therefor of interest when combined with imaging techniques.

Future work will focus on the translation of the current work to in vivo experiments.

ACKNOWLEDGEMENTS

This research is funded by Panaxium and was conducted in the scope of the electroporation in Biology and Medicine (EBAM) European Associated Laboratory (LEA).

REFERENCES

[1] T. Someya, et. al., 'The rise of plastic bioelectronics', *Nature*, 2016, 540, 379-385.

[2] M. Sessolo, et. al., 'Easy-to-Fabricate Conducting Polymer Microelectrode Arrays', *Adv. Mater*, 2013, 25 (15), 2135-2139.

Exhausting and killing glioblastoma by electropulsation

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INTRODUCTION

Glioblastoma is the most common brain cancer and has still a poor prognosis despite the development of innovative new therapies.

Human glioblastoma U87 cell line is characterized by the expression of voltage-gated ion channels at the plasma membrane which makes these cells sensitive to external electric fields and hence excitable [1]. Like neurons, the cell membrane of U87 cells can therefore be depolarized, however, their repolarization time is much longer (min vs ms) and significantly consumes ATP [2].

Taking advantage of the biological and electrical properties of U87 cells, we want to repetitively stimulate U87 cells with electrical pulses in order to deplete cellular ATP storage and trigger cell death by apoptosis. This technique called electropulsation is a fundamental different phenomenon than electroporation as it does not electroporate the cell plasma membrane but activates the voltage-gated ion channels. Electropulsation will be performed using PEDOT:PSS covered gold interdigitated electrodes. PEDOT:PSS is a biocompatible, conductive polymer with unique properties that facilitates a good interface between cells and electronics and improves stimulation response [3].

WORK IN PROGRESS AND PERSPECTIVES

We first need to determine the parameters (cell stimulation threshold, pulse length, number of pulses) for electropulsation. This work is in progress. Then, we can determine the parameters required to trigger a depletion of the ATP storage in the cell. Burke et al. [2] already showed that the U87 membrane repolarization occurred within 15 to 20 min. A defined number of pulses every 5 to 10 min will have an important impact on the cellular ATP. The evolution of ATP will be followed in real-time using the PercevalHR ATP fluorescent biosensor which senses the changes in the ATP:ADP ratio [4].

If glioblastoma U87 cells will be sensitive to electropulsations, non-excitable healthy cells will not. Healthy excitable cells like neurons will be stimulated but their membranes are used to be depolarized and the treatment will have no repercussion on the cell physiology.

These in vitro data will be essential for setting up in vivo protocols for killing glioblastoma selectively and, in the future, also other types of cancers.

ACKNOWLEDGEMENTS

We thank Panaxium and EDF grant ATPulseGliome for the financial support.

REFERENCES

[1] T. Ducret, A-M. Vacher and P. Vacher, "Voltage-dependent ionic conductances in the human malignant astrocytoma cell line U87-MG", Molecular Membrane Biology, vol. 20, pp. 329-343, 2003.

[2] R. C. Burke, S. M. Bardet, L. Carr, S. Romanenko, D. Arnaud-Cormos, P. Leveque and R. P. O'Connor, "Nanosecond pulsed electric fields depolarize transmembrane potential via voltage-gated K^+, Ca^{2+} and TRPM8 channels in U87 glioblastoma cells", Biochimica et Biophysica Acta, vol. 1859, pp. 2040-2050, 2017.

[3] J. Rivnay, R. M. Owens and G. G. Malliaras, "The Rise of Organic Bioelectronics", Chemistry of Materials, vol. 26, pp. 679-685, 2014.

[4] M. Tantama, J. R. Martinez-François, R. Mongeon and G. Yellen, "Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio", Nature Communication, vol. 4, article 2550, 2013.

Investigating the activation of voltage gated calcium channels by uni- and bipolar nanosecond electric pulse stimulation

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INTRODUCTION

Pulsed electric field (PEF) damages cell membrane and initiates multiple cellular effects such as activation of cell death pathways, cell shrinkage, swelling, etc. [1,2]. As the nanosecond (ns) pulses create defects in the cell plasma membrane, ion channels located there likely become one of the targets [3,4]. In this work, we studied the response of voltage-gated calcium channels (VGCC) to 300 ns uni- and bipolar nsPEF stimuli**.**

METHODS AND MATERIALS:

We assembled Cav1.3 L-type channel by transfection in Human Embryonic Kidney cells (HEK293) with pDNAs coding for alpha, beta, and alpha-delta channel subunits. Also, we used pDNA coding for fluorescent protein mCherry, which served as an indicator for the successful transfection. 48 hrs before experiment the cells were seeded on laminin-coated coverslips. For experiments, they were transferred onto the stage of a confocal microscope. Ca^{2+} entry was assessed by emission of Fluo-4, a fluorescent indicator for Ca2+. The cells containing active channels (VGCC+) were identified by Ca^{2+} entry after membrane depolarization with a buffer containing 145 mM KCL.

nsPEF stimulation was done using a pair of tungsten electrodes separated by 150-250 microns. The electrodes were placed above and at the sides of a mixed group of VGCC+ and VGCC- cells. We performed time-lapse recording for 100 s with the pulse delivered at 15 sec. We tested both uni- and bipolar 300 ns pulses at 1.4, 1.8 and 2.3 kV/cm. The second phase always had half of the amplitude of the first one and was delivered right after first phase with no gap between them. In parallel experiments, calcium currents and membrane conductance were measured by a patch-clamp method.

RESULTS:

Increased Ca^{2+} transients in cells were observed at 1.8 and 2.3 kV/cm but not at 1.4 kV/cm (Fig.1). This was also correlated with patch clamp measurements where the membrane conductance was increased after pulse delivery at 1.8 and 2.3 kV/cm but not at 1.4 kV/cm in both VGCC+ and VGCC- cells. Calcium transients were larger in VGCC+ cells than in VGCC- cells thus pointing to VGCC channel activation by nsPEF. When comparing uni- and bipolar pulses, we observed smaller Ca^{2+} transients in bipolar group at 2.3 kV/cm, and no transients at 1.8 kV/cm or 1.4 kV/cm.

CONCLUSION:

We observed the activation of voltage-gated calcium channels in response to nsPEF stimulation. Electroporative membrane depolarization is the most likely cause of channel activation. Calcium transients can be attenuated by pulses with reverse polarity. The mechanisms behind the activation of channels and the attenuation effects are yet to be studied in detail.

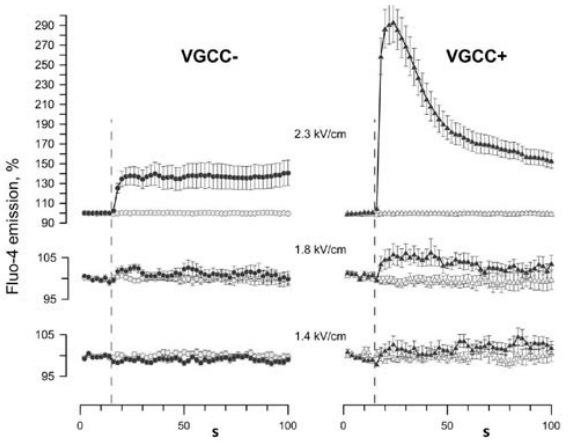

Figure 1: Ca^{2+} transients in VGCC+ and VGCC- cells after nsPEF stimulation (black symbols). The cells were exposed to one 300 ns unipolar pulse at 15 s (vertical dashed lines). Open symbols indicate sham exposure. Data are presented as mean±SEM with 9-38 cells per group.

ACKNOWLEDGEMENTS: This study was supported by *AFOSR-MURI* grant FA9550-15-1-0517 (to AGP)

REFERENCES:

[1] Pakhomov, A.G. *et al.* Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). *Bioelectromagnetics* 28, 655-63 (2007).

[2] Nesin, O.M., Pakhomova, O.N., Xiao, S. & Pakhomov, A.G. Manipulation of cell volume and membrane pore comparison following single cell permeabilization with 60- and 600-ns electric pulses. *Biochim Biophys Acta* 1808, 792-801 (2011).

[3] Nesin, V., Bowman, A.M., Xiao, S. & Pakhomov, A.G. Cell permeabilization and inhibition of voltage-gated Ca(2+) and Na(+) channel currents by nanosecond pulsed electric field. *Bioelectromagnetics* 33, 394-404 (2012).

[4] Nesin, V. & Pakhomov, A.G. Inhibition of voltage-gated Na (+) current by nanosecond pulsed electric field (nsPEF) is not mediated by Na (+) influx or Ca(2+) signaling. *Bioelectromagnetics* 33, 443-51 (2012).

Cold Atmospheric Plasma and Pulsed Electric Field Combination Potentiates Anti-Tumour Effect in a Three Dimensional Tissue Model.

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INTRODUCTION

In the last decade, cold atmospheric plasma (CAP), an ionized gas composed of heat, reactive species, charged particles and photons, has been proposed as a new tool for several biological and medical applications [1]. Among them, anti-cancer effect of CAP and plasma-activated liquids has been reported on different types of cancer *in vitro* and *in vivo* [2,3]. In parallel, electrical fields are well-known for their potential to reversibly or irreversibly permeabilize the cell membrane [4]. Cell electropermeabilization can be used as a therapeutic tool. In this context, the aim of the project is to bring a better understanding of the effect of CAP on cancer cells, and to potentiate this effect by combining CAP treatment with electric pulses (EP).

EXPERIMENTAL STRATEGY

In order to study the response of the cells in a 3D tumour model, spheroids of human colorectal cancer cells line HCT116 expressing the GFP were generated by cell aggregation method. CAP jet was generated by 10 kV square pulses of 1µs at 10 kHz and helium gas (3L/min). Plasma-activated PBS (PA-PBS) was generated by a 2 minutes exposure to the CAP jet. 5 days old spheroids were submitted either to EP (600V/cm, 8 pulses of 100µs at 1Hz), or EP combined with PA-PBS.

RESULTS

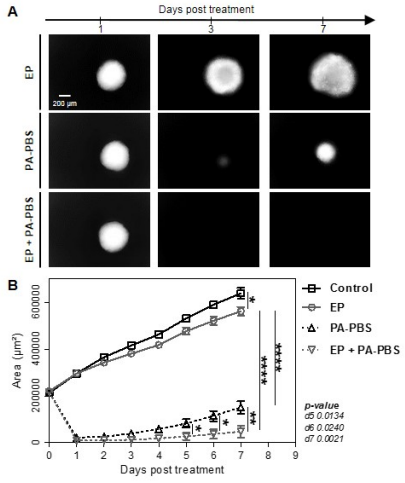

Figure 1: Spheroid growth follow-up after treatment. **A**: Fluorescence microscopy images. **B:** Growth curve plotted from GFP fluorescent area.

After being electro-pulsed and incubated for 4 hours with the PA-PBS, spheroids were placed in culture medium. Growth and morphological changes were assessed by live-microscopy (Figure 1.A). Fluorescence images show that when combined with EP, PA-PBS's negative effect on spheroids viability is irreversible. Growth curves of viable spheroids (GFP positive) measured up to 5 days after treatment show significant differences (t-test) in the growth patterns between spheroids treated with PA-PBS and PA-PBS combined with EP (Figure 1.B).

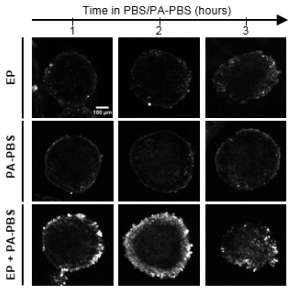

Figure 2: Caspase 3/7 detection in spheroids over treatment time.

To go further, we investigated the cell death mechanism triggered by PA-PBS and EP. For this purpose, the kinetics of caspases 3/7 activation was followed over time by confocal fluorescence microscopy (**Figure 2**). A concentric activation of caspases is visible only after 1h of treatment with EP + PA-PBS and signal highly increased with time until entire spheroid at 3h. However, EP and PA-PBS alone displayed very weak signal at the border of the spheroids, even after 3h. A more sustained and earlier caspase activation is obtained in spheroids treated with both EP and PA-PBS, resulting in a faster disassembly of the spheroids. All together, these encouraging results highlight a synergistic effect of the two physical methods as potential anti-cancer treatment.

AKNOWLEDGEMENT

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REFERENCES

[1] G. Fridman et al."Applied plasma medicine" in *Plasma Processes and Polymers*. 5(6): p. 503-533, 2008.

[2] D. Yan et al. "Cold atmospheric plasma, a novel promising anti-cancer treatment modality" in *Oncotarget,* Vol. 8, (No. 9), pp: 15977-15995, 2017.

[3] N.K. Kaushik et al. "Biological and medical application of plasma-ativated media, water and solutions" in *Biol. Chem, 2018.*

[4] J. Teissié et al. "Mechanism of cell membrane electropermeabilisation" in *Biochemica et Biophysica Acta*, 1724(3):270-80, 4727-473, 2005.

The general view of multidrug resistance in cancers

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INTRODUCTION

It is assessed that 9,6 million people will die due to cancer in 2018. Each type of cancer requires a specific treatment strategy such as surgery, radiotherapy or chemotherapy [1]. Chemotherapy is especially effective for metastatic tumors. The issue is more complicated because cells have an ability to become resistant to a variety of cytotoxic drugs, as evinced by lower drug concentration inside a cell as a result of increased drug efflux.

This phenomenon is called multidrug resistance (MDR) and it is a significant obstacle in a successful chemotherapy [2]. Most tumors consist of both drug-resistant and drug-sensitive cells, but chemotherapy causes only the death of drug-sensitive cell [3].

The drug-resistant cells present molecular "pumps" on their membranes, often in higher expression [4], which are able to expel drugs used in chemotherapy from interior [3].

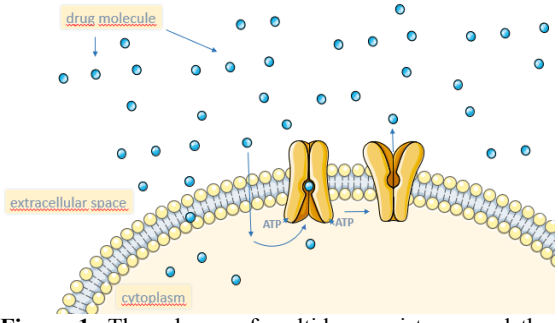

Figure 1: The scheme of multidrug resistance and the P-glycoprotein. Modified from: R. Weinberg "The biology of cancer" pp. 834 and Servier Medical Art website, http://smart.servier.com/

There are 49 mammalian ABC transporter molecules having ATP- binding cassettes [4]. The most common pumps found in cancer cells are P-glycoprotein and multidrug resistance-associated proteins (MRP) [3]. Those proteins can also occur in normal human adrenal, colon, kidney and liver cells [3]. As a result of the localization of cells in an organism**,** or type of cell differentiation**,** cells show resistance to different drugs even before treatment [5]. This phenomenon is called intrinsic multidrug resistance. Collaterally in acquired resistance cells can gain resistance to drugs as a consequence of therapy.

One of the methods to overcome both types of the cell resistance *in vivo* and *in vitro* is electrochemotherapy. Application of high voltage current electric pulses causes permeabilization of membranes triggering to increase of drug uptake and therefore enhance its cytotoxicity [6,7].

Understanding the multidrug resistance phenomena is crucial to effective treatment and improving clinical protocols [8].

ACKNOWLEDGES

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REFERENCES

[1] http://www.who.int/news-room/fact-sheets/detail/cancer [accessed: 20/09/2018 8:46]
[2] M. M. Gottesman, T. Fojo, S. E. Bates, "Multidrug resistance in cancer: role of ATP-dependent transporters", Nature Reviews. Cancer, vol. 2, pp. 48-58, 2002
[3] F. Thiebaut, T. Tsuruo, H. Hamada, M.M. Gottesman, I. Pastan, M.C. Willingham, "Cellular localization of the multidrug-resistance gene product", Proceedings of the National Academy of Sciences, vol. 84, pp. 7735-7738, 1987
[4] R. Weinberg "The biology of cancer", second edition, Garland Science, Taylor & Francis Group, LLC, pp. 833-834, 2014
[5] A.A. Stavrovskaya "Cellular Mechanisms of Multidrug Resistance of Tumor Cells", Biochemistry (Moscow), vol. 65, pp. 95-106, 2000
[6] M. Cemazar, G. Sersa, D. Miklavcic "Electrochemotherapy with Cisplatin in the Treatment of Tumor Cells Resistant to Cisplatin", Anticancer Research, vol. 18, pp. 4463-4466, 1998
[7] M. Cemazar, D. Miklavcic, L.M. Mir, J. Belehradek, Jr, M. Bonnay. D. Fourcault, G. Sersa, "Electrochemotherapy of tumours resistant to cisplatin: a study in a murine tumour model", European Journal of Cancer, vol. 37, pp. 1166-1172, 2001
[8] B. A. Teicher, Cancer Drug Resistance. New Jersey: Humana Press, 2006.

The efficiency of photodynamic therapy mediated by curcumin against human amelanotic melanoma in vitro

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INTRODUCTION

In recent years, new active substances of natural origin have been discovered with proven anticancer activity that interferes and affects with the metabolism of tumor cells. One of these compounds is curcumin - a component of turmeric. Curcumin has immunomodulatory, anti-cancer effects and is a potent photosensitizer, thus showing strong potential for use in oncotherapy [1].

METHODS

In the present study, the combination of curcumin was investigated in PDT using human melanoma amelanotic cell line (C32) and normal human fibroblasts (HF) from primary culture as control cells. The cells were treated with curcumin at the following concentrations 5 - 50 μM for 24 and 48 hours. Then, the cells were irradiated with blue light (20 J/cm^2) for 5 minutes and incubated for 24 hours. The efficacy of photodynamic effect was evaluated by the viability assay (MTT) (Figure 1 and 2).

RESULTS AND FIGURES

The obtained results suggest that curcumin may be a potent alternative to commonly used cytostatics. Depending on the curcumin concentration, the cell survival ranged from 18.95% of control cells after incubation with 50 μM curcumin, to 0.91% of control cells after PDT.

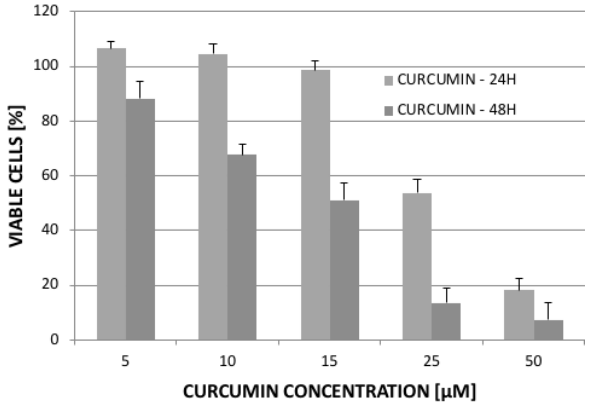

Figure 1: The evaluation of curcumin cytotoxicity after 24 and 48 hours of incubation on melanoma cells of the C32 cell line (the results are represented as a percentage of control (untreated – 0 μM) cells [%]).

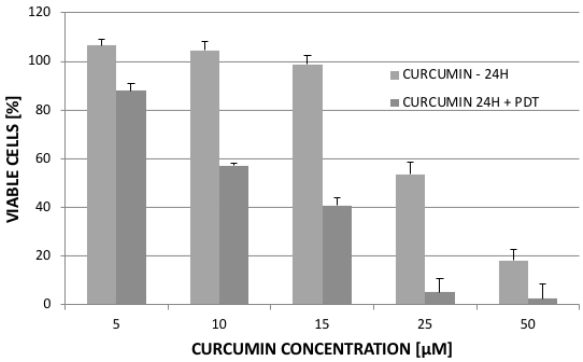

Figure 2: The comparison of the cytotoxic effect of curcumin and PDT with curcumin after 24 hours of incubation on C32 cell line (the results are presented as a percentage of control (untreated – 0 μM) cells [%]).

CONCLUSION

This study showed that PDT with curcumin as a photosensitizer appears to represent an efficient alternative for the treatment of amelanotic melanoma. Moreover, the electroporation method is planned in further studies, to increase the bioavailability of curcumin in human melanoma C32 cells. Thus, the proposed protocol seems to be promising in the amelanotic melanoma which is extremely resistant to standard chemo- and radiotherapy.

ACKNOWLEDGEMENTS

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REFERENCES

[1] Bimonte S, Barbieri A, Leongito M, Piccirillo M, Giudice A, Pivonello C, de Angelis C, Granata V, Palaia R, Izzo F: Curcumin AntiCancer Studies in Pancreatic Cancer, Nutrients, 2016, 8(7).

The influence of photodynamic therapy with curcumin as a photosensitizer on human Glioblastoma multiforme SNB19 cells

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INTRODUCTION

Glioblastoma multiforme (GBM) is a highly invasive (WHO grade IV) brain tumor that has a very poor prognosis for patients (median survival 14.2 months) [1]. In the study we treated glioblastoma multiforme with curcumin (CUR), as it was proved to reduce proliferation of several types of cancer before [2]. Moreover, it was proven that curcumin can be used as a photosensitizer in photodynamic therapy (PDT) [3]. The main advantage of PDT is its selectivity and control via manipulation of light source.

OBJECTIVES

Main aim of the study was to measure and analyze the cytotoxic effect of curcumin alone and in combination with irradiation on glioblastoma cells and optimization of an effective concentration to obtain photocytotoxic effect on cancer cells.

MATERIAL AND METHODS

The research was performed on human Glioblastoma cell line (SNB19). The cells were grown in sterile condition in culture bottles as a monolayer. The SNB-19 cells where incubated in the presence of different concentration of curcumin. The photodynamic protocol was performed by irradiating cells with fiber lamp with blue filter (20 J/cm^2) for 5 minutes. To measure the cell viability the mitochondrial activity was tested by MTT cytotoxicity assay after 24h and 48h. The same tests were performed after PDT procedure and both efficacies were compared.

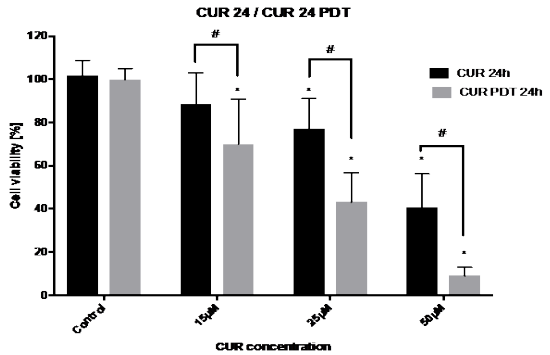

Figure 1. Cell viability after 24h incubation and PDT fallowing 24h and 48h incubation with different curcumin concentrations. All data are presented as means ±SD (n≥12).

RESULTS

The curcumin-based treatment decreased cell viability as compared with the untreated cells. PDT with curcumin as a photosensitizer additionally increased cytotoxic effect of the cancer cells.

CONCLUSIONS

As results show, properly chosen parameters have the cytotoxic effect on glioblastoma cells. We conclude that curcumin alone and with photodynamic therapy may have a positive influence on tumor therapy. Further research on glioma and normal cells is required to get more information about direct cellular effect and potential side effects of curcumin and PDT.

ACKNOWLEDGMENT

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REFERENCES

[1] Ostrom, Quinn T., *et al.* "CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2007–2011." Neuro-oncology 16.suppl_4 (2014): iv1-iv63.

[2] Wilken, Reason, *et al.* "Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma." *Molecular cancer* 10.1 (2011): 12.

[3] Ahn, Jin-Chul, *et al.* "Combination treatment with photodynamic therapy and curcumin induces mitochondria-dependent apoptosis in AMC-HN3 cells." *International journal of oncology*41.6 (2012): 2184-2190.

Peritumoural Gene Electrotransfer of Interleukin 12 Combined with Ionizing Radiation in TS/A Mouse Tumour Model

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INTRODUCTION

Gene electrotransfer (GET) of plasmid DNA encoding an immunostimulatory cytokine interleukin 12 (IL-12) has shown promising results in preclinical studies as well as veterinary and human clinical trials [1]. However, when applied peritumourally it has yet to be combined with radiotherapy. Ionising radiation (IR) is one of the main treatment modalities in clinical practice whose immunological aspects are being constantly revisited. Although it is already being combined with various immunotherapies in clinic, many factors remain unknown [2]. In this study we focus on the antitumour effectiveness of combining IR with IL-12 GET, which act as a local ablative technique and an immunological boost, respectively. The selected tumour model was the murine mammary carcinoma cell line, TS/A. Due to its low mutational load it could be regarded as an immunologically 'cold' tumour [3].

METHODS

The antitumour effect of the therapeutic combination of IR with IL-12 GET was evaluated by measuring the tumour growth delay of mouse mammary adenocarcinoma TS/A tumors in BALB/c mice. TS/A tumours were induced by a subcutaneous injection of $2x10^6$ cells on the back of the mouse. When the tumour volume reached 40 mm^3 the tumours were irradiated with 5, 10 or 15 Gy at a dose rate of 1,7 Gy/min. Immediately after, mice in therapeutic groups were injected peritumourally with pORF-mIL-12-ORT (50 µg) or in plasmid control groups with a control plasmid DNA pControl (50 µg). Using a non-invasive multielectrode array without its central pin, low voltage pulses (60 V/cm, 150 ms) were applied as described previously [4]. Tumour growth was monitored three times a week by measuring the tumours using a Vernier calliper until the tumour volume reached 250 mm3 as described previously [4].

RESULTS

IL-12 GET did not significantly contribute to the growth delay of TS/A tumours caused by ionizing radiation in lower doses. However, at the highest dose of 15 Gy, a significant difference ($P < 0,05$) was observed between the control group using pControl and the therapeutic group using pORF-mIL-12-ORT (Figure 1).

CONCLUSIONS

A minimal antitumour effect of the therapeutic combination of IR and IL-12 GET was observed in the immunologically 'cold' TS/A tumour model. The combined treatment of tumour irradiation and peritumoural immunostimulation was not able to convert a 'cold' tumour into a 'hot' tumour. Considering the results of this study, the next phase will be investigating the antitumour effect of combining IR with IL-12 GET in an immunologically 'hot' tumour model, which is a more promising tumour model due to a greater involvement of the immune component in battling cancer.

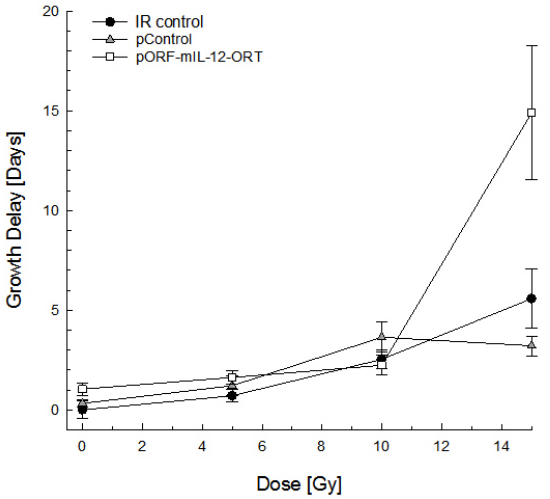

Figure 1: Tumour growth delay curve of TS/A tumours after the irradiation with 5, 10 or 15 Gy only (IR control) or combined with GET of plasmid DNA pControl (plasmid control group) or pORF-mIL-12-ORT (therapeutic group), * pORF-mIL-12-ORT *vs.* pControl at 15 Gy.

ACKNOWLEDGMENTS

This study was funded by ARRS and conducted in the scope of LEA-EBAM.

REFERENCES

[1] U. Kamensek, M. Cemazar, U. Lampreht Tratar, K. Ursic, G. Sersa, "Antitumor in situ vaccination effect of TNFα and IL-12 plasmid DNA electrotransfer in a murine melanoma model," *Cancer Immunol, Immun*, vol. 67(5), pp. 785-795, 2018.

[2] H. Scheithauer, C. Belka, K. Lauber, U.S. Gaipl, "Immunological aspects of radiotherapy," *Radiat Oncol*, vol. 9:185, 2014.

[3] A. Rosato, S. Dalla Santa, A. Zoso, S. Giacomelli, G. Milan, B. Macino, V. Tosello, P. Dellabona, P.L. Lollini, C. De Giovanni, P. Zanovello, "The cytotoxic T-lymphocyte response against a poorly immunogenic mammary adenocarcinoma is focused on a single immunodominant class I epitope derived from the gp70 Env product of an endogenous retrovirus," *Cancer Res*, vol. 63 (9), pp. 2153-63, 2003.

[4] S. Kos, T. Blagus, M. Cemazar, J. Jelenc, G. Sersa, "Utilization of Multi-array Electrodes for Delivery of Drugs and Genes in the Mouse Skin," in IFMBE Proceedings. Springer, Singapore, 2016, vol. 53, pp. 321-3.

Platform for Treatment Planning in Irreversible Electroporation Therapy

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INTRODUCTION

Irreversible electroporation (IRE) is a non-thermal ablation procedure for the treatment of solid tumours. By applying strong but short electric pulses, the cell membrane permeability to ions and macromolecules rises. When an electric field threshold is surpassed, homoeostasis is lost, and that leads to IRE. This procedure only affects the cell membrane, leaving the extracellular matrix unaffected. Numerical modelling can be used for predicting the ablation volume, although an accurate model is needed due to the complexity of tissues.

The main goal of this project is to develop a treatment planning platform for IRE. In contrast to similar past developments [1], this project pursues a platform entirely based on open access software.

METHODS

We are developing an IRE treatment planning platform. We aim at releasing a product that clinicians can use with a friendly interface. IRE planning is distributed in three main steps. The first one is to segment the regions of interest (e.g., organs, tumour). Then, the user can insert the electrodes in the desired position, and decide the voltage that will be delivered. Finally, a 3D model (mesh) is built and the electric field distribution is computed. The work-flow is shown in Fig. 1.

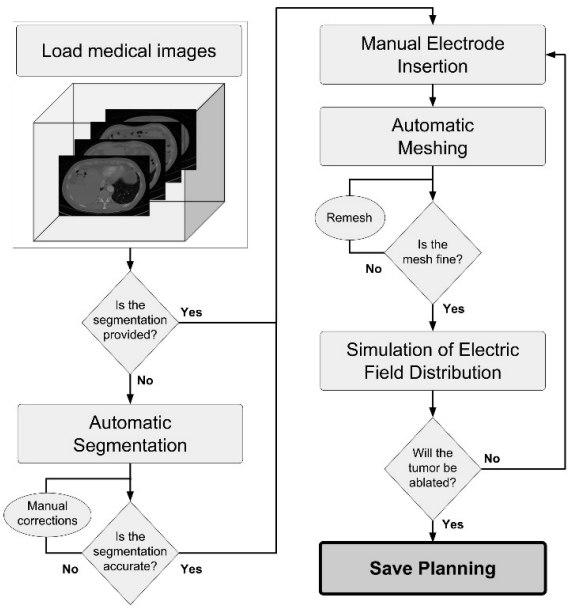

Figure 1: Work-flow diagram of the platform.

A heterogeneous model is built from the segmentations. The different conductivities among tissues will have a significant role on the computation of the electric field distribution [2]. It has been demonstrated that conductivity changes due to the electroporation phenomenon must be taken into account for accurate treatment planning [3]. In addition, a rise in temperature also increases the conductivity in physiological fluids. These non-linearities are considered when obtaining the electric field distribution, computed by means of the finite element method (FEM).

We are building this platform on top of the Medical Imaging Interaction Toolkit (MITK), which is in charge of image processing, and visualization. We are using the Computational Geometry Algorithms Library (CGAL) to extract a volumetric mesh from the segmentations and Elmer to compute the electric field distribution.

RESULTS

In an early prototype, we have assayed the treatment planning procedure of two cases in which the tissues were already segmented. One case corresponded to a liver tumour, with a model considering healthy liver, tumour, bile ducts and vessels tissues. The second case corresponded to a brain tumour. This model was built considering white matter, grey matter, cerebrospinal fluid and tumour tissues. The size of the tumours was 34mm and 14mm, respective-ly. Both treatment plans showed full coverage of the tumour with minimal damage to nearby structures. The computation time for the liver case was 47s for meshing and 162s for FEM solving. For the brain case, the times were 18s and 44s, respectively.

CONCLUSIONS

We have developed a platform for IRE treatment planning, reporting two cases were planning is feasible. The next step is to check these plans with actual cases, to compare if the ablated volume observed on post-treatment images correlates with the planned ablation volume.

REFERENCES

[1] D. Pavliha, B. Kos, M. Marčan, A. Županič, G. Serša, and D. Miklavčič, "Planning of Electroporation-Based Treatments Using Web-Based Treatment-Planning Software," J. Membr. Biol., vol. 246, no. 11, pp. 833–842, Nov. 2013.

[2] R. Qasrawi and A. Ivorra, "Impact of liver vasculature on electric field distribution during electroporation treatments: An anatomically realistic numerical study," in IFMBE Proceedings, vol. 45, Springer, Cham, 2015, pp. 573–576.

[3] A. Ivorra, L. M. Mir, and B. Rubinsky, "Electric field redistribution due to conductivity changes during tissue electroporation: Experiments with a simple vegetal model," in IFMBE Proceedings, vol. 25, no. 13, Springer, Berlin, Heidelberg, 2009, pp. 59–62.

Numerical study of irreversible electroporation treatment of liver tumors in the vicinity of metallic surgical clips

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INTRODUCTION

Irreversible electroporation (IRE) is a novel minimally invasive tumor ablation technique, which has shown promise for ablation of deep-seated tumors, such as primary and metastatic tumors in the liver. However, patients that undergo IRE treatment of liver tumors often have metallic surgical clips present due to previous treatment. In this study we numerically evaluated the potential influence of metallic surgical clips within the ablation zone on the efficiency of IRE treatment of colorectal liver metastases.

MATERIALS AND METHODS

Nine clinical cases of liver tumors, treated with IRE, were used for this study – four cases with metallic surgical clips present within the ablation zone (up to 1 cm from tumor) and five cases without clips. For each case a patient specific anatomically correct numerical model was built based on patients' medical images and the IRE treatment was reconstructed using treatment data from the IRE device. A numerical framework, previously designed for treatment planning of electroporation-based treatments was used for modelling and numerical computations [1].

The analysis was based on computations of electric field distribution in tumor volume and ablation safety margin. Tissue heating was also considered. In cases, where clips were present within the ablation zone, an additional set of computations was performed in which the metallic clips were omitted from calculations (Table 1). This enabled direct comparison of the effect of metallic clips on electric field. Electric field distribution was calculated for each case. Cell kill probability due to IRE and thermal damage was calculated, using the statistical Peleg-Fermi model [2] and Arrhenius equation, respectively.

RESULTS

Ablation was considered successful, if the electric field strength in tissue exceeded the threshold for IRE (500 V/cm). Additionally, calculated cell kill probability in ablated tissue should be at least 0.9 (90 %).

Numerical computations showed distortions in electric field only in the immediate vicinity of the metallic clips less than a millimeter away. The presence of clips did not significantly affect the electric field coverage nor cell kill in tumors (Table 1). The effect of metallic clips on electric field coverage and cell viability was more pronounced in the safety margins, where both values were usually lower by a few percentage points when metallic clips were present.

Due to high number of delivered pulses during IRE treatment thermal damage was also observed in a significant volume of the target tissue. However, cell kill volume due to IRE is much higher than cell kill volume caused by thermal damage, which indicates that the success of IRE treatment is not dependent on thermal damage (Table 1).

Table 1: Electric field coverage (above 500 V/cm) and cell kill in tumor volume for nine studied tumor cases

Tumor case	Electric field coverage (%)		IRE cell kill (%)		Thermal cell kill (%)	
	no clips	clips	no clips	clips	no clips	clips
1	94,57	94,63	93,61	93,87	86,74	86,68
2	59,32	58,94	55,70	55,51	51,90	52,09
3	71,26	71,18	79,53	81,03	59,34	59,84
4	87,98	87,35	96,89	96,53	45,66	44,75
5	75,36	/	60,87	/	00,00	/
6	98,71	/	95,76	/	68,63	/
7	87,04	/	95,37	/	35,19	/
8	81,44	/	95,62	/	29,90	/
9	99,48	/	99,48	/	90,63	/

CONCLUSIONS

Our numerical study shows that the presence of metallic surgical clips within the ablation zone does not significantly affect the IRE treatment outcome in terms of tumor coverage and cell kill. However, a decrease in electric field and cell kill within a few millimeters from the clips was observed in ablation safety margin, which can potentially be critical for treatment success, since micrometastases can be present in vicinity of the tumor/clips.

ACKNOWLEDGEMENTS

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REFERENCES

[1] B. Kos *et al.*, "Careful treatment planning enables safe ablation of liver tumors adjacent to major blood vessels by percutaneous irreversible electroporation (IRE)," *Radiol. Oncol.*, vol. 49, no. 3, pp. 234–241, Sep. 2015.

[2] J. Dermol and D. Miklavčič, "Mathematical Models Describing Chinese Hamster Ovary Cell Death Due to Electroporation In Vitro," *J. Membr. Biol.*, vol. 248, no. 5, pp. 865–881, Oct. 2015.

Cell death enhancement in vitro by electroporation mediated calcium delivery

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INTRODUCTION

The different methods of tumour treatment were developed based on the tumour type, stage and level, to reduce the high mortality rate of cancer patients. In the recent years, it has been shown that the method of electroporation (EP) can be successfully applied to cause cancer cell death leading to tumour loss. EP occur when the cell membrane is affected with electric field, thus causing a temporal increase of transmembrane potential leading to the formation of temporal pores in the affected cell membrane. As a result, the permeability of the affected cell membrane to hydrophilic small molecules (such as anticancer drugs) is increased [1]. The enhanced effect of transfer of anticancer drugs by using electroporation has been widely implemented for cancer treatment both *in vitro* and *in vivo*. Therefore, the electrotransfer of anticancer drug to cells can be applied in the clinics. Electrochemotherapy, a method for high voltage electric pulse assisted intratumoral chemotherapeutic drug delivery, has been accepted in the European clinics as an efficient palliative treatment for the metastatic surface tumours [2].

Calcium ion delivery via electroporation has been recently suggested as a chemotherapeutic-free alternative to bleomycin or cisplatin used in conventional electrochemotherapy, reducing the cost and potential side effects of the treatment while retaining high efficiency and selectivity towards tumour cells [3]. The sudden increase in intracellular calcium concentration leads to cell death via numerous processes such as ATP depletion, increased activity of lipases and proteases, production of reactive oxygen species, the opening of non-specific pores, etc. [4]. However, the mechanism behind the calcium electroporation remains unclear to this day. The electroporation parameters, that lead to the optimal efficiency of calcium electrotransfer, also remain to be determined.

RESULTS

In this study, we analysed the Chinese Hamster Ovary (CHO) cell viability and metabolic activity after calcium electroporation by using clonogenic and MTT assays, respectively. Cells were electroporated using from 1 to 4 high voltage (1200 V/cm, 100 µs, 1 Hz) electric pulses in the presence of $CaCl_2$ at various concentrations (0.1 – 5 mM).

Cell viability and metabolic activity was evaluated during the time of 24 hours post electric pulse delivery. We show the decrease in cell viability from 20 to100 %. Such viability change depends on the electroporation parameters and the concentration of calcium ions in the pulsed medium. Additionally, we have found that the low calcium ion concentrations (up to 0.5 mM) stimulated cell viability (Figure 1), as compared to calcium untreated cells. In conclusion, our study indicates the modulation of cell viability, using calcium electroporation.

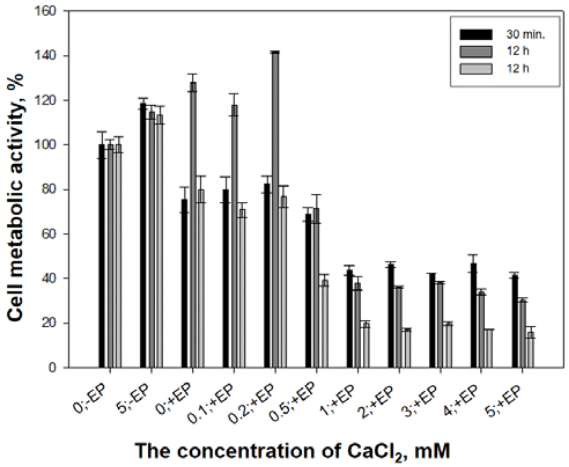

Figure 1: Metabolic activity changes in CHO cells at different Ca^{2+} concentrations after 1200 V/cm, 100 µs, 4 HV pulses using the MTT assay (mean ± SEM, n = 6).

REFERENCES

[1] A. J. Nickoloff, "Animal cell electroporation and electrofusion protocols", *Methods in Molecular Biology*, vol. 48, pp. 3-24, 1995.

[2] S. Gregor, M. Čemažar, D. Miklavčič, "Antitumor Effectiveness of Electrochemotherapy with cis-Diamminedichloroplatinum (II) in Mice", Cancer Research, vol. 55, pp. 3450-3455, 1995.

[3] H. Falk, L. W. Matthiessen, G. Wooler, J, "Calcium electroporation for treatment of cutaneous metastases; a randomized double-blinded phase II study, comparing the effect of calcium electroporation with electrochemotherapy", *Acta Oncol*, vol. 57, pp. 311-319, 2018

[4] S.K. Frandsen., M. B. Krüger, U. M. Mangalanathan, T. Tramm, F. Mahmood, I. Novak, J. Gehl, " *Cancer Res*. ", vol. 15, pp. 4389-4401, 2017.

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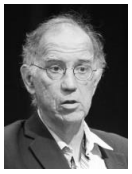

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APPENDIX

Recommendation papers on how to report on electroporation research:

Campana LG, Clover AJP, Valpione S, Quaglino P, Gehl J, Kunte C, Snoj M, Čemažar M, Rossi CR, Miklavčič D, Serša G. **Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review.** *Radiol. Oncol. 50*: 1-13, 2016. 149

Raso J, Frey W, Ferrari G, Pataro G, Knorr D, Teissié J, Miklavčič D. **Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes.** *Innov. Food Sci. Emerg. Technol. 37*: 312-321, 2016. 163

Čemažar M, Serša G, Frey W, Miklavčič D, Teissié J. **Recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples.** *Bioelectrochemistry 122*: 69-76, 2018. 173

review

Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review

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Disclosure: DM holds patents on electrochemotherapy that have been licensed to IGEA S.p.a. and is also a consultant to IGEA. The other co-authors have nothing to disclose.

Background. Electrochemotherapy is becoming a well-established treatment for malignancies of skin and non-skin origin and its use is widening across Europe. The technique was developed and optimized from solid experimental and clinical evidence. A consensus document is now warranted to formalize reporting results, which should strengthen evidence-based practice recommendations. This consensus should be derived from high quality clinical data collection, clinical expertise and summarizing patient feedback. The first step, which is addressed in this paper, aims to critically analyze the quality of published studies and to provide the recommendations for reporting clinical trials on electrochemotherapy.

Methods. The quality of reporting in published studies on electrochemotherapy was analyzed in order to produce procedure specific reporting recommendations. A comprehensive literature search of studies published from 2006 to 2015 was performed followed by qualitative analysis of manuscripts assessing for 47 quality criteria grouped into four major clusters: (1) trial design, (2) description of patient population, (3) description of treatment delivery and patient outcome, (4) analysis of results and their interpretation. The summary measure during literature assessment was the proportion of studies fulfilling each manuscript quality criteria.

Results. A total of 56 studies were screened, from the period 2006 to 2015, of which 33 were included in the qualitative analysis, with a total of 1215 patients. Overall, the quality of reporting was highly variable. Twenty-four reports (73%) were single-center, non-comparative studies, and only 15 (45%) were prospective in nature (only 2 of them were entered into a clinical trials registry). Electrochemotherapy technique was consistently reported, with most studies (31/33) adhering closely to published standard operating procedures. The quality of reporting the patient population was variable among the analyzed studies, with only between 45% and 100% achieving dedicated quality criteria. Reporting of treatment delivery and patient outcome was also highly variable with studies only fulfilling between 3% and 100%. Finally, reporting study results critically varied, fulfilling from 27% to 100% of the quality criteria. Based on the critical issues emerging from this analysis, recommendations and minimal requirements for reporting clinical data on electrochemotherapy were prepared and summarized into a checklist.

Conclusions. There is an increasing body of published clinical data on electrochemotherapy, but more high quality clinical data are needed. Published papers often lack accurate description of study population, treatment delivery as well as patient outcome. Our recommendations, provided in the form of a summary checklist, are intended to ameliorate data reporting in future studies on electrochemotherapy and help researchers to provide a solid evidence basis for clinical practice.

Key words: electrochemotherapy; clinical trials, recommendations

Introduction

Electrochemotherapy is becoming a well-established non-thermal ablative technique for malignancies of skin and non-skin origin.[1,2] The medical applications of electrochemotherapy are based on the principle of electroporation, which dates back to 1982, when sequences of electric pulses were applied to deliver naked DNA molecules within mouse lyoma cells.[3] Preclinical studies carried out by several research groups, coupled with technical developments, culminated in the clinical application of electroporation during the early 1990s.[4-13] These initial data on electroporative uptake of molecules are viewed as seminal for various biotechnological and medical applications.[14,15] The principle of electrochemotherapy is the use of electroporation to enhance chemotherapeutic drug delivery. Two agents, bleomycin or cisplatin, can achieve a several fold increase in their intracellular availability, and consequently cytotoxicity, when the tumor tissue is exposed to reversible electroporation and transient cell membrane permeabilization, thus achieving an optimal intratumor drug distribution.[7,16-18] Electrochemotherapy has proven effective for the treatment of different tumor histotypes, including both skin and non-skin cancers, as well as for the palliation of metastases involving cutaneous and subcutaneous tissues.[19-22] The treatment of primary skin tumors is largely restricted to multifocal cutaneous tumors, most notably some selected cases of basal cell carcinoma, when tumor anatomical location and patient medical conditions contraindicate more aggressive treatments.[23]

The publication of the European Standard Operating Procedures of Electrochemotherapy (ESOPE) in 2006 facilitated a broad acceptance of electrochemotherapy for treatment of cutaneous tumors and metastases.[24] Over a number of years, several clinical reports have confirmed its effectiveness. Interestingly, the vast majority of studies used the Standard Operating Procedure (SOP) as a guideline for electrochemotherapy. The availability of SOP allowed for reproducibility and improvement of results in the clinical practice. Several large follow up series confirmed the efficiency of electrochemotherapy. A recent meta-analysis of the use of electrochemotherapy in the treatment of cutaneous metastasis places it well amongst other, more established, treatment options.[2] Recently, electrochemotherapy has also been recognized by the National Institute for Health and Care Excellence (NICE) as an integral part of the multidisciplinary treatment for patients with skin metastases of non-skin origin and melanoma (NICE interventional procedure guidance IPG 446, http://www.nice.org.uk/guidance/ipg446). More recently, electrochemotherapy has been introduced into the treatment of deep-seated and endoluminal tumors.[25-28] The first clinical report on visceral metastases indicates its effectiveness, and suggests a possible role of electrochemotherapy for the treatment of liver metastases, especially when located close to major blood vessels and when not manageable with surgery or other ablative techniques.[29]

Overall, literature data from Web of Science database indicate a steady increase in number of publications and their citations under the key word "electrochemotherapy" (Figure 1A,B) and "clinical electrochemotherapy" (Figure 1C,D). Despite a steady increase in the number of published reports, a higher quality and standardization of reported studies is needed to improve and support a truly evidence based practice. In our study we only included papers published after 2006, specifically only to include reports published after the Standard Operating Procedures (SOP).[24]

The purpose of this recommendation paper is to provide practical recommendations in order to improve the precision of reported clinical studies on electrochemotherapy (a summary checklist is provided as Supplementary file). This, in turn, we hope will stimulate the scientific community to report research using these guidelines to give comprehensive reports on areas including study design, definition of study endpoints, patient selection criteria, treatment plan and outcome assessment. The adoption of more precision in reporting will enable researchers and clinicians to perform more meaningful outcome comparisons with other ablative techniques, to clarify the direction for future research, and to produce more evidence-based practice. It is our hope that these advancements may improve patient selection, resource allocation, and ultimately patient outcome.

This report was prepared based on initiative of the Steering Committee of the COST TD 1104 Action (www.electroporation.net) and in response to a general call for increased awareness and concern for low quality reporting practice[30]; moreover, it has been prepared by the committee within the Working group of Medical applications of electroporation, in COST action TD 1104 EP4Bio^{2}Med, and is included in the series of publications addressing the same topic in preclinical research in electroporation as well as in the pulsed electric fields for industrial purposes.[31]

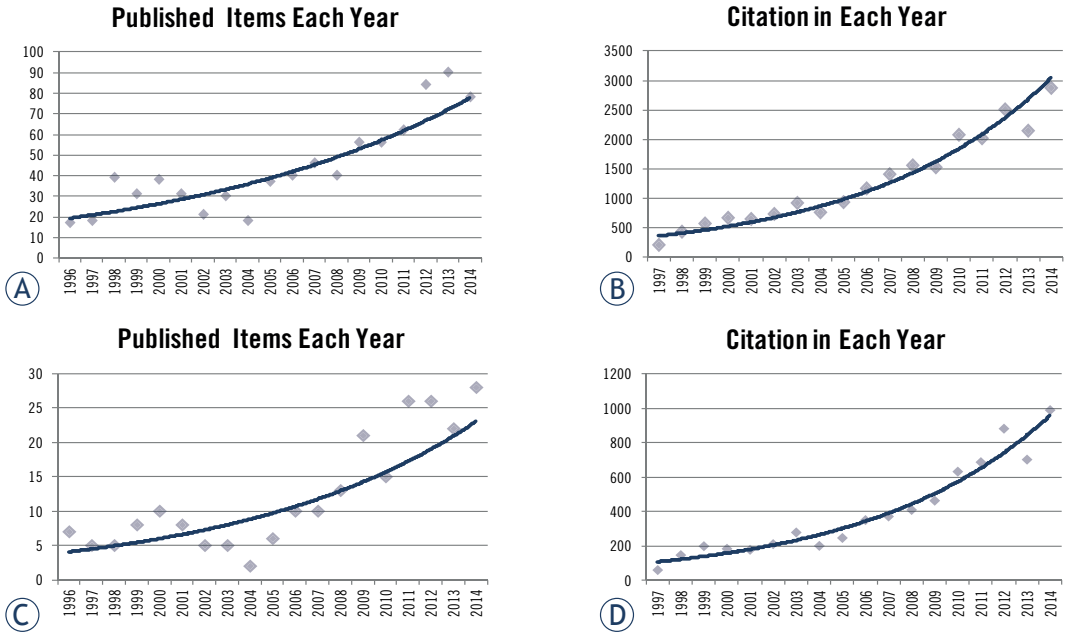

FIGURE 1. Search in Web of Science demonstrates a steady increase in number of publications under the key word "electrochemotherapy" **(A,B)** as well under the "electrochemotherapy, clinical" **(C,D)**. The Meta data indicate the expanding field.

Several guidelines exist with the aim of assuring sound research practices, and improving the quality of clinical trials and, ultimately, allow for generalizable results. At a basic level, Good Clinical Practice (GCP) represents an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. At a higher level, dedicated guidelines and recommendations have been developed according to the specific type of study performed. For instance, the STROBE statement (www.strobe-statement.org) indicates a checklist for details that should be reported in observational trials; the Consolidated Standards of Reporting Trials (CONSORT) statement (www.consort-statement.org/consort-statement/) provides guidance for reporting the aim, methods, results and implications of randomized controlled trials; the PRISMA statement (www.prisma-statement.org) indicates preferred reporting for systematic reviews; finally, the REporting recommendations for tumor MARKer prognostic studies (REMARK, www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-marker-prognostic-studies-remark/) suggest guidelines to provide relevant information about study design, preplanned hypotheses, patient and specimen characteristics, assay methods, and statistical analyses when evaluating tumor markers in oncology. In addition, these guidelines provide helpful suggestions on how to present data and important elements to include in discussions. Although these guidelines provide a fundamental guidance for conducting a valid clinical trial and reporting generalizable findings, nonetheless it is recognized that there is a need for specialty-specific guidelines and that these guidelines will lead to improvement in the quality of reports and to higher impact publications.[32,33] In the field of electrochemotherapy, comprehensive meta-analyses or Cochrane style reviews of efficiency are hampered by the lack of some relevant clinical data in published reports. Therefore, we evaluated the published papers on clinical electrochemotherapy and identified possible pitfalls in data reporting. On this basis, we prepared recommendations for improving the quality of future studies and fostering further rational development of electrochemotherapy.

Systematic review and qualitative analysis of publications

Methods

The initial step was to identify and access all published trials evaluating the efficacy of electrochem-

TABLE 1. Manuscript quality criteria

Manuscript quality criteria			
Trial design	**Description of Patient population**	**Treatment delivery and outcome assessment**	**Analysis of results and interpretation**
1. Prospective trial	1. Setting (curative / palliative)	1. Type of anaesthesia	1. Summary of trial endpoints
2. Trial registration		2. Drug route and dosages	
3. Comparative trial	2. Demographic data (in tabular form)	3. Pulse generator	2. Predictive factors
4. Mention of trial design		4. EP parameters	
5. Multicenter study	3. No of tumors	5. Electrode description	3. Other patient outcome parameters
6. Mention of sponsor		6. Tumor safety margins indicated	
7. Trail hypothesis and sample size	4. Tumor location	7. Deviation from SOPs	4. Results interpretation
8. Informed consent	5. Tumor histotype	9. Criteria for retreatment	5. Comparison to historical controls
9. EC approval		8. Tumor coverage with EP	
10. Structured abstract		10. Total No of ECT sessions	
11. Rationale of the trial	6. Tumor size	11. ECT sessions required[a]	6. Future directions
12. A priori inclusion criteria		12. Toxicity criteria	
13. Follow-up dates	7. Visceral mts indicated	13. Response criteria	7. COI statement
14. Statistical methods		14. Evaluation of tumor control	
15. Software used	8. Concomitant treatments	15. ECT success[b]	
16. C.I., p-values		16. Keep track of patients lost to follow-up	

C.I. = confidence intervals; COI = conflict of interest statement; EC = Ethic Committee; EP = electric pulses (including number, duration and amplitude); mts = metastases; SOPs = Standard Operating Procedures.

[a] Number of electrochemotherapy (ECT) sessions required for achieving response (either complete or partial) on baseline tumors

[b] Decision rule for determining ECT success

otherapy in the treatment of tumors including skin cancers, cutaneous/subcutaneous metastases from other histotypes, deep-seated tumors or visceral metastases.

From October 4 to 10, 2015, we conducted a comprehensive literature assessment that included searches of Medline (EBSCO), Pubmed (NLM), Web of Science and Embase. The search terms used were "electrochemotherapy", "electrochemotherapy" AND "clinical trial". We limited our search to humans. Articles published from January 2006 to September 30, 2015 were retrieved. We included studies on the clinical application of electrochemotherapy regardless of study design (both prospective and retrospective) patient population, tumors histotype and anatomical location or electrochemotherapy treatment protocol. However, treatment outcome had to include tumor response and follow-up tumor control evaluation, procedural morbidity and toxicity or patient quality of life. Two of the authors (LGC and SV) and an external collaborator with experience in clinical trials independently screened the retrieved studies based on the title, key words, and abstract to exclude non-relevant and non-English written studies. After completion of all searches, duplicates were removed and only the most recent report from follow-up series was included in order to avoid overlapping series. Both retrospective and prospective studies were included, while case reports and small series were excluded because of their intrinsic lower level of evidence (the minimum number of patients was arbitrarily set at 9). Published reviews on electrochemotherapy were similarly excluded, but their reference list was reviewed in order to identify possible additional studies. Studies whose main purpose was unrelated to electrochemotherapy efficacy and biological studies (*i.e.*, those exploring immune effects of treatment) were also excluded, unless clear and standardized description of patient outcome was retrievable from the manuscript. Studies that did not meet the inclusion criteria were discarded during the initial review. When uncertainty existed in the abstract evalu-

ation, we retrieved and assessed the full text. A third author (GS) resolved differing opinions. Full text of the included articles was independently reviewed by two of the authors using a predefined checklist quality criteria. These quality criteria were discussed and agreed among the authors in a series of operative meetings which were hold during the 1st World Congress on Electroporation in Portoroz, Slovenia, between September 6 to 10 2015 and were also based on deliberations at the Recommendation paper workshop organized by COST TD1104 on 28th March 2014 in Copenhagen, Denmark. The checklist was also adapted from similar reporting standard guidelines in the field of neuro-oncology, isolated limb perfusion and in phase II cancer trials.[34-36] As a result, we had a final count of 47 quality criteria that were clustered into four domains: trial design, description of patient population, treatment delivery and outcome assessment, and analysis of results and their interpretation (Table 1). The summary measure during literature assessment was the proportion of studies fulfilling each manuscript quality criteria.

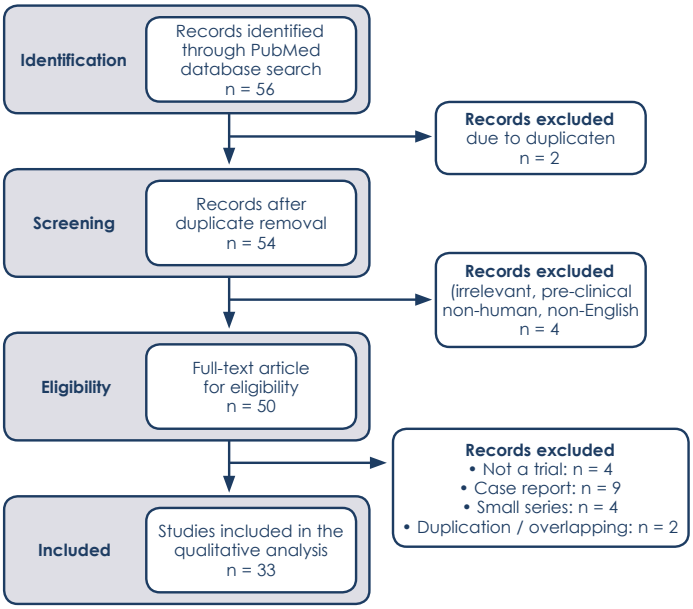

FIGURE 2. PRISMA flow diagram of identification, screening, eligibility and inclusion of studies.

Results

A total of 56 papers were initially identified. Of these, only 33 reports were finally retained in the qualitative synthesis; the reasons for exclusion of the remaining reports are listed in Figure 2.

A summary of the studies included in the final analysis is presented in Table 2.[20-22,29,37-65] The total number of patients across all studies was 1215. Electrochemotherapy protocol was following the SOP as defined in ESOPE study in all but two cases.[40,65]

The majority (24/33) of reports were single-center studies. There were 24 tumor-specific studies (melanoma, n=8; breast cancer, n=5; head and neck squamous cell carcinoma, n=4; Kaposi sarcoma, n=3; pancreatic cancer, n=1; colorectal cancer, n=1; soft tissue sarcomas, n=1; vaginal squamous cell cancer, n=1) and 9 studies including heterogeneous histologies. Response assessment was based on clinical evaluation in all except 3 studies on pancreatic cancer[41], liver metastases from colorectal cancer[29], and chest wall recurrence from breast cancer[57], where response assessment was radiological (ultrasound scan, magnetic resonance imaging, computed tomography, or fluorine-18-deoxyglucose PET-CT scan). Details of the quality criteria used to assess trial design are presented in Figure 3. Less than half (15/33, 45%) of studies

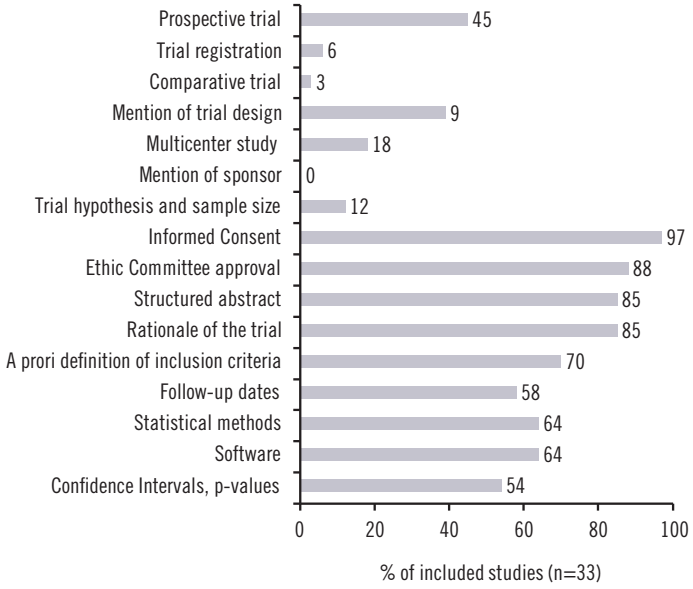

FIGURE 3. Assessment of published studies according to **quality criteria concerning trial design**.

were prospective and only two of them (6%) were entered into a publicly accessible clinical trials registry.[29,57] Eighteen percent (6/33) of papers represented the report of a multicenter study. There was a single comparative trial (an internally controlled

TABLE 2. Trials identified included in the qualitative analysis

Study, year	Setting	No of pts	Tumor histotype	ECT protocol
Rotunno, 2015 [37]	Two-center, Italy	55	non-melanoma SC	ESOPE
Cabula, 2015 [38]	Multi-center, Italy	125	BC	ESOPE
Mozzillo, 2015 [39]	Single-center, Italy	15	melanoma	ESOPE
Landstrom, 2015 [40]	Single-center, Sweden	19	HNSCC	Other [a]
Granata, 2015 [41]	Single-center, Italy	13	pancreatic cancer	ESOPE
Kreuter, 2015 [42]	Multi-center, Germany	56	various	ESOPE
Quaglino, 2015 [43]	Multi-center, Europe	121	various	ESOPE
Mir-Bonafé, 2015 [44]	Single-center, Spain	31	melanoma	ESOPE
Campana, 2014 [45]	Single-center, Italy	39	HNSCC	ESOPE
Ricotti, 2014 [46]	Single-center, Italy	30	melanoma	ESOPE
Campana, 2014 [47]	Single-center, Italy	55	BC	ESOPE
Edhemovic, 2014 [29]	Single-center, Slovenia	16	CRC-liver mts	ESOPE [b]
Seccia, 2014 [48]	Single-center, Italy	9	HNSCC	ESOPE
Campana, 2014 [50]	Two-center, Italy	34	STS	ESOPE
Solari, 2014 [51]	Single-center, Italy	39	various	ESOPE
Di Monta, 2014 [52]	Single-center, Italy	19	KS	ESOPE
Caracò, 2013 [49]	Single-center, Italy	60	melanoma	ESOPE
Perrone, 2013 [53]	Single-center, Italy	9	V-SCC	ESOPE
Benevento, 2012 [54]	Single-center, Italy	12	BC	ESOPE
Mevio, 2012 [55]	Single-center, Italy	15	HNSCC	ESOPE
Campana, 2012 [20]	Single-center, Italy	35	BC	ESOPE
Latini, 2012 [56]	Single-center, Italy	18	KS	ESOPE
Matthiessen, 2012 [57]	Single-center, Denmark	12	BC	ESOPE
Gargiulo, 2012 [58]	Single-center, Italy	52	non-melanoma SC	ESOPE
Campana, 2012 [21]	Single-center, Italy	85	melanoma	ESOPE
Curatolo, 2012 [59]	Two-center, Italy	23	KS	ESOPE
Kis, 2011 [60]	Single-center, Hungary	9	melanoma	ESOPE
Matthiessen, 2011 [22]	Two-center, Denmark-UK	52	various	ESOPE
Skarlatos I, 2011 [61]	Multi-center, Greece	52	various	ESOPE
Campana, 2009 [62]	Single-center, Italy	52	various	ESOPE
Quaglino, 2008 [63]	Single-center, Italy	14	melanoma	ESOPE
Larkin, 2007 [64]	Single-center, Ireland	30	various	ESOPE
Gaudy, 2006 [65]	Single-center, France	12	melanoma	Other [c]

BC = breast cancer; ECT = electrochemotherapy; CRC-liver mts = colorectal cancer liver metastases; HNSCC = head and neck squamous cell cancer; KS = Kaposi's sarcoma; SC = skin cancer; STS = soft tissue sarcomas; V-SCC = vaginal squamous cell cancer

[a] Intratumoral BLM injection (1000 IU/cm^3 and tumor electroporation by means of six 1100 V/cm square wave pulses with 0.1 ms duration

[b] In this trial, the ESOPE protocol was integrated by the application of variable geometry electrodes for the treatment of deep visceral metastases.

[c] Intratumoral BLM injection (concentration, 4 mg/mL; dose, 1 mg/cm^3 of tumor volume was followed, after 10 minutes, by the application of electric pulses (six 100 µsec-long pulses, 4 pulses/sec, electric field >600V/cm

study with intrapatient randomization of melanoma metastases to intralesional bleomycin versus intralesional bleomycin followed by electric pulses)[65]; a formal sample size calculation or analysis of "intent-to-treat" population was found in only 4/33 (12%) studies.[20,50,57,65]

Details of the quality criteria used to assess the description of patient population are presented in Figure 4. Treated tumors were described in detail in most reports: number of tumors, 94%; tumor location, 100%; tumor histotype, 100%; tumor size, 91%. On the other hand, additional clinical information was less frequently reported: study setting -palliative/curative-, 54%; presence of visceral metastases, 54%; concomitant oncologic treatments, 45%.

Details of the manuscript quality criteria used to assess the description of treatment delivery and response assessment are presented in Figure 5. Treatment details were accurately described in most reports: type of anaesthesia, 32/33 (97%); drugs, 33/33 (100%); pulse generator, 33/33 (100%); electrode types, 31/33 (93%); electric pulse parameters, 32/33 (97%). The criteria for response assessment were clearly stated in 29/33 (88%) of studies, while toxicity criteria were indicated in only 14/33 (42%) of papers.

Details of the quality criteria used to assess the analysis of results and their interpretation are presented in Figure 6. The majority of reports included a critical analysis: interpretation of results, 33/33 (100%); comparison to historical control, 25/33 (76%); indication of possible future directions, 33/33 (100%); conflict of interest statement, 27/33 (82%). On the contrary, only a minority of them fulfilled other specific quality criteria: summary of primary and secondary endpoints, 13/33 (39%); indication of predictive factors, 9/33 (27%); additional patient outcome parameters, 9/33 (27%).

Based on the results of this analysis, the consensus between authors was to recommend some minimal requirements for reporting clinical data in future studies.

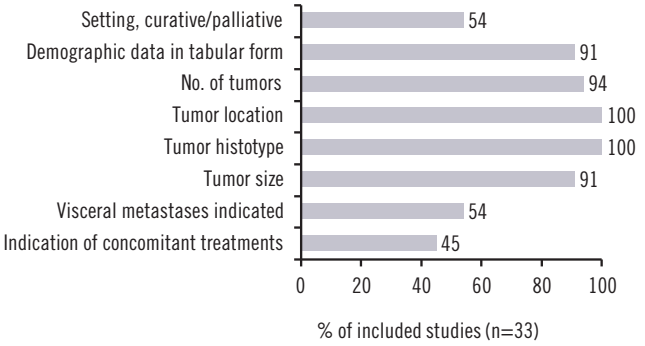

FIGURE 4. Assessment of published studies according to **quality criteria concerning description of patient population**.

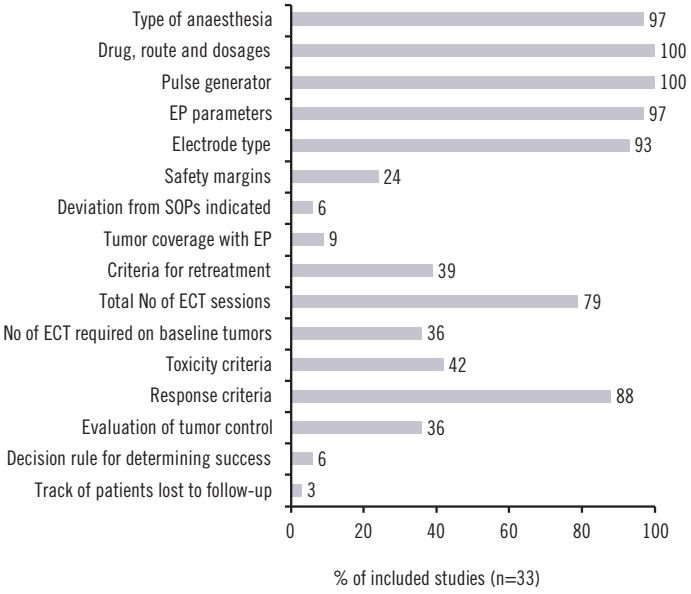

FIGURE 5. Assessment of published studies according to **quality criteria concerning treatment delivery and outcome assessment**.

ECT = electrochemotherapy; EP = electric pulses.

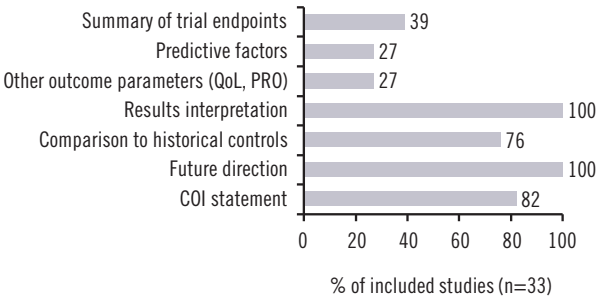

FIGURE 6. Assessment of published studies according to **quality criteria concerning analysis of results and interpretation.**

COI = conflict of interest statement; PRO = patient reported outcomes; QoL = quality of life.

Recommendations and minimal requirements for reporting clinical trial results on electrochemotherapy

Trial design

Any consolidation of the evidence base of electrochemotherapy requires that reports adhere strictly to research reporting standards and are the result of well-designed clinical trials. Much of these

topics are covered by STROBE (STrengthening the Reporting of Observational studies in Epidemiology, http://www.strobe-statement.org/) checklist and CONSORT (CONsolidated Standards of Reporting Trials, http://www.consort-statement.org/checklists/view/32-consort/66-title) guidelines which should be adhered to as much as possible when reporting observational studies and randomized controlled trials, respectively. Incorporation of these electrochemotherapy guidelines will further improve the quality of the reports. So far, only phase I-II single-arm trials have been reported, with the exception of a single small-sized study, which included an intra-patient randomization of tumors to direct bleomycin injection or bleomycin injection followed by electroporation.[65] It is likely that improving the evidence base will involve conducting properly designed, prospective comparative - possibly randomized - clinical trials in order to perform accurate analyses of the advantages of electrochemotherapy against other ablative procedures or alternative local treatments. Of utmost importance, future trials should aim to be prospective and preferably multicentric, with clearly defined endpoints and inclusion criteria. It is also advisable that all trials should be registered at publicly accessible clinical trials registries, (*e.g.*, clinicaltrials.gov, ISRCTN registry at http://www.isrctn.com, WHO registry at www.apps.who.int/trialsearch, or similar) and approved by institutional review boards or respective national bodies. Finally, according to the current requirements of most scientific journals – which refer to the recommendation of the International Committee of the Medical Journal Editors (ICMJE, http://www.icmje.org/), manuscripts should conform to well-defined general principles and include, for example, a statement about patient informed consent, modalities of study conduct, as well as authors conflicts of interest.

Key elements of trial design:

- Explanation of the rationale of the study
- Description of trial design and sponsorship
- Indication of trial endpoints
- Indication of inclusion and exclusion criteria
- Trial approval and registration
- Informed consent statement

Description of patient population

Electrochemotherapy was initially used with palliative intent. First trials demonstrated remarkable efficiency in the treatment of skin metastases from malignant melanoma.[21,60,63] Subsequently, electrochemotherapy was also evaluated for the treatment of other tumor histotypes (*e.g.*, non-melanoma skin cancers and cutaneous metastases from other tumor histotypes) with equally high success.[20,37-38,47,57-58] Reports of small series indicate also its possible usefulness in the treatment of primary basal cell carcinomas[23] and a clinical trial is currently ongoing comparing the effectiveness of electrochemotherapy to standard surgical resection and is due to report 5 year follow up data next year (EudraCT Number: 2010-019260-37). A particular advantage of electrochemotherapy is that it is a reliable alternative treatment option for patients who have exhausted more conventional oncological treatments or are judged unfit for or refuse repetitive surgical interventions.[47] Therefore, future reports need to include detailed description of patient's demographic and clinical data including detailed description of previous treatments. A detailed description of tumor location, histotype as well as number and size of the electrochemotherapy target and non-target lesions is paramount. Authors should also specify whether targeted lesions had previously received irradiation or not, whether visceral metastases are present and whether the treatment is intended as palliative or curative. Additionally, since electrochemotherapy is finding its place among other oncologic treatments, and will be increasingly used also in combination with them, an accurate record of concomitant treatments is also advisable.[66]

Key elements of patient population:

- Patient demographic data (in tabular form)
- Setting - palliative or curative
- Tumor histology
- Disease stage (lymph node or visceral metastases)
- Description of target lesions treated with electrochemotherapy (anatomical location, number and size)
- Previous local treatments
- Concomitant oncological treatments
- Adjuvant and / or following oncological treatments

Treatment information

The treatment is applied by performing a procedure conjugating the administration of a drug and local application of electric pulses. In one "session"

or "cycle" a single or several tumor nodules can be treated. Since the procedures can be repeated on the same and also on newly emerged tumor nodules, patient treatment may require one or more sessions of electrochemotherapy. Therefore, reports should clearly indicate how many sessions (or cycles) were needed for the treatment of baseline tumors and, overall, for patient management. If retreatment is necessary, the indication should be clear, detailing previous response and disease status in target and non-target tumors. In order to ensure the maximum efficacy, electrochemotherapy needs two key elements: the presence of a cytotoxic agent within tumor tissue and the adequate coverage of tumor with electric pulses above the threshold of reversible membrane electroporation.[67] The results of the ESOPE study and the adoption of SOP that were prepared within the ESOPE project (QLK-2002-02003) were of great importance for the development of electrochemotherapy.[68,24] In fact, they provided practical guidelines and standardization of the procedure. The clinical data evaluation demonstrated that the use of guidelines and a standardized protocol enabled to reach the same level of effectiveness also in the centers without previous experience with electrochemotherapy.[17] The ESOPE study provided evidence for electrochemotherapy in the treatment of skin metastases of different histotypes.[68] It included use of bleomycin or cisplatin as chemotherapeutics, different routes of administration (intravenous or intratumoral) and the use of either local or general anaesthesia. The pulse parameters (number, sequence and amplitudes) for different electrodes were however well defined within the Cliniporator project.[45] A specific electric pulse generator has been consistently used with different electrodes, according to the size, depth and anatomical location of treated tumors.

As confidence with the procedure has developed, treatment indications have also widened. The first studies were based on patients with tumors less than 3 cm, however lesions greater than 3 cm are now routinely treated[57,62,69], representing a natural development of the field based on success with smaller tumors for which the ESOPE guidelines were prepared. As such, there is a need to adapt and revise the SOP and this is already underway. Furthermore, new producers of electric pulse generators are coming to the market, and new electrodes with different design for different treatment settings are emerging. All these changes will make the clinical data evaluation even more challenging. First of all, to address this topic, future reports will need to state the type of anesthesia used (local or general; drugs and doses), the chemotherapeutic agent, drug concentration and dose used, which both depend on the route of administration. The duration of bolus injection, as well as time interval between the drug administration and application of electric pulses, should be specified. The type of electric pulse generator as well as the type of electrodes and their manufacturers should be reported. Additional information should include if the pulse generator is under software control and the specification of the version of that software. If new types of electrodes are used, a detailed description of the design and the sequence and amplitude of pulses is needed. It must be clearly stated whether applied electrodes are needle or plate, the distance between the electrodes, their shape and size, the amplitude of applied electric pulses, their duration, number and repetition frequency. Furthermore, the total number of pulse deliveries, as well as the time interval required for electrode applications after drug injection, should be specified. Additionally, the report of adequate or inadequate coverage of the tumor as well as the way the pulses are applied (e.g., from the margins to the tumor centre or if the pulses were applied in 4+4 (perpendicular) configuration each time) would also be advisable, when possible (Figure 7).[70]

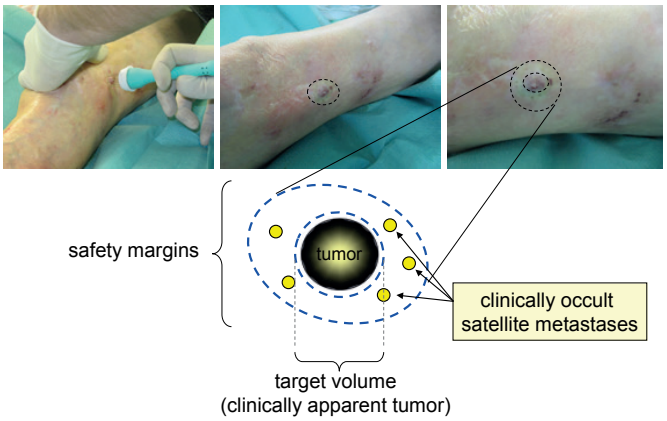

FIGURE 7. Importance of covering whole tumor area along with safety margins. Reporting of the type of electrode applied is essential.

Electrochemotherapy has a high therapeutic index, therefore after successful treatment minimal damage is observed on normal surrounding tissues. During treatment, it is also possible for the treating physician to include a safety margin around the target tumor, depending on tumor size, biologic aggressiveness and propensity for

developing satellite lesions such as in the case of malignant melanoma or soft tissue sarcomas. In order to improve reporting, the information about the safety margins and their extent should also be reported. In addition, electrochemotherapy can be repeated several times (however there is a ceiling for the total lifetime dose of bleomycin), according to tumor response and disease behavior.[62,63] This fundamental aspect is not covered by the currently available SOP. For providing a more informative report, data regarding repetitive treatments should be included, along with the description on what basis the retreatment was performed and at what time interval.

Key elements on treatment information:

- Indication of electroporation protocol (adherence to SOP or other)
- Type of anesthesia
- Drug (producer)
- Drug details (dose, concentration and route of administration
- Time interval between drug administration and application of electric pulses
- Technical details of the electric pulse generator, including type, manufacturer and version of software, if applicable
- Information about the electrodes used, for respective tumor(s)
- Number of electric pulses application per tumor
- Inclusion of a report on electrical parameters (n, T, U, I, f)*
- Adequacy of tumor treatment (treatment application success rate)
- Extent of the safety margins treated
- Number of treatment sessions (with interval between sessions)

* Legend: n = number; T = duration of pulses; U = voltage amplitude applied; I = measured current; f = pulse repetition frequency

Outcome assessment

The early studies on electrochemotherapy antitumor activity have carefully evaluated the response of treated tumors. Response assessment was initially performed by the bidimensional WHO criteria.[71] According to these criteria, baseline and post-treatment tumor size is determined by bidimensional measurements *e.g.* the sum of the two longest diameters in the perpendicular dimensions. The tumor response to treatment is divided into four categories (complete response, partial response, stable disease, progressive disease, according to the change from baseline tumor measurement).

Indeed, most past studies were focused on tumor response and on patient early outcomes. Nevertheless, a number of reports indicate that the disease locally relapsed or progressed elsewhere, but only few reports indicated the value of electrochemotherapy in the local management of patient symptoms. Hence, the clinical benefit for patients, especially in the palliative setting, where preservation of quality of life (QoL) and evaluation of patient reported outcome (PRO) are crucial, should be based on dedicated assessments and described.

The new RECIST (Response Evaluation Criteria in Solid Tumors) version 1.1 criteria, with some adaptations, have proven a suitable tool for response assessment of superficial tumors[21,72], whereas for the setting of treatment of deep-seated tumors (*i.e.*, electrochemotherapy application on liver metastases) the modified RECIST criteria represent the most appropriate and standardized method for the evaluation of tumor response.[73] In general, for standard electrochemotherapy on superficial tumors, the RECIST 1.1 criteria, which are based on one-dimensional measurements, seem even more practical and offer highly concordant response assessment compared with the bidimensional WHO criteria.[74]

So far, most of the published papers do not report on any serious treatment related adverse event after electrochemotherapy. Nevertheless, the process surrounding the determination, recording and reporting of adverse events remains moderately challenging especially for the clinician who may not be involved in drug or device-related research. Nevertheless, it is important to understand the basic definitions of adverse events reporting in order to ensure that the proper information is collected in clinical protocols. Moreover, a comprehensive patient observation and a detailed report of all types and grades of toxicities are essential for providing a comprehensive report of treatment outcome, not only in the early, but also in the long-term follow-up. In this way, only large cohorts of patients will enable in-deep view of long-term toxicity and more detailed analyses of treatment-related adverse events according to different patients subgroup, as demonstrated by a recently published report on electrochemotherapy-related pain.[43] For this purpose, Common Terminology Criteria for Adverse Events (CTCAE v4.0) is widely accepted throughout the oncology community as the standard classification and severity grading scale for

adverse events. Unfortunately, most of the studies conducted so far do not report consistently on this crucial aspect.

Key elements of treatment outcome assessment:

- Time of response assessment
- Standardized response evaluation criteria (e.g. WHO, RECIST 1.1, mRECIST)
- Time to local and systemic disease progression
- Standardized toxicity criteria (e.g. CTCAE v4.0)
- Quality of Life (QoL), patient reported outcomes (PRO)
- Track of patients lost to follow-up

Analysis and interpretation of the results

A clear summary of the trial endpoints is essential. In fact, the field is moving beyond simply reporting on tumor control, as treatment now includes, in some instances, also primary tumors. Here it is important to report and discuss other parameters, such as time to local/systemic progression and, if possible, also the patient survival time and QoL as well. Such data will increase the evidence level of electrochemotherapy effectiveness, and consolidate a role for electrochemotherapy outside the palliative setting and into a confirmed primary treatment modality.

It has been clearly demonstrated that tumor size is the most reliable predictive factor for response in patients who underwent electrochemotherapy.[21,22,69] In future, detailed reports including data on previous local therapies (*e.g.*, radiation) as well as on local (within electrochemotherapy field) tissue status (*e.g.*, presence of lymphedema or fibrosis) and concomitant/adjuvant oncologic treatments would allow for the identification of other reliable predictive indicators for response.

Key elements for analysis and interpretation of the results:

- Summary of trial endpoints
- Additional patient outcome parameters (e.g., QoL, PRO)
- Predictive factors
- Results interpretation
- Future research directions

Conclusions

Electrochemotherapy represents an effective treatment option for an increasing number of cancer patients with superficial tumors. Nevertheless, to further improve its evidence basis, it will be crucial to raise the quality of future reports.

In this study, we have highlighted some relevant aspects of clinical data reporting, with the aim of improving the quality of future studies in the field of electrochemotherapy. Although a large amount of data are published so far, clinical research needs to adopt detailed and accurate reporting as well as moving from small, non-comparative series to well-designed, possibly randomized, clinical trials. Despite the encouraging results indicated, the vast majority of included reports are case series from single institutions. Although there was a wide consensus to use previously published SOP for the treatment protocol, these studies often present a variety of designs and reporting methods, thus limiting the understanding of patient selection, treatment effect, toxicity and overall patient outcome. Of note, published studies often lack sufficient procedural as well as patient data. These shortcomings represent a major hurdle to performing systematic reviews or meta-analysis, which may provide a more robust evaluation of treatment effectiveness and, ultimately, encourage wider acceptance of electrochemotherapy in the clinical practice.

Our study has some limitations. We identified a set of manuscript quality criteria from available literature and we have expanded this list by including additional, procedure-specific criteria that were discussed and agreed among the authors. The list of 47 quality criteria that were used for reviewing published reports represents an arbitrary selection of criteria performed by a relatively small number of authors. There is potential for selection bias in the inclusion of papers for analysis, as the initial screen was based on broad, non-selective inclusion criteria. However, we feel that these were widely inclusive and fitting in order to develop the proposed recommendations. Nevertheless, we believe that our suggestions largely cover the most crucial aspects, which are required to improve the quality of clinical practice and future research: trial design and conduction, definition of study endpoints, patient selection, treatment delivery, patient management and follow-up, standardization of outcome assessment. Our recommendations are open to a broader discussion with the community users of electrochemotherapy and, possibly, to further improvements in line with other interventional

oncology procedures.[75,76] Electrochemotherapy requires standardization of terminology and reporting criteria to facilitate effective communication among researchers and appropriate comparison between different treatment technologies. As such, investigators involved in this field should be familiar with these recommendations and use them for future study design and conduction, treatment application as well as data reporting. We envision that the adoption of these recommendations will further improve the quality of future studies and allow more meaningful comparisons of outcome data of patients treated with electrochemotherapy (Supplementary file).

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References

1. Mali B, Jarm T, Snoj M, Sersa G, Miklavcic D. Antitumor effectiveness of electrochemotherapy: A systematic review and meta-analysis. *EJSO* 2013; **39:** 4-16.
2. Spratt DE, Spratt EAG, Wu SH, DeRosa A, Lee NY, Lacouture ME, et al. Efficacy of skin-directed therapy for cutaneous metastases from advanced cancer: A meta-analysis. *J Clin Oncol* 2014; **32:** 3144-55.
3. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1982; **1:** 841-5.
4. Glass LF, Pepine ML, Fenske NA, Jaroszeski M, Reintgen DS, Heller R. Bleomycin-mediated electrochemotherapy of metastatic melanoma. *Arch Dermatol* 1996; **132:** 1353-7.
5. Heller R, Jaroszeski MJ, Glass LF, Messina JL, Rapaport DP, DeConti RC, et al. Phase I/II trial for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy. *Cancer* 1996; **77:** 964-71.
6. Mir LM, Belehradek M, Domenge C, Orlowski S, Poddevin B, Belehradek J Jr., et al. Electrochemotherapy, a new antitumor treatment: first clinical trial. *C R Acad Sci III* 1991; **313:** 613-18.
7. Mir LM, Orlowski S, Belehradek J Jr., Paoletti C. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. *Eur J Cancer* 1991; **27:** 68-72.
8. Giraud P, Bachaud JM, Teissie J, Rols MP. Effects of electrochemotherapy on cutaneous metastases of human malignant melanoma. *Int J Rad Oncol Biol Phys* 1996; **36:** 1285.
9. Rols MP, Bachaud JM, Giraud P, Chevreau C, Roche H, Teissie J. Electrochemotherapy of cutaneous metastases in malignant melanoma. *Melanoma Res* 2000; **10:** 468-74.
10. Mir LM, Glass LF, Sersa G, Teissie J, Domenge C, Miklavcic D, et al. Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. *Brit J Cancer* 1998; **77:** 2336-42.
11. Glass LF, Fenske NA, Jaroszeski M, Perrott R, Harvey DT, Reintgen DS, et al. Bleomycin-mediated electrochemotherapy of basal cell carcinoma. *J Am Acad Dermatol* 1996; **34:** 82-6.
12. Heller R, Jaroszeski MJ, Reintgen DS, Puleo CA, DeConti RC, Gilbert RA, et al. Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. *Cancer* 1998; **83:** 148-57.
13. Belehradek M, Domenge C, Luboinski B, Orlowski S, Belehradek J Jr., Mir LM. Electrochemotherapy, a new antitumor treatment. First clinical phase I-II trial. *Cancer* 1993; ***72:*** 3694-700.
14. Eisenstein M. A shock to the system. *Nat Meth* 2006; **3:** 66.
15. Kotnik T, Frey W, Sack M, Haberl Meglič S, Peterka M, Miklavčič D. Electroporation-based applications in biotechnology. *Trends Biotechnol* 2015; **33:** 480-8.
16. Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M. Electrochemotherapy in treatment of tumours. *EJSO* 2008; **34:** 232-40.
17. Miklavcic D, Mali B, Kos B, Heller R, Sersa G. Electrochemotherapy: from the drawing board into medical practice. *BioMedical Engineering OnLine* 2014; **13:** 29.
18. Bureau MF, Gehl J, Deleuze V, Mir LM, Scherman D. Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer. *Biochim Biophys Acta* 2000; **1474:** 353-9.
19. Quaglino P, Mortera C, Osella-Abate S, Barberis M, Illengo M, Rissone M, et al. Electrochemotherapy with intravenous bleomycin in the local treatment of skin melanoma metastases. *Ann Surg Oncol* 2008; **15:** 2215-22.
20. Campana LG, Valpione S, Falci C, Mocellin S, Basso M, Corti L, et al. The activity and safety of electrochemotherapy in persistent chest wall recurrence from breast cancer after mastectomy: a phase-II study. *Breast Cancer Res Treat* 2012; **134:** 1169-78.
21. Campana LG, Valpione S, Mocellin S, Sundararajan R, Granziera E, Sartore L, et al. Electrochemotherapy for disseminated superficial metastases from malignant melanoma. *Brit J Surg* 2012; **99:** 821-30.
22. Matthiessen LW, Chalmers RL, Sainsbury DC, Veeramani S, Kessell G, Humphreys AC, et al. Management of cutaneous metastases using electrochemotherapy. *Acta Oncol* 2011; **50:** 621-9.
23. Salwa SP, Bourke MG, Forde PF, O'Shaughnessy M, O'Sullivan ST, Kelly EJ, et al. Electrochemotherapy for the treatment of ocular basal cell carcinoma; a novel adjunct in the disease management. *J Plast Reconstr Aesthet Surg* 2014; **67:** 403-6.
24. Mir LM GJ, Sersa G, Collins CG, Garbay JR, Billard V, et al. Standard operating procedures of the electrochemotherapy: Instructions for the use of bleomycin or cisplatin administered either systemically or locally and electric pulses delivered by the Cliniporator by means of invasive or non-invasive electrodes. *EJC Suppl* 2006; **4:** 14-25.
25. Miklavcic D, Snoj M, Zupanic A, Kos B, Cemazar M, Kropivnik M, et al. Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. *BioMedical Engineering OnLine* 2010; **9:** 10.
26. Edhemovic I, Gadzijev EM, Brecelj E, Miklavcic D, Kos B, Zupanic A, et al. Electrochemotherapy: a new technological approach in treatment of metastases in the liver. *Tecnol Cancer Res Treat* 2011; **10:** 475-85.
27. Miklavcic D, Sersa G, Brecelj E, Gehl J, Soden D, Bianchi G, et al. Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. *Med Biol Eng Comput* 2012; **50:** 1213-25.
28. Soden D, Larkin J, Collins C, Piggott J, Morrissey A, Norman A, et al. The development of novel flexible electrode arrays for the electrochemotherapy of solid tumour tissue. (Potential for endoscopic treatment of inaccessible cancers). *Conf Proc IEEE Eng Med Biol Soc* 2004; **5:** 3547-50.
29. Edhemovic I, Brecelj E, Gasljevic G, Marolt Music M, Gorjup V, Mali B, et al. Intraoperative electrochemotherapy of colorectal liver metastases. *J Surg Oncol* 2014; **110:** 320-7.
30. Journals unite for reproducibility. *Nature* 2014; **515:** 7.
31. Miklavcic D. Network for development of electroporation-based technologies and treatments: COST TD1104. *J Membrane Biol* 2012; **245:** 591-8.
32. Khan AA, Clover AJ. New guidelines for reporting observational studies and their implications for plastic surgery (STROBE). *J Plast Reconstr Aesthet Surg* 2009; **62:** 155-6.

33. Al-Benna S, Clover J. The role of the journal impact factor: choosing the optimal source of peer-reviewed plastic surgery information. *Plast Reconstr Surg* 2007; **119:** 755-6.

34. Chang SM, Reynolds SL, Butowski N, Lamborn KR, Buckner JC, Kaplan RS, et al. GNOSIS: guidelines for neuro-oncology: standards for investigational studies-reporting of phase 1 and phase 2 clinical trials. *Neuro Oncol* 2005; **7:** 425-34.

35. Trabulsi NH, Patakfalvi L, Nassif MO, Turcotte RE, Nichols A, Meguerditchian AN. Hyperthermic isolated limb perfusion for extremity soft tissue sarcomas: systematic review of clinical efficacy and quality assessment of reported trials. *J Surg Oncol* 2012; **106:** 921-8.

36. Mariani L, Marubini E. Content and quality of currently published phase II cancer trials. *J Clin Oncol* 2000; **18:** 429-36.

37. Rotunno R, Marenco F, Ribero S, Calvieri S, Amerio P, Curatolo P, et al. Electrochemotherapy in non-melanoma head and neck skin cancers: a three centers experience and literature review. *G Ital Dermatol Venereol* 2015; in press

38. Cabula C, Campana LG, Grilz G, Galuppo S, Bussone R, De Meo L, et al. Electrochemotherapy in the treatment of cutaneous metastases from breast cancer: A multicenter cohort analysis. *Ann Surg Oncol* 2015; **22 (Suppl 3):** 442-50.

39. Mozzillo N, Simeone E, Benedetto L, Curvietto M, Giannarelli D, Gentilcore G, et al. Assessing a novel immuno-oncology-based combination therapy: Ipilimumab plus electrochemotherapy. *Oncoimmunology* 2015; **4(6):** e1008842.

40. Landstrom FJ, Reizenstein J, Adamsson GB, Beckerath M, Moller C. Long-term follow-up in patients treated with curative electrochemotherapy for cancer in the oral cavity and oropharynx. *Acta Otolaryngol* 2015; **135:** 1070-8.

41. Granata V, Fusco R, Piccirillo M, Palaia R, Petrillo A, Lastoria S, et al. Electrochemotherapy in locally advanced pancreatic cancer: Preliminary results. *Int J Surg* 2015; **18:** 230-6.

42. Kreuter A, van Eijk T, Lehmann P, Fischer M, Horn T, Assaf C, et al. Electrochemotherapy in advanced skin tumors and cutaneous metastases - a retrospective multicenter analysis. *J Dtsch Dermatol Ges* 2015; **13:** 308-15.

43. Quaglino P, Matthiessen LW, Curatolo P, Muir T, Bertino G, Kunte C, et al. Predicting patients at risk for pain associated with electrochemotherapy. *Acta Oncologica* 2015; **54:** 298-306.

44. Mir-Bonafe JM, Vilalta A, Alarcon I, Carrera C, Puig S, Malvehy J, et al. Electrochemotherapy in the treatment of melanoma skin metastases: a report on 31 cases. *Actas Dermosifiliogr* 2015; **106:** 285-91.

45. Campana LG, Mali B, Sersa G, Valpione S, Giorgi CA, Strojan P, et al. Electrochemotherapy in non-melanoma head and neck cancers: a retrospective analysis of the treated cases. *Brit J Oral Maxillofac Surg* 2014; **52:** 957-64.

46. Ricotti F, Giuliodori K, Cataldi I, Campanati A, Ganzetti G, Ricotti G, et al. Electrochemotherapy: an effective local treatment of cutaneous and subcutaneous melanoma metastases. *Dermatol Ther* 2014; **27:** 148-52.

47. Campana LG, Galuppo S, Valpione S, Brunello A, Ghiotto C, Ongaro A, et al. Bleomycin electrochemotherapy in elderly metastatic breast cancer patients: clinical outcome and management considerations. *J Cancer Res Clin Oncol* 2014; **140:** 1557-65.

48. Seccia V, Muscatello L, Dallan I, Bajraktari A, Briganti T, Ursino S, et al. Electrochemotherapy and its controversial results in patients with head and neck cancer. *Anticancer Res* 2014; **34:** 967-72.

49. Caraco C, Mozzillo N, Marone U, Simeone E, Benedetto L, Di Monta G, et al. Long-lasting response to electrochemotherapy in melanoma patients with cutaneous metastasis. *BMC Cancer* 2013; **13:** 564.

50. Campana LG, Bianchi G, Mocellin S, Valpione S, Campanacci L, Brunello A, et al. Electrochemotherapy treatment of locally advanced and metastatic soft tissue sarcomas: Results of a non-comparative phase II study. *World J Surg* 2014; **38:** 813-22

51. Solari N, Spagnolo F, Ponte E, Quaglia A, Lillini R, Battista M, et al. Electrochemotherapy for the management of cutaneous and subcutaneous metastasis: a series of 39 patients treated with palliative intent. *J Surg Oncol* 2014; **109:** 270-4.

52. Di Monta G, Caraco C, Benedetto L, La Padula S, Marone U, Tornesello ML, et al. Electrochemotherapy as "new standard of care" treatment for cutaneous Kaposi's sarcoma. *EJSO* 2014; **40:** 61-6.

53. Perrone AM, Galuppi A, Cima S, Pozzati F, Arcelli A, Cortesi A, et al. Electrochemotherapy can be used as palliative treatment in patients with repeated loco-regional recurrence of squamous vulvar cancer: a preliminary study. *Gynecol Oncol* 2013; **130:** 550-3.

54. Benevento R, Santoriello A, Perna G, Canonico S. Electrochemotherapy of cutaneous metastastes from breast cancer in elderly patients: a preliminary report. *BMC Surg* 2012; **12 (Suppl 1):** S6.

55. Mevio N, Bertino G, Occhini A, Scelsi D, Tagliabue M, Mura F, et al. Electrochemotherapy for the treatment of recurrent head and neck cancers: preliminary results. *Tumori* 2012; **98:** 308-13.

56. Latini A, Bonadies A, Trento E, Bultrini S, Cota C, Solivetti FM, et al. Effective treatment of Kaposi's sarcoma by electrochemotherapy and intravenous bleomycin administration. *Dermatol Ther* 2012; **25:** 214-8.

57. Matthiessen LW, Johannesen HH, Hendel HW, Moss T, Kamby C, Gehl J. Electrochemotherapy for large cutaneous recurrence of breast cancer: a phase II clinical trial. *Acta Oncol* 2012; **51:** 713-21.

58. Gargiulo M, Papa A, Capasso P, Moio M, Cubicciotti E, Parascandolo S. Electrochemotherapy for non-melanoma head and neck cancers: clinical outcomes in 25 patients. *Ann Surg* 2012; **255:** 1158-64.

59. Curatolo P, Quaglino P, Marenco F, Mancini M, Nardo T, Mortera C, et al. Electrochemotherapy in the treatment of Kaposi sarcoma cutaneous lesions: a two-center prospective phase II trial. *Ann Surg Oncol* 2012; **19:** 192-8.

60. Kis E, Olah J, Ocsai H, Baltas E, Gyulai R, Kemeny L, et al. Electrochemotherapy of cutaneous metastases of melanoma--a case series study and systematic review of the evidence. *Dermatol Surg* 2011; **37:** 816-24.

61. Skarlatos I, Kyrgias G, Mosa E, Provatopoulou X, Spyrou M, Theodorou K, et al. Electrochemotherapy in cancer patients: first clinical trial in Greece. *In Vivo* 2011; **25:** 265-74.

62. Campana LG, Mocellin S, Basso M, Puccetti O, De Salvo GL, Chiarion-Sileni V, et al. Bleomycin-based electrochemotherapy: clinical outcome from a single institution's experience with 52 patients. *Ann Surg Oncol* 2009; **16:** 191-9.

63. Quaglino P, Mortera C, Osella-Abate S, Barberis M, Illengo M, Rissone M, et al. Electrochemotherapy with intravenous bleomycin in the local treatment of skin melanoma metastases. *Ann Surg Oncol* 2008; **15:** 2215-22.

64. Larkin JO, Collins CG, Aarons S, Tangney M, Whelan M, O'Reily S, et al. Electrochemotherapy: aspects of preclinical development and early clinical experience. *Ann Surg* 2007; **245:** 469-79.

65. Gaudy C, Richard MA, Folchetti G, Bonerandi JJ, Grob JJ. Randomized controlled study of electrochemotherapy in the local treatment of skin metastases of melanoma. *J J Cutan Med Surg* 2006; **10:** 115-21.

66. Valpione S, Campana LG, Pigozzo J, Chiarion-Sileni V. Consolidation electrochemotherapy with bleomycin in metastatic melanoma during treatment with dabrafenib. *Radiol Oncol* 2015; **49:** 71-4.

67. Miklavcic D, Corovic S, Pucihar G, Pavselj N. Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. *EJC Suppl* 2006; **4:** 45-51.

68. Marty M SG, Garbay JR, Gehlc J, Collinsd CG, Snoj M et al. Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. *EJC Suppl* 2006; **4:** 3-13.

69. Mali B, Miklavcic D, Campana LG, Cemazar M, Sersa G, Snoj M, et al. Tumor size and effectiveness of electrochemotherapy. *Radiol Oncol* 2013; *47:* 32-41.

70. Sersa G, Cemazar M, Semrov D, Miklavcic D. Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice. *Bioelectroch Bioener* 1996; **39:** 61-6.

71. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. *Cancer* 1981; **47:** 207-14.

72. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45:** 228-47.

73. Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Disease* 2010; **30:** 52-60.

74. Choi JH, Ahn MJ, Rhim HC, Kim JW, Lee GH, Lee YY, et al. Comparison of WHO and RECIST criteria for response in metastatic colorectal carcinoma. *Cancer Res Treat* 2005; **37:** 290-3.

75. Goldberg SN, Grassi CJ, Cardella JF, Charboneau JW, Dodd GD, Dupuy DE, et al. Image-guided tumor ablation: Standardization of terminology and reporting criteria. *J Vasc Interv Radiol* 2009; **20:** S377-S90.

76. Callstrom MR, York JD, Gaba RC, Gemmete JJ, Gervais DA, Millward SF, et al. Research reporting standards for image-guided ablation of bone and soft tissue tumors. *J Vasc Interv Radiol* 2009; **20:** 1527-40.

Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review

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CHECKLIST

Recommendations and minimal requirements for reporting clinical trial results on electrochemotherapy (key elements)

Trial design:

- ☐ Explanation of the rationale of the study
- ☐ Description of trial design and sponsorship
- ☐ Indication of trial endpoints
- ☐ Indication of inclusion and exclusion criteria
- ☐ Trial approval and registration
- ☐ Informed consent statement

Patient population:

- ☐ Patient demographic data (in tabular form)
- ☐ Setting - palliative or curative
- ☐ Tumor histology
- ☐ Disease stage (lymph node or visceral metastases)
- ☐ Description of target lesions treated with electrochemotherapy (anatomical location, number and size)
- ☐ Previous local treatments
- ☐ Concomitant oncological treatment
- ☐ Adjuvant and / or following oncological treatments

Treatment information:

- ☐ Indication of electroporation protocol (adherence to SOP or other)
- ☐ Type of anesthesia
- ☐ Drug (producer)
- ☐ Drug details (dose, concentration, route of administartion)
- ☐ Time interval between drug administration and application of electric pulses
- ☐ Technical details of the electric pulse generator, including type, manufacturer and version of software, if applicable
- ☐ Information about the electrodes used, for respective tumor(s)
- ☐ Number of electric pulses application per tumor
- ☐ Inclusion of a report on electrical parameters (n, T, U, I, f)*
- ☐ Adequacy of tumor treatment (treatment application success rate)
- ☐ Extent of the safety margins treated
- ☐ Number of treatment sessions (with interval between sessions)

* Legend: n = number; T = duration of pulses; U = voltage amplitude applied; I = current measured; f = pulse repetition frequency

Treatment outcome assessment:

- ☐ Time of response assessment
- ☐ Standardized response evaluation criteria (*e.g.* WHO, RECIST1.1, mRECIST)
- ☐ Time to local and systemic disease progression
- ☐ Standardized toxicity criteria (*e.g.* CTCAE v4.0)
- ☐ Quality of Life (QoL), patient reported outcomes (PRO)
- ☐ Track of patients lost to follow-up

Analysis and interpretation of results:

- ☐ Summary of trial endpoints
- ☐ Additional outcome parameters (*e.g.*, QoL, PRO)
- ☐ Predictive factors
- ☐ Results interpretation
- ☐ Future research directions

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Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes

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ABSTRACT

The application of pulsed electric field (PEF) technology as a non-thermal cell membrane permeabilization treatment, was widely demonstrated widely to be effective in microbial inactivation studies, as well as to increase the rates of heat and mass transfer phenomena in food and biotechnological processes (drying, osmotic treatment, freezing, extraction, and diffusion). Nevertheless, most published papers on the topic do not provide enough information for other researchers to assess results properly. A general rule/guidance in reporting experimental data and most of all exposure conditions, would be to report details to the extent that other researchers will be able to repeat, judge and evaluate experiments and data obtained. This is what is described in the present recommendation paper. *Industrial relevance:* Pulsed electric field (PEF) treatment is a promising technology that has received considerable attention in food and biotechnology related applications food and biotechnology related applications of PEF include:

i) "cold" pasteurization of liquid foods and disinfection of wastewater by microbial inactivation
ii) PEF-assisted processing (drying, extraction or expression)

1. Introduction

Pulsed electric field (PEF) treatment is considered to be a promising technology that has in the last years received considerable attention in food and biotechnology related applications in the last years (Haberl, Miklavčič, Serša, Frey, & Rubinsky, 2013; Kotnik et al., 2015; Puértolas, Luengo, Álvarez, & Raso, 2012). The treatment bases on the application of electric pulses of high voltage and short duration (μs–ms) (Mahnič-Kalamiza, Vorobiev, & Miklavčic, 2014) to biomaterials of plant or animal origin, or suspensions of microorganisms placed between two electrodes. As a result, the biological material is exposed to an electric field whose intensity depends on the voltage across the electrodes, as well as on the geometry of both the electrodes and the interelectrode space containing the material to be treated. PEF impact causes membrane permeabilization, also synonymously termed as electroporation, leading to an increased permeability of the membrane to ions and molecules (Kotnik, Kramar, Pucihar, Miklavčič, & Tarek, 2012).

Depending on the intensity of the treatment applied (external electric field, single pulse duration, treatment time) and the cell characteristics (size, shape, orientation in the electric field), the viability of the electroporated cell can be preserved by recovering the membrane integrity, or the electroporation can permanently lead to cell death. Cell size differences between plant and microbial cells, give a wide range of treatment intensity: 0.5–1.5 kV/cm for induction of stress responses and reversible electroporation, 1.0–3.0 kV/cm for irreversible permeabilization in plant or animal tissues, and 15–40 kV/cm for microbial inactivation. Reversible "electroporation" is a procedure usually used in molecular biology and clinical biotechnological applications in vivo to gain access to the cytoplasm in order to introduce or deliver in vivo drugs, oligonucleotides, antibodies, plasmids, etc. (Miklavčič, Mali,

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Kos, Heller, & Serša, 2014; Yarmush, Golberg, Serša, Kotnik, & Miklavčič, 2014; Zorec, Préat, Miklavčič, & Pavšelj, 2013). However most of food and biotechnology related applications of PEF are based on irreversible permeabilization of the cell membranes and mainly include: i) "cold" pasteurization of liquid foods and disinfection of wastewater by microbial inactivation (Frey, Gusbeth, & Schwartz, 2013; Saldaña, Álvarez, Condón, & Raso, 2014); ii) PEF-assisted processing (drying, osmotic dehydration, freeze-drying, freezing, thawing, extraction or expression) for improving food quality, accelerating heat transfer processes, as well as enhancing the mass transfer efficiency of water, solutes (e.g., osmotic agents, cryoprotectants), juices, or high added value compounds from matrices of biological origin, such as plant tissues, suspension of microbial or algae cells, food waste and by-products generated during food processing, or agricultural and forestry residues (Donsì, Ferrari, & Pataro, 2010; Goettel, Eing, Gusbeth, Straessner, & Frey, 2013; Jalte, Lanoiselle, Lebovka, & Vorobiev, 2009; Mahnič-Kalamiza et al., 2014; Parniakov, Lebovka, Bals, & Vorobiev, 2015, 2016a,b; Phoon, Galindo, Vicente, & Dejmek, 2008; Puértolas et al., 2012; Sack et al., 2008; Wiktor et al., 2013).

Technical limitations impeded the exploitation of PEF at an industrial level during many years. The lack of reliable and viable industrial equipment was indeed a critical factor to support up-scaling of the volumes to be treated (from mL to m^3) (Sack et al., 2010; Toepfl, 2012). Large treatment volumes required a shift from the well-established batch methodologies used in basic science towards the flow processes, which is nowadays possible due to the recent developments in pulsed power generators. Other critical aspects that have contributed to limit the spread of PEF technology, are the poor description of the operating protocols, the control and monitoring of the pulse parameters, and the lack of a standardized way of reporting treatment conditions. As the first commercial applications of PEF technology are now available (Golberg et al., 2016), more details on the reports published on the new innovative research are required to improve the reproducibility of treatments when used at industrial level. Lack of such information is a barrier for the development and wide use of the technology.

Variability in results obtained in different laboratories on PEF research may be a consequence of a number of reasons including differences in PEF equipment and PEF treatment conditions. Additionally, in microbial inactivation works and in algae processing, the growth or cultivation conditions of the microorganisms, the treatment medium and the recovery conditions, can play an important role to the outcome of the process. Furthermore, pre and post-treatment conditions can considerably influence the efficiency of the PEF-assisted mass transfer processing.

Different aspects of experimental procedures (biological and engineering) must be described in sufficient details to allow the work to be reproduced in other laboratories. All data must be obtained by paying attention to statistical detail in the planning stage. If a sufficiently large number of replicates are not organized before the experiment is undertaken, biological variation is not eliminated satisfactorily. Replicate design has been recognized to be important to biological experiments for a considerable time (Dhand, 2014; McNutt, 2014).

This recommendation paper has been prepared based on initiative of the Steering Committee of the COST TD 1104 Action (www.electroporation.net), due to increased concern and awareness of low quality reporting practice. Specifically, it has been adopted by the committee within the Working group of Food and Biotechnology of electroporation, in COST action TD 1104 EP4Bio2Med (Miklavčič, 2012), and is in the series of publications that addresses the same topic in Preclinical research in electroporation as well as in the field of medical use of electroporation (Campana et al., 2016).

The objective of this paper is to provide recommendation guidelines on the key information that should be reported in studies regarding the application of PEF for microbial inactivation or PEF-assisted processing in food and biotechnological field. These guidelines are intended to facilitate the comparison of data, to create a reliable basis for a better understanding on the influence of different factors on the PEF efficacy, as well as on the involved mechanisms. It can also be expected that this report may help new researchers in the field to obtain data which are repeatable, reproducible and free from methodological errors.

2. Pulsed electric field (PEF) processing

2.1. Processing parameters

The most typical process parameters that characterize PEF technology are amplitude of electric pulses (U), electric field strength (E), treatment time (t), pulse shape, pulse width (τ), number of pulses (n), pulse specific energy (W) and pulse repetition frequency (f).

The electric field strength and the treatment time are the main process parameters that define the PEF treatment intensity.

Electric field strength refers to the field strength locally present in the treatment chamber during the sample treatment, and depends on the voltage applied between the electrodes, geometry of the treatment chamber, and the spatial distribution of dielectric properties of the material between the electrodes. For parallel plate electrode configuration of batch or continuous treatment chambers, apart from some edge effects (Donsì, Ferrari, & Pataro, 2007), the electric field is homogeneous within the interelectrode space (Fig. 1), and can be estimated by dividing the voltage (U) measured across the electrodes of the treatment chamber by the electrode distance (L):

$$E = \frac{U}{L} \tag{1}$$

In contrast, other chamber configurations, such as co-linear electrode configuration (Fig. 1), suffer from a non-uniform distribution of the electric field in the treatment zone, where the actual field strength is often lower than the estimation predicted by Eq. (1). Therefore, in such cases, several approaches based on numerical simulation procedures have been considered for obtaining a more accurate estimation of the actual field strength applied, such as those based on a graph showing the field strength distribution along the central axis of the treatment zone (Toepfl, Heinz, & Knorr, 2007), determination of the lowest electric field strength for the entire volume of the treatment zone (Meneses, Jaeger, Moritz, & Knorr, 2011) or considering different volume elements and calculation of an average field strength for the entire volume of the treatment zone (Gerlach et al., 2008).

Treatment time refers to the number of pulses applied multiplied by the pulse width (or pulse duration):

$$t = n \cdot \tau \tag{2}$$

where τ depends on the pulse shape. As it is shown in Fig. 2, the pulse shapes commonly used in PEF treatments are either exponential or square-wave pulses, unipolar or bipolar. Voltage and current waveforms of the electric pulses delivered in the treatment chamber, should be monitored continuously using high voltage and fast high current probes located as close as possible to the treatment chamber, in order to precisely define the treatment intensity. Generally, in fact, the voltage output from the pulse power is lower than the voltage measured in the treatment chamber, especially for chamber configuration characterized by a low intrinsic electrical resistance.

Thus, in order to accurately describe processing conditions, pulse characteristics, including peak voltage, pulse shape, pulse width, and pulse polarity, should be reported. To this purpose, a snapshot of the monitored pulses (voltage, current) delivered to the treatment chamber, should be provided, which implies that a digitized recording is included in the set-up of the PEF system.

Pulse duration, or *pulse width,* for a square pulse is the time that the voltage is kept at the maximum value (peak voltage) (Reberšek, Miklavčič, Bertacchini, & Sack, 2014). In the case of exponential decay

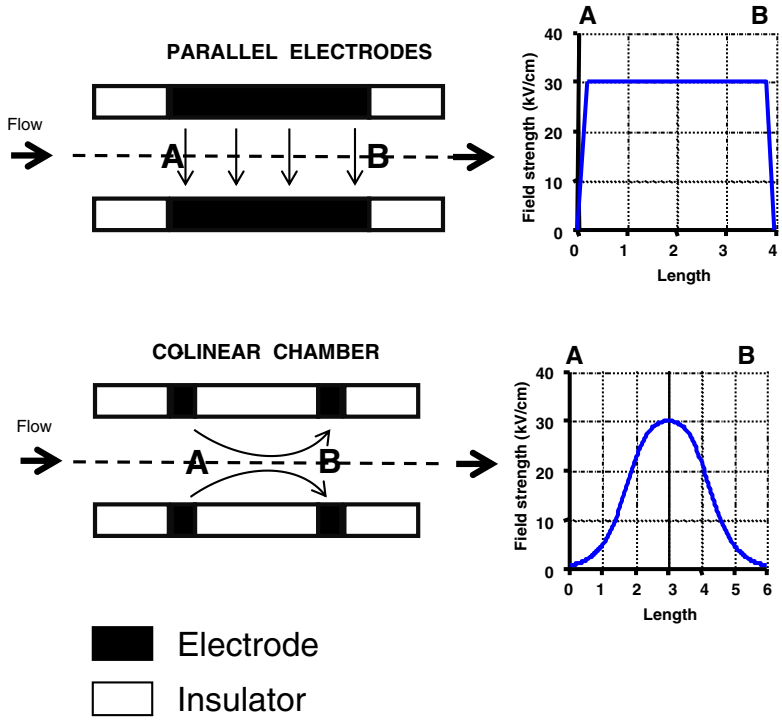

Fig. 1. Schematics of parallel plate and co-linear treatment chamber configuration with qualitative distribution of the electric field in the treatment zone.

pulses, the pulse width is defined as the time needed to decrease the voltage to 37% of its peak value (Fig. 2).

Frequency and protocol of application of the series of pulses should be also documented. Frequency indicates the number of pulses applied by unit of time, and it is reported in Hz (number of pulses/s). The specification of the pulse frequency is important, since it determines the amount of electrical energy delivered per unit of time on the product placed in the treatment chamber, which, in turn, affects the temperature increase of the processed product due to Joule effect. Moreover, pulse frequency has been proved to be, among others, a key factor affecting the extent of the unavoidable electrochemical reaction which occurs at the electrode-liquid interface of the treatment chamber, especially those involving the migration of metal from the electrodes into the biological matrices (Kotnik, Miklavčič, & Mir, 2001; Pataro, Barca, Donsì, & Ferrari, 2015a,b; Pataro, Falcone, Donsì, & Ferrari, 2014). This is a very important issue, since the metal released may affect microbial inactivation or may further react with the biomaterials present in the bulk, also after the application of the pulse treatment, as well as negatively affect the efficiency of the PEF treatment with time, and reduce the electrode lifetime (corrosion).

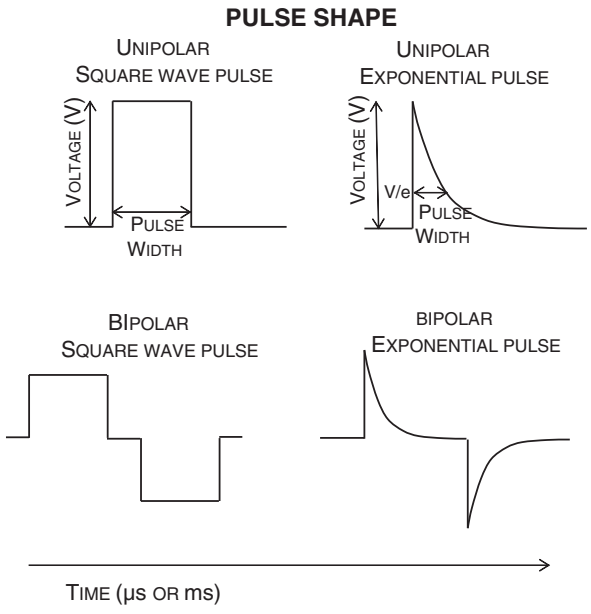

Fig. 2. Pulse shapes commonly used in PEF treatments.

In addition to pulse frequency, pulse protocol should be also described in detail. For batch treatment, number of pulses applied per each train, number of trains of pulses and time interval between two consecutive trains, should be reported. For continuous flow treatment, the number of recirculation of the treated product through the PEF chamber, should also be specified. Moreover, it is worth remembering that, in batch treatment the number of electric pulses to be applied is set directly by the user. In continuous flow process, instead, it is a function of the pulse frequency and residence time (t_r) of the product in the treatment chamber, which depends on the flow rate (F) and volume (v) of the treatment zone, according to the following equation:

$$n = t_r \cdot f = \frac{v}{F} \cdot f \tag{3}$$

The *energy density* or *specific energy per pulse* (W, in kJ/kg/pulse) is the electrical energy received by the treated product in the PEF chamber per each pulse. It depends on the electrical properties of the treated product and on the pulse shape (including peak voltage and pulse width). The electrical properties of treated product are changing —

conductivity is increasing for two reasons: membrane electroporation resulting in increased conductivity and due to diffusion of ions from cells to water/media, usually of low conductance at the beginning of the treatment. Due to the losses through the connections and the components of the discharge circuit, the value of W is generally different from the energy output from the pulse generator. Moreover, waveforms of voltage and current can considerably deviate from the ideal square or exponential shapes. Therefore, according to Eq. (4), the specific energy input per pulse W should be evaluated by the integral over time of the recorded waveforms of voltage and current at the treatment chamber.

$$W = \frac{1}{m}\int_0^{\infty}\frac{U(t)^2}{R}dt = \frac{1}{m}\int_0^{\infty}U(t)\cdot I(t)dt \tag{4}$$

where m is the mass of treated sample, $U(t)$ and $I(t)$ are, respectively, the voltage across and current through the treatment chamber load at time t. R (in Ω) is the electrical resistance of the treatment chamber, which can be calculated according to the following equation:

$$R = \frac{1}{\sigma}\frac{L}{A} \tag{5}$$

where σ is the electrical conductivity of the treated product (S/m) and A is the electrode area (m^2).

The *total specific energy input* (W_T, kJ/kg) can be calculated by multiplying W with the number of pulses applied:

$$W_T = W \cdot n \tag{6}$$

Electric field strength and total specific energy input (instead of treatment time) have been suggested as suitable parameters enabling to compare the data obtained under distinct conditions and equipments (Heinz, Alvarez, Angersbach, & Knorr, 2002). Particularly, total specific energy input should be preferred instead of treatment time especially when exponentially decay pulses are applied. Furthermore, the specification of the total energy input will also give an estimation of the energy consumption due to the PEF process.

Temperature is also a critical parameter that influences the efficacy of PEF treatment. Several reports described an enhanced microbial inactivation or cell degree permeabilization upon increasing the PEF treatment temperature (Lebovka, Praporscic, Ghnimi, & Vorobiev, 2005; Saldaña et al., 2010). This PEF-temperature synergy is likely due to the fact that membrane of biological cells become more fluid and their mechanical resistance decreases with increasing the processing temperature (Coster & Zimmermann, 1975), making the cell membrane more prone to electroporation.

On the other hand, the dissipation of the electrical energy delivered to the product during PEF treatment increases the temperature of the product, which in turn increases the electrical conductivity and may modify product viscosity. As a consequence, the increment of the product temperature may lower the resistance of the treatment chamber, leading to a decrease of the applied field strength, unless the external voltage is not increased accordingly. In addition, in continuous processes, flow rate and residence time of the processed product in the treatment chamber may also change as a result of the product temperature increase. Moreover, temperature increase may also lead to an overestimation of the effectiveness of the treatment due to the sensitizing effect of the temperature on the PEF resistance of biological cells (Heinz, Toepfl, & Knorr, 2003). It has been demonstrated that using static parallel electrode treatment chamber with temperature-controlled electrodes allows to obtain data on microbial inactivation at different temperatures at quasi-isothermal conditions preventing the artefacts caused by temperature increase when no temperature control of electrodes is used (Saldaña et al., 2010).

Temperature increase, as a consequence of Joule heating, is enhanced at higher electric fields, total specific energy, frequencies and pulse widths. Therefore, optimal processing conditions for studying the influence of these factors on the outcome of the PEF process should be chosen by minimizing the related heating effects, for instance by using treatment chambers in which it is possible to cool the electrodes (Saldaña et al., 2010). In any case, in batch treatments the initial temperature of the product as well as the final temperature after the application of the PEF treatment should be documented. In continuous flow processes, the temperature of the product entering the treatment chamber (inlet temperature) and that at the exit of the treatment chamber (outlet temperature) should be measured and reported (Meneses, Jaeger, & Knorr, 2011). An adequate description of the methods used for pre-heating the product before entering the PEF treatment chamber, as well as for cooling the treated product at the exit of the PEF chamber, should also be provided. Moreover, it is also necessary to specify the location of the temperature sensors in relation to the treatment chamber. When the experimental setup consists in several continuous flow treatment chambers connected in series, the temperature sensors should be located immediately before and after each treatment chamber, especially when the treated product is cooled in heat exchangers placed in between two consecutive treatment chambers. Temperature sensors whose measurement is not influenced by the electric field should be used. If a direct temperature measurement is not possible, the resulting temperature increase of the treated product can be estimated based on a calculation of the total specific energy and assuming adiabatic heating, i.e. all electrical energy is converted to heat.

2.2. PEF equipment

An appropriate description of the PEF generator and treatment chamber used to conduct the experiments should be provided. For commercial equipment, the name of the supplier company and the model should be specified. If the PEF generator is a laboratory prototype or specially fabricated unit, an adequate description of the components (power supply, capacitors, switches, transformers, etc), electrical configuration, measurement and data acquisition systems, and any other pertinent information that characterizes the equipment to reproduce exposure of sample to pulsed electric field should be provided. Laboratory studies on either microbial inactivation or improving mass transfer phenomena by PEF may be conducted in batch or in continuous flow treatment chambers that should be described in details.

The two most used treatment chamber designs considered for application of PEFs in continuous flow are parallel plate electrodes and co-linear configurations (Fig. 1). Parallel plate electrode configuration is the simplest in design and consists of a rectangular parallelepiped shape channel of insulating material with two electrodes on opposite sides. As previously reported, this configuration typically provides uniform electric field in the treatment zone, with the applied electric field being perpendicular to the product flow. However, because it is characterized by a large electrode surface and low intrinsic electrical resistance, it generally operates at high current, which also may facilitate the triggering of undesired electrochemical phenomena at the electrode-liquid food interface of the PEF chamber (electrode corrosion). In the co-linear configuration, couples of tubular electrodes are spaced with insulator spacer tubes. The product is treated as it flows from one electrode to the other, parallel to the electric field. Such configuration has advantageous fluid dynamics, highly desiderate for food processing and convenient for cleaning in place, as well as a high intrinsic resistance due to the low effective area of the cross section of the tubular electrodes. Thus, this device typically operates at lower current than the parallel plate configuration, which makes it suitable for limiting the occurrence of electrode reactions, as well as for the connection of multiple co-linear units in parallel from the electrical viewpoint. The main problem of this configuration is in-homogeneity in the electric field strength and temperature distribution in the treatment zone during PEF processing. Therefore, an adequate chamber design is required in order to ensure more uniform distribution of the electric field

(generally sufficiently high ratio between electrode gap and area, as well as rounded edges of the electrodes, are recommended).

A further cause of treatment inhomogeneity in both parallel plate electrode and co-linear treatment chamber, which is most important in microbial inactivation studies, is the existence of laminar flow into the treatment zone. This is because the higher flow rate required to promote turbulent flow conditions needs a higher pulse modulator power, as well as a higher commutation rate of the switching devices, in order to deliver the required amount of energy per volume element. Therefore, the use of the highest possible flow rate (Pataro, Senatore, Donsí, & Ferrari, 2011), as well as the generation of turbulent flow by modifying the treatment chamber geometry or by inserting a grid in front of the treatment zone have been suggested to improve the treatment uniformity (Meneses, Jaeger, Moritz and Knorr, 2011). Thus, in addition to the electrical parameters, also the flow conditions (rates, laminar or non-laminar) should be reported in studies regarding PEF applications (Jaeger, Meneses, & Knorr, 2009).

As already recommended for power supplies, the name of the supplier company and the model number should be specified for commercial treatment chambers. If the treatment chamber is a prototype or specially fabricated, an adequate description is required. A schematic drawing of the treatment chamber details, which describes the geometrical shape of insulator and electrode along the boundary to the material to be treated, should be provided. Additionally, the material of the electrodes and insulators, and the most relevant sizes such as electrode gap, surface area or dimensions of the electrodes (e.g., diameter of the tube for co-linear configurations), treatment volume, i.e. the volume where the specified electric field strength is present, should be reported.

PEF processing for industrial applications requires continuous flow processing, thus the results obtained in batch treatments need to be validated in a continuous flow installation before they can be successfully implemented on a large scale. Recently, there has been considerable progress in the development of both pulse generators and continuous flow treatment chambers design that are essential for scaling up the technology for industrial applications (Huang & Wang, 2009). In this frame, however, further studies based on the development and application of characteristic (dimensionless) numbers are necessary. This would lead to a more targeted approach for industrial scale-up and application of the PEF technology.

A detailed knowledge or a good estimation of the values assumed to be the critical process parameters inside the treatment chamber during processing of biological matrices is required. However, the small dimensions of the treatment chambers may make impossible to perform adequate measurements of the process parameters inside the treatment chamber with the corresponding probes without perturbation of the flow, temperature, and electric field distribution (Jaeger et al., 2009). Therefore, it is recommended to use numerical simulation techniques to provide information on the spatial and temporal distribution of the electric field strength, temperature, and flow velocity inside the treatment chambers. Moreover, it is worth noting that a numerical approach would also allow the use of local and time resolved information, which could help in obtaining insight in the mechanisms of action of the PEF technology, with respect to an analyses based only on integral values.

3. Microbial inactivation by PEF

Many studies on microbial inactivation by PEF have been conducted and reported in the literature. The technology of PEF follows general principles, however the numerous factors affecting microbial inactivation by PEF, the broad experimental conditions used by different research groups and the diversity of equipment available limits the comparison of results, the standardization of experimental procedures used in different laboratories and obtaining solid conclusions in this topic.

Due to the difficulty to standardize experimental procedures used in different laboratories, information that should be provide for researchers in any study of microbial inactivation by PEF are shown in Table 1.

3.1. Microorganism and culture conditions

It has been observed that there is a great variation in the sensitivity of different strains of the same species of bacteria to PEF treatments (Lado & Yousef, 2003; Saldaña et al., 2009). Therefore the strain of the

Table 1
Recommended information to be reported in studies on microbial inactivation by PEF.

Microorganism culture and recovery conditions	*Genus, species and strain of the microorganisms* *Culture conditions* Initial inoculum o Description of the procedure for microbial cultivation o Growth medium composition, growth temperature, incubation time and growth phase (exponential or stationary) *Recovery conditions* o Time and storage conditions between treatment and microbiological analysis o Description of the procedure for enumerating microorganisms o Composition of the recovery medium, incubation time and incubation temperature
PEF equipment	*PEF generator* o For commercial: equipment name of the supplier company and model o For prototypes: adequate description of the components, electrical configurations, electrical specifications *Treatment chamber* o For commercial: equipment name of the supplier company and model o For prototypes: adequate description (e.g., configuration of the electrodes, material of electrodes and insulators, dimensions, volume, gap) *Auxiliary devices* o Pump o Heat exchangers o Voltage and current o Temperature probe o Measurement/data acquisition system
Processing parameters	*Batch treatments* o Pulse amplitude (voltage and current), electric field strength, pulse energy, number of pulses, pulse shape, pulse width, pulse protocol treatment time, frequency, initial and final temperature *Continuous flow treatments* o Pulse amplitude (voltage and current), electric field strength, pulse energy, number of pulses for each treatment chamber, pulse shape, pulse width, pulse protocol treatment time, frequency, mass flow, residence time, inlet and outlet temperature for each treatment chamber
Treatment medium properties	o Composition o pH o a_w o Electrical conductivity

Table 2
Recommended information to be reported in studies of PEF-assisted processing for improving heat and mass transfer phenomena in food and biotechnological processes.

Raw material	*Origins, variety, maturation and storage conditions of plant matrices and cell (microbial, algae) suspensions* *Plant matrices* o Variety o Geographical origin o Degree of ripeness o Moisture content o Storage conditions (temperature, humidity, storage time) *Cells suspension* *Microbial cell* (see Table 1) *Algae cells* o Genus, species, strain, source of supply of the microalgae o Description of the bioreactor and cultivation procedure o Growth medium composition, growth temperature and time, growth phase (exponential or stationary) o Biomass concentration
Upstream process	*Equipment for raw material pre-treatments and characterization* o For commercial equipment: name of the supplier company and model o For prototypes: adequate description and operating mode *Characterization of pre-treated raw material* o Size, shape, particle size distribution (slicing/mechanical grinding) o Temperature, time (pre-heating) o Moisture content o Electrical conductivity of solid and liquid phase o Cell concentration or inoculum size (for microbial cells) o Biomass concentration (for algae cells) o Biomaterial (plant)/treatment medium ratio
PEF equipment	*PEF generator* o For commercial: equipment name of the supplier company and model o For prototypes: adequate description of the components, electrical configuration, electrical specifications *Treatment chamber* o For commercial: equipment name of the supplier company and model o For prototypes: adequate description (e.g., configuration of the electrodes, material of electrodes and insulators, dimensions, volume, gap) *Auxiliary devices* o Pump o Heat exchangers o Voltage and current probes o Temperature probe o Measurement/data acquisition system
Processing parameters	*Batch treatments* o Pulse amplitude (voltage and current), electric field strength, pulse energy, number of pulses, pulse shape, pulse width, pulse protocol treatment time, frequency, initial and final temperature *Continuous flow treatments* o Pulse amplitude (voltage and current), electric field strength, pulse energy, number of pulses for each treatment chamber, pulse shape, pulse width, pulse protocol treatment time, frequency, mass flow, residence time, inlet and outlet temperature for each treatment chamber
Treatment medium properties	o Composition o pH o a_w o Electrical conductivity
Downstream process	*Extraction by mechanical pressing* o Type of press (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode) o Pressing procedure (Pressure, time, pressing cycles) *Extraction with solvent* o Type of extractor (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode) o Type of Solvent (composition, pH) o Temperature and time o Solid/solvent ratio o Shaking conditions *Purification of the extracts* o Centrifugation (revolution per unit of time, time, temperature) o Filtration (type and size of filter) o Concentration (pressure, temperature) *Thermal drying* o Type of dryer (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode) o Initial temperature and moisture content of sample before drying o Hot air properties (e.g., temperature, humidity, velocity) o Drying time o Degree of dehydration *Osmotic dehydration* o Type of osmotic dryer (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode) o Type of osmotic solution o Concentration of the osmotic agent o Pressure o Dehydration temperature and time o Solid/liquid ratio o Shaking conditions o Degree of dehydration

(continued on next page)

Table 2 (*continued*)

Freeze-drying
o Type of freeze-dryer (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode)
o Initial temperature and moisture content of sample before freeze-drying
o Freeze-drying temperature, pressure, and time
o Degree of dehydration
Freezing
o Type of freezer (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode)
o Initial temperature and moisture content of sample before freezing
o Freezing temperature, pressure, and time
o Air velocity
o Type and concentration of cryoprotectant
Thawing
o Type of thawing chamber (for commercial equipment: name of the supplier company and model; for prototypes: adequate description and operating mode)
o Initial temperature of sample before thawing
o Thawing temperature and time
o Air velocity

microorganism used should be reported including the name with genus, species and strain number. It should be desirable that that the strain or strains used in the study should be available for other researchers.

The preparation of the microbial culture and the storage conditions can significantly affect the microbial sensitivity to PEF. Therefore, the cultivation of the microorganism should be standardized to minimize its influence on variability between repeated experiments either from day to day, or from test period to test period. Initial inoculum, growth medium composition, growth temperature, time of incubation and growth phase of the cells used for inactivation experiments should be reported.

Several studies indicate that microorganisms at the exponential phase of growth are more PEF sensitive than those at the stationary phase (Álvarez, Pagán, Raso, & Condón, 2002; Rodrigo, Ruíz, Barbosa-Cánovas, Martínez, & Rodrigo, 2003; Wouters, Dutreux, Smelt, & Lelieveld, 1999). This higher microbial sensitivity could be related to the larger size of cells in the exponential phase or to the manifestation of an alternative sigma factor when microorganisms enter in the stationary phase resulting in the expression of a number of genes that confer stress resistance, as well altered metabolism, structural and morphological changes (Somolinos, García, Mañas, Condón, & Pagán, 2008). On the other hand, reported data indicate that cells, grown at temperatures lower than the optimal one, are more PEF sensitive that those grown at the optimal temperature (Álvarez et al., 2002; Ohshima, Akuyama, & Sato, 2002). Lipid composition variations in the cytoplasmic membrane induced by modifications of the growth temperature have been suggested as the origin of the distinct PEF sensitivity. At lower growth temperatures the degree of fatty acid insaturations of the phospholipids of the cell membrane raises which could increase the fluidity of the bacterial cell membrane and increased its sensitivity to electroporation.

3.2. Treatment medium

The treatment medium used for the inactivation studies should be well defined to allow reproduction in other laboratories. Composition of the treatment medium should be reported and factors that may affect microbial inactivation such as pH, conductivity, activity of water (osmolarity), as well as presence of preservatives should be measured and reported.

3.3. Inactivation studies

For inactivation studies it has been recommended a minimum of three replicate sets per trial repeated on separated days in order to be able to measure the experimental error as well as differences in response due to biological variability has been suggested (Balasubramaniam, Ting, Stewart, & Robbins, 2004). For testing microbial resistance to a lethal treatment such as PEF, acquisition of multiple data points along the time for a given electric field strength is preferred because they give more information than end-point measurements based on the inactivation produced by a given treatment. The acquisition of multiple data points permits the elaboration of the survival curves in which the logarithmic of survivors is plotted against inactivation time for a given treatment intensity. Survival curves can be described by mathematical models (Dermol & Miklavčič, 2015). Modelling kinetics data obtained under different experimental conditions and developing of predictive models are very useful tools for quantifying the influence of different factors on microbial inactivation by PEF, as well as to define equivalent treatment conditions to achieve a given level of inactivation.

3.4. Recovery conditions

Quantification of the survivors after the treatment is one of the most important factors in estimating the efficacy of an inactivation technique such as PEF. It is important to use procedures that recover the greatest number of microorganisms. Recovery medium, incubation time and temperature during incubation should be reported because they have a significant effect on the number of microorganisms recovered after the PEF treatment. The time and storage conditions between treatment and microbiological analysis should also be reported.

Comparison of cell counts of PEF treated samples on selective and nonselective media is the most conventional technique to detect sublethal injury. Sublethally injured population fails to survive and multiply in harsh environments tolerant by untreated cells (Mackey, 2000). If the existence of sub-lethal injured microorganisms is detected by adding selective agents in the recovery medium it is necessary to establish previously the maximum concentration of the selective agent that has not inhibitory effect on untreated cells. The selective agent and the concentration used for detecting sub-lethal injured microorganisms need to be given. Generally longer incubation times are required when microorganism are plated on selective media because inactivation may be overestimated when the incubation time is the same in nonselective and selective media.

4. PEF-assisted processing for improving mass transfer phenomena in food and biotechnological processes

The application of PEF as a mild cell disintegration technique for improving food quality, accelerate heat transfer process, as well as mass transfer efficiency of target compounds from matrices of biological

origin, demonstrated its efficiency especially in extraction by solvent diffusion or pressing, as well as in drying, osmotic dehydration, freeze-drying, freezing, and freeze-thawing (Barba et al., 2015; Bobinaitė et al., 2014; Eing, Goettel, Straessner, Gusbeth, & Frey, 2013; Jalte et al., 2009; Parniakov et al., 2015, 2016a,b; Wiktor et al., 2013).

However, similarly to the application of PEF for microbial inactivation, it is difficult to compare between studies of different groups, due to the large number of parameters, which are interrelated, as well as the large variety of experimental conditions and equipment used by several researchers.

Moreover, no or very few systematic studies are available, to date, taking into account the entire production process including complex interactions between raw material properties, their changes after pre-treatment, cell disintegration by PEF, and downstream processes, which are all of relevance on the outcome of the entire process (Jaeger, Schulz, Lu, & Knorr, 2012).

For these reasons, in Table 2 we summarize the main information regarding raw material characteristics, the upstream processes (e.g., grinding, slicing, heating, concentration), the PEF process (e.g., electric field strength, energy input, pulse characteristics), as well as downstream processes (e.g., extraction, drying, freezing, purification) that should be reported in published papers.

This information is essential to allow standardization of experimental procedures and reproducibility of the experiments in view of the utilization of bench-scale data on PEF assisted processing to define processing conditions in commercial size equipment.

4.1. Raw material

Information on raw materials is very important since it can contribute to define the optimal processing conditions as well as the properties of the final product.

Therefore, for the case of raw material of plant origin, information such as geographical origin, variety, degree of ripeness, moisture content, as well as storage time and conditions (e.g., temperature, air humidity) before processing should be reported. Similarly, in the case of cell (microbial, algae) in suspension, detailed description of the genus, species, strain number and source of supply, as well as growth or cultivation conditions should be provided, as reported in detail in the Section 3.1 and Tables 1 and 2.

4.2. Upstream process

Raw materials are typically pre-treated before PEF-assisted processing with the aim of softening or hydrating biomaterial, reducing the particle size, increasing the surface–volume-ratio, or to induce mixture densification. For example, raw materials of plant origin are typically subjected to peeling, slicing, mechanical grinding or pre-heating. In the case of cell suspensions, a concentration step of the biomass could be required.

Therefore, detailed information on the features of the equipment used (name of the manufacturer and model) and processing conditions should be specified.

Moreover, when plant material is pre-treated, information on the textural properties, size, shape and particle size distribution of the mash after grinding, pre-heating time and temperature, moisture content of the plant tissue, electrical conductivity of solid and liquid phase, solid/liquid ratio, as well as any other physical and chemical-physical properties of relevance for the next processing steps, should be provided. In the case of cell suspensions, the type, pH and electrical conductivity of suspending medium, as well as the cell concentration (for studies on microbial cells), should be reported. For microalgae processing the component yield per kg of biomass (dry weight) in suspension is in focus. Thus, the content of biomass (dry weight) in the treated suspension is a mandatory value to be reported in experimental studies. Also component yields have to be related to the processed biomass dry weight, as usual in the microalgae processing community.

Finally, it is worth noting that raw material pre-treatment may also cause partial or total cell disintegration (Jaeger et al., 2012). Therefore, the impact of conventional pre-treatment on cell membrane disruption should be reported in order to be discriminated from that of the PEF treatment.

4.3. PEF equipment and processing conditions

As previously reported, an appropriate description of the PEF generator, treatment chamber and auxiliary devices (e.g., voltage, current and temperature probes, pump, heat exchangers) should be provided. In addition, in order to allow a proper comparison between data of different authors, electric field strength and total specific energy input W_T [kJ/kg] should be chosen as parameters to describe the treatment intensity. Moreover, also (initial) voltage applied, pulse shape, pulse width, number of pulses or treatment time, frequency, pulse protocol, initial and final temperature for batch processes, and mass flow, residence time, inlet and outlet temperature for continuous flow chamber, should be also specified.

4.4. Downstream process

The design and the operating mode of the equipment to be used for processing of the electroporated matrices, may play an important role for the exploitation of the potential benefits that may result from PEF pre-treatment. In addition, the results achieved from the PEF-assisted processing investigations that are typically used to compare data obtained from different studies, are generally collected after the characterization of the final product. It is, therefore, crucial to provide detailed information on the equipment (manufacturer, model), the experimental conditions, the protocol analysis and methods used in the downstream process (Table 2).

For example, in the case of the extraction processes by mechanical pressing, a detailed description of the type of press as well as the pressing conditions (e.g., pressure, pressing time, and number of pressing cycle) should be reported. When the extraction process following the PEF pre-treatment is carried out by using solvents, a detailed description of the type of extractor, as well as information on the type of solvent (e.g., composition, pH), the temperature and extraction time, the solid/solvent ratio, and shaking conditions, should be reported. If the extract solution requires a further purification stage before analyses, detailed information on the type of the devices and protocols of purification adopted, should be also reported.

In PEF assisted drying processes (thermal drying, osmotic dehydration, freeze-drying) information on the type of dryer, initial temperature and moisture content of the biomaterial, thermodynamic properties of hot air (e.g, temperature, humidity) and air velocity, type and concentration of osmotic agent, as well as temperature, pressure and time of drying, should be included.

Finally, when PEF is used to assist freezing/thawing processes, a detailed description of the type of freezing/thawing chamber, as well as initial temperature and moisture content of sample before freezing/thawing, processing conditions (temperature, pressure, time, air velocity), as well as type and concentration of cryoprotectant (if applicable), should be reported.

5. Conclusions

In this paper, basic principles of PEF technology and its application in food and biotechnological processes have been reviewed, and the main problems that a researcher may encounter when conducting experiments with the PEF technology, have been described. This paper provides recommendations for standardization of research methodology, as well as key information that should be reported in studies regarding

the application of PEF technology, in order to be able to compare data obtained in various laboratories. It is expected that this paper will contribute to improve the current state of knowledge on electroporation mechanisms, and to identify the critical factors affecting electroporation, with final objective of extending the commercial exploitation of PEF processing in the food and biotechnological industries.

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References

Álvarez, I., Pagán, R., Raso, J., & Condón, S. (2002). Environmental factors influencing the inactivation of *Listeria monocytogenes* by pulsed electric fields. *Letters in Applied Microbiology*, *35*, 489–493.

Balasubramaniam, V. M., Ting, E. Y., Stewart, C. M., & Robbins, J. A. (2004). Recommended laboratory practices for conducting high-pressure microbial inactivation experiments. *Innovative Food Science & Emerging Technologies*, *5*, 299–306.

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., et al. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773–798.

Bobinaitė, R., Pataro, Lamanauskas, N., Šatkauskas, S., Viškelis, P., & Ferrari, G. (2014). Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products. *Journal of Food Science and Technology*. http://dx.doi.org/10.1007/s13197-014-1668-0.

Campana, L. G., Clover, A. J. P., Valpione, S., Quaglino, P., Gehl, J., Kunte, C., ... Sersa, G. (2016). Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review. *Radiology and Oncology*, *50*(1), 1–13.

Coster, H. G. L., & Zimmermann, U. (1975). The mechanism of electrical breakdown in the membranes of *Valonia utricularis*. *Journal of Membrane Biology*, *22*, 73–90.

Dermol, J., & Miklavčič, D. (2015). Mathematical models describing Chinese hamster ovary cell death due to electroporation in vitro. *Journal of Membrane Biology*, *248*, 865–881.

Dhand, R. (2014) Editorial Nature 5 1 5: 7

Donsì, F., Ferrari, G., & Pataro, G. (2010). Applications of pulsed electric field treatments for the enhancement of mass transfer from vegetable tissue. *Food Engineering Reviews*. http://dx.doi.org/10.1007/s12393-010-9015-3.

Donsì, G., Ferrari, G., & Pataro, G. (2007). Inactivation kinetics of *Saccharomyces cerevisiae* by pulse electric field in a batch treatment chamber: The effect of electric field unevenness and initial cells concentration. *Journal of Food Engineering*, *78*, 784–792.

Eing, C., Goettel, M., Straessner, R., Gusbeth, C., & Frey, W. (2013). Pulsed electric field treatment of microalgae-benefits for microalgae biomass processing. *IEEE Transactions on Plasma Science*, *41*, 2901–2907.

Frey, W., Gusbeth, C., & Schwartz, T. (2013). Inactivation of pseudomonas putida by pulsed electric field treatment: A study on the correlation of treatment parameters and inactivation efficiency in the short-pulse range. *Journal of Membrane Biology*, *246*, 769–781.

Gerlach, D., Alleborn, N., Baars, A., Delgado, A., Moritz, J., & Knorr, D. (2008). Numerical simulations of pulsed electric fields for food preservation: A review. *Innovative Food Science & Emerging Technologies*, *9*, 408–417.

Goettel, M., Eing, C., Gusbeth, C., Straessner, R., & Frey, W. (2013). Pulsed electric field assisted extraction of intracellular valuables from microalgae. *Algal Research-Biomass Biofuels and Bioproducts*, *2*, 401–408.

Golberg, A., Sack, M., Teissie, J., Pataro, G., Pliquett, U., Saulis, G., ... Frey, W. (2016). Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development. *Biotechnology for Biofuels*, *9*, 94. http://dx.doi.org/10.1186/s13068-016-0508-z.

Haberl, S., Miklavčič, D., Serša, G., Frey, W., & Rubinsky, B. (2013). Cell membrane electroporation – Part 2: The applications. *IEEE Electrical Insulation Magazine*, *29*(1), 29–37.

Heinz, V., Alvarez, I., Angersbach, A., & Knorr, D. (2002). Preservation of liquid foods by high intensity pulsed electric fields – Basic concepts for process design. *Trends in Food Science & Technology*, *12*, 3–4.

Heinz, V., Toepfl, S., & Knorr, D. (2003). Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. *Innovative Food Science & Emerging Technologies*, *4*, 167–175.

Huang, K., & Wang, J. (2009). Designs of pulsed electric fields treatment chambers for liquid foods pasteurization process: A review. *Journal of Food Engineering*, *95*, 227–239.

Jaeger, H., Meneses, N., & Knorr, D. (2009). Impact of PEF treatment inhomogeneity such as electric field distribution flow characteristics and temperature effects on the inactivation of *E. coli* and milk alkaline phosphatase. *Innovative Food Science & Emerging Technologies*, *10*, 470–480.

Jaeger, H., Schulz, M., Lu, P., & Knorr, D. (2012). Adjustment of milling, mash electroporation and pressing for the development of a PEF assisted juice production in industrial scale. *Innovative Food Science & Emerging Technologies*, *14*, 46–60.

Jalte, M., Lanoiselle, J. L., Lebovka, N., & Vorobiev, E. (2009). Freezing of potato tissue pretreated by pulsed electric fields. *Food Science and Technology*, *42*(2), 576–580.

Kotnik, T., Frey, W., Sack, M., Haberl-Meglič, S., Peterka, M., & Miklavčič, D. (2015). Electroporation-based applications in biotechnology. *Trends in Biotechnology*, *33*, 480–488.

Kotnik, T., Kramar, P., Pucihar, G., Miklavčič, D., & Tarek, M. (2012). Cell membrane electroporation — Part 1: The phenomenon. *IEEE Electrical Insulation Magazine*, *28*(5), 14–23.

Kotnik, T., Miklavčič, D., & Mir, L. M. (2001). Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses. Part II. *Reduced electrolytic contamination, Bioelectrochemistry*, *54*, 91–95.

Lado, B. H., & Yousef, A. E. (2003). Selection and identification of a *Listeria monocytogenes* target strain for pulsed electric field processing optimization. *Applied and Environmental Microbiology*, *69*, 2223–2229.

Lebovka, N. I., Praporscic, I., Ghnimi, S., & Vorobiev, E. (2005). Temperature enhanced electroporation under the pulsed electric field treatment of food tissue. *Journal of Food Engineering*, *69*, 177–184.

Mackey, B. M. (2000). Injured bacteria. In M., T. C., & G. W. (Eds.), *The microbiological safety and quality of food. Vol. I.* (pp. 315–341). Gaithersburg, Maryland: Aspen Publisher, Inc.

Mahnič-Kalamiza, S., Vorobiev, E., & Miklavčic, D. (2014). Electroporation in food processing and Biorefinery. *The Journal of Membrane Biology*, *247*, 1279–1304. http://dx.doi.org/10.1007/s00232-014-9737-x.

McNutt, M. (2014). Journals unite for reproducibility. *Science*, *346*(6210), 679. http://dx.doi.org/10.1126/science.aaa1724.

Meneses, N., Jaeger, H., & Knorr, D. (2011a). Minimizing thermal impact by application of electrode cooling in a co-linear PEF-treatment chamber. *Journal of Food Science*, *76*, 536–543.

Meneses, N., Jaeger, H., Moritz, J., & Knorr, D. (2011b). Impact of insulator shape, flow rate and electrical parameters on inactivation of *E. coli* using a continuous co-linear PEF system. *Innovative Food Science & Emerging Technologies*, *12*, 6–12.

Miklavčič, D. (2012). Network for development of electroporation-based technologies and treatments. *The Journal of Membrane Biology*, *245*, 591–598.

Miklavčič, D., Mali, B., Kos, B., Heller, R., & Serša, G. (2014). Electrochemotherapy: From the drawing board into medical practice. *Biomedical Engineering Online*, *13*, 29. http://dx.doi.org/10.1186/1475-925X-13-29.

Ohshima, T., Akuyama, K., & Sato, M. (2002). Effect of culture temperature on high-voltage pulsed sterilization of *Escherichia coli*. *Journal of Electrostatics*, *55*, 227–235.

Parniakov, O., Bals, O., Lebovka, N., & Vorobiev, E. (2016a). Effects of pulsed electric fields assisted osmotic dehydration on freezing-thawing and texture of apple tissue. *Journal of Food Engineering*, *183*, 32–38.

Parniakov, O., Lebovka, N., Bals, O., & Vorobiev, E. (2015). Effect of electric field and osmotic pre-treatments on quality of apples after freezing-thawing. *Innovative Food Science & Emerging Technologies*, *29*, 23–30.

Parniakov, O., Lebovka, N., Bals, O., & Vorobiev, E. (2016b). Pulsed electric field assited vacuum freeze-drying of apple tissue. *Innovative Food Science & Emerging Technologies*, *35*, 52–57.

Pataro, G., Barca, G. M. J., Donsì, G., & Ferrari, G. (2015a). On the modelling of electrochemical phenomena at the electrode-solution interface in a PEF treatment chamber: Methodological approach to describe the phenomenon of metal release. *Journal of Food Engineering*, *165*, 34–44.

Pataro, G., Barca, G. M. J., Donsì, G., & Ferrari, G. (2015b). On the modelling of the electrochemical phenomena at the electrodesolution interface of a PEF treatment chamber: Effect of electrical parameters and chemical composition of model liquid food. *Journal of Food Engineering*, *165*, 45–51.

Pataro, G., Falcone, M., Donsì, G., & Ferrari, G. (2014). Metal release from stainless steel electrodes of a PEF treatment chamber: Effects of electrical parameters and food composition. *Innovative Food Science & Emerging Technologies*, *21*, 58–65.

Pataro, G., Senatore, B., Donsí, G., & Ferrari, G. (2011). Effect of electric and flow parameters on PEF treatment efficiency. *Journal of Food Engineering*, *105*, 79–88.

Phoon, P. Y., Galindo, F. G., Vicente, A., & Dejmek, P. (2008). Pulsed electric field in combination with vacuum impregnation with trehalose improves the freezing tolerance of spinach leaves. *Journal of Food Engineering*, *88*(1), 144–148.

Puértolas, E., Luengo, E., Álvarez, I., & Raso, J. (2012). Improving mass transfer to soften tissues by pulsed electric fields: Fundamentals and applications. In M. P., & T. R. (Eds.), *Annual review of food science and technology. Vol. 3.* (pp. 263–282). Palo Alto: Annual Reviews.

Reberšek, M., Miklavčič, D., Bertacchini, C., & Sack, M. (2014). Cell membrane electroporation – Part 3: The equipment. *IEEE Electrical Insulation Magazine*, *30*(3), 8–18.

Rodrigo, D., Ruíz, P., Barbosa-Cánovas, G. V., Martínez, A., & Rodrigo, M. (2003). Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. *International Journal of Food Microbiology*, *81*, 223–229.

Sack, M., Eing, C., Berghofer, T., Buth, L., Stangle, R., Frey, W., & Bluhm, H. (2008). Electroporation-assisted dewatering as an alternative method for drying plants. *IEEE Transactions on Plasma Science*, *36*, 2577–2585.

Sack, M., Sigler, J., Frenzel, S., Eing, C., Arnold, J., Michelberger, T., & Muller, G. (2010). Research on industrial-scale electroporation devices fostering the extraction of substances from biological tissue. *Food Engineering Reviews*, *2*, 147–156.

Saldaña, G., Álvarez, I., Condón, S., & Raso, J. (2014). Microbiological aspects related to the feasibility of PEF technology for food pasteurization. *Critical Reviews in Food Science and Nutrition*, *54*, 1415–1426.

Saldaña, G., Puértolas, E., Álvarez, I., Meneses, N., Knorr, D., & Raso, J. (2010). Evaluation of a static treatment chamber to investigate kinetics of microbial inactivation by pulsed electric fields at different temperatures at quasi-isothermal conditions. *Journal of Food Engineering*, *100*, 349–356.

Saldaña, G., Puértolas, E., López, N., García, D., Álvarez, I., & Raso, J. (2009). Comparing the PEF resistance and occurrence of sublethal injury on different strains of *Escherichia coli*, *Salmonella* Typhimurium, *Listeria monocytogenes* and *Staphylococcus aureus* in media of pH 4 and 7. *Innovative Food Science & Emerging Technologies*, *10*, 160–165.

Somolinos, A., García, D., Mañas, P., Condón, S., & Pagán, R. (2008). Effect of environmental factors and cell physiological state on pulsed electric field resistance and repair capacity of various strains of *Escherichia coli*. *International Journal of Food Microbiology*, *124*, 260–267.

Toepfl, S. (2012). Pulsed electric field food processing — Industrial equipment design and commercial applications. *Stewart Postharvest Review*, *2*, 1–7.

Toepfl, S., Heinz, V., & Knorr, D. (2007). High intensity pulsed electric fields applied for food preservation. *Chemical Engineering and Processing*, *46*, 537–546.

Wiktor, A., Iwaniuk, M., Sledz, M., Nowacka, M., Chudoba, T., & Witrowa-Rajchert, D. (2013). Drying kinetics of apple tissue treated by pulsed electric field. *Drying Technology*, *31*(1), 112–119.

Wouters, P. C., Dutreux, N., Smelt, J. P. P., & Lelieveld, H. L. M. (1999). Effects of pulsed electric fields on inactivation kinetics of *Listeria innocua*. *Applied and Environmental Microbiology*, *65*, 5354–5371.

Yarmush, M. L., Golberg, A., Serša, G., Kotnik, T., & Miklavčič, D. (2014). Electroporation-based technologies for medicine: Principles, applications, and challenges. *Annual Review of Biomedical Engineering*, *16*, 295–320.

Zorec, B., Préat, V., Miklavčič, D., & Pavšelj, N. (2013). Active enhancement methods for intra- and transdermal drug delivery: A review. *Zdravniški Vestnik*, *82*, 339–356.

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Recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples☆

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ABSTRACT

Electric field-induced membrane changes are an important approach in the life sciences. However, the developments in knowledge and translational applications face problems of reproducibility. Indeed, a quick survey of the literature reveals a lack of transparent and comprehensive reporting of essential technical information in many papers. Too many of the published scientific papers do not contain sufficient information for proper assessment of the presented results. The general rule/guidance in reporting experimental data should require details on exposure conditions such that other researchers are able to evaluate, judge and reproduce the experiments and data obtained. To enhance dissemination of information and reproducibility of protocols, it is important to agree upon nomenclature and reach a consensus on documentation of experimental methods and procedures. This paper offers recommendations and requirements for reporting on applications of electric pulse delivery for electroporation of biological samples in life science.

1. Introduction

This manuscript outlines a proposal for defined nomenclature and guidance for reporting of materials and methods related to the use of electroporation of biological samples in the life sciences, for both in vitro and in vivo applications. This paper is presented in support of the Electroporation-Based Technologies and Treatments courses (EBTT) (http://2017.ebtt.org/) that supply participants with sufficient theoretical background and practical knowledge for effective use of electroporation in their working environments. This work is intended as a complete set of advice and suggestions on the information that should be included in scientific papers to fully describe the results.

As a general rule/guidance in reporting, experimental data should contain details on exposure conditions such that that other researchers are able to evaluate, judge and reproduce the experiments and data obtained. This type of reporting is necessary for future systematic reviews and/or meta-analyses, which are studies that systematically assess previous research and derive conclusions that cannot be extracted from single studies [1–4]. The outcomes of meta-analyses can lead to further advances in the field of electroporation-based technologies [2,5]. Offering adequate description of materials and methods used in the study presents an apparent contradiction with the trend towards papers that are more concise and shorter in length, but many journals now offer publication of additional (Supplemental) material online. We should all embrace these options.

Such a conclusion on the need for recommendation and guidance on reporting materials and methods is shared by many practitioners [6–8]. Common agreement exists that recommendations are needed to improve the consistency and quality of reporting in life science articles [9–11]. The Global Biological Standards Institute (GBSI) presented a report making a case for biological standards in the life sciences. In interviews in the life science community, working with irreproducible data and/or results was emphasized as a serious issue. The conclusion of the GBSI was that "there is a need for more well-defined and consistently used standards, both material (reference reagents and chemicals) and written (optimal practices and methodologies). This need is urgent because life science research is increasing in its complexity. Due to economic pressure, conclusions must be rapidly available for translational opportunities, mostly for medical applications. To facilitate interpretation and improve the reliability of published results in life and health sciences, we need to report key protocol details more systematically, examine the statistics more closely and offer additional ways for authors to be transparent about these matters. If researchers detailed their exploratory studies more accurately, late-stage trials would be better planned and executed" [12].

The current recommendation guidelines target all electroporation-based applications of delivery of electric pulses to cells and tissues (in which most work is performed with batch processes and electrodes are

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held at a fixed position during pulse delivery). This scope includes biomedical pre-clinical applications of electroporation. This manuscript is also a result of the discussions within the COST TD 1104 action "European Network for Development of Electroporation Based Technologies and Treatments", starting with comments from the steering committee in Salerno in 2012, where members noticed that in many presentations, participants did not supply sufficient information for other researchers to properly assess the results. A workshop was subsequently organized in Copenhagen (2014) to discuss issues related to terminology and reproducibility issues (http://www.electroporation.net/Events/COST-TD1104-Management-Committee-Meeting-and-WG-Meeting-Symposium-March-27-28th). Presenters and discussants identified major problems related to reporting of electric pulse delivery for electroporation of biological samples. Finally, during the 1st World Congress on Electroporation held in Portoroz, Slovenia during September 6–10, 2015 the decision was made to prepare recommendation papers specific to electrochemotherapy [13], food processing [14], and life sciences. Selected critical issues were raised in discussions during the COST TD 1104 action [15] that should be further highlighted

a- Emphasis on good practice in experimental design
b- Modeling based on data obtained on selected reference cells and tissues
c- Unification of terminology or a need to supply clear-cut descriptions of the wording.

In this paper, the above principles for reporting are presented using examples and critical details discussed in several Appendixes. We have conceptualized this recommendation paper to make it as short and comprehensive as possible. Therefore, more extensive descriptions are added in an appendix. Furthermore, a one-page summary has been prepared and is included at the end of this paper. This summary can be used as a checklist for authors when writing manuscripts. This work is in line with CONSORT (Consolidated Standards of Reporting Trials) that offers "a standard way for authors to describe how trials are designed, analyzed and interpreted" [12].

2. Terminology

The definition of terms and their explanations are often missing, and therefore, persistent confusion and misuse of terminology is rife in the literature. The consequence is poor reproducibility of results, which is a critical problem for the description of the biological effects of pulsed electric fields, a scientific discipline that is both multidisciplinary and interdisciplinary. Different terminologies are found in physical chemistry and in life science that allow confusion in the reports.

Pulsed electric field treatment (PEF treatment, electropulsation, electroporation) is the process of exposing cells in suspension or tissue to electric pulses. Electric field exposure can be applied via direct, capacitive or inductive coupling [16–18]. This paper focuses predominantly on research that applies direct coupling (direct conductive contact of the electrodes with the sample). With respect to cell suspensions and plant tissue in suspension, this process can be applied either in batch or in continuous mode [19–21]. The major consequence of PEF treatment is permeabilization of the plasma membrane (enhanced transmembrane transport). One underlying hypothesis of the basic effects of membrane permeabilization is formation of defects (known as "pores", hence electroporation) [22]. An accurate definition is required because increasingly more new expressions are used (EP (electropulsation, electropermeabilization, electroporation), ECT (electrochemotherapy), PEF (pulsed electric field), electrogene therapy, GET (gene electrotransfer), electroextraction, electrofusion, electrochemoembolization). As an example, we use electrochemotherapy because this term is already widespread. As an analogy, one could use chemotherapy locally potentiated by electric pulses. We recommend using gene electrotransfer (GET) rather than electrogene transfer/therapy (EGT), although the latter is currently more frequently used.

Increased membrane permeability should refer to a given X molecule, which may be small or large. In most experiments, it is the transport of the given X molecule that is assayed, which is different from increased membrane conductivity, i.e., increased current and increased suspension conductivity due to increases in ionic leakage and heating [23,24], but it remains an associated phenomenon, where transport of X is the key parameter in the assay [22,25].

3. Physical parameters

It is important to standardize the reporting information related to what is currently referred to as a PEF session, such as a train of pulses or pulsing frequency vs. pulse repetition frequency. When these terms are used, it is unclear as to the intended meaning.

Certain PEF parameters are under direct control of the settings of the electric pulse generator and the definition of the applicators (electrodes). A key definition is that one should report the voltage actually applied between/delivered to the electrodes. The electric field is a more complex parameter that depends on the geometry of the experimental system and on the heterogeneity of the sample (tissue or cell density) in terms of conductivity and permittivity [26–29]. Furthermore, whether single or multiple pulses were delivered should be clearly reported. Electropermeabilization is a dynamic process in which local time-dependent changes in the tissue conductivity occur [30]. This process results in a redistribution of the field during the pulse application [31,32].

3.1. Electric pulse generators

The technology that supports pulse generators is complex [33,34]. The type of generator should be described with the specification of whether it is a commercial model or an in-house/built set-up. Because few pulse generators report/offer reliable and accurate measurement of U (voltage) and I (current) [35], monitoring of the pulses with digitized recording (and display of the graph) is an important step in ensuring that pulse delivery was obtained as requested from the settings of the generator. The electrical properties of the sample between the electrodes might affect the current delivered (conductivity change). The electric charge stored in the generator might fall short of that needed and affects the profile of the voltage (in the case of long pulses, i.e., tens of ms, high frequency). It is recommended that treatment parameters and experimental profiles, e.g., voltage and current waveforms, are stored for later re-evaluation. A precise description of this step is required [36]. Authors should report how the voltage (and current) was measured, i.e., where and with which instrument(s), and a schematic drawing of the measurement circuit/setup is helpful.

3.2. Electrodes

Because the electric field at a point E(x, y, z) is equal to the negative gradient of the electric potential, it is strongly dependent on the geometry of the electrodes to which the voltage is applied [37]. Thus, the geometry of electrodes and the sample/tissue treated should be described. The design of the electrodes (cuvette, plate, needles, wires, etc.), including the composition (material) of the electrodes, should be stated because electrochemical reactions occur during application of the electric pulses that could affect the sample and consequently the results [38,39].

However, if the equipment is a manufactured product, the authors should list the reference if the requested info is provided freely on the web. However, the details on the placement and penetration of needle electrodes should also be supplied to enable reproduction of the experiment (Appendix 1) and/or to evaluate the electric field distribution via numerical modeling [40] for comparison with other studies.

For use of arrays (using hexagonal electrodes or other types of multi-array electrodes), a complete description of the complex geometry should be included together with the sequence of pulses delivered

[41] because it was stipulated that the sequence of pulses might have a significant influence on the outcome of treatment [42–44]. This point is highly important in the case of non-penetrating electrodes or in applications in which the limited depth of penetration to the skin presents a major advantage by focusing on the effects on the epidermis [45–47].

3.3. Pulse duration

A clear definition of the temporal pulse parameters is required, such as the duration of square wave pulses or the decay time constant for exponentially decaying pulses generated by capacitor discharge systems [17].

The decay time constant characterizes the time elapsed until the pulse voltage value decays to 1/e = 0.3678 of the pulse maximum (Annex 2). Determination of the decay time constant is only applicable for resistive and capacitive discharge circuits. For bell-shaped voltage waveforms (caused in most instances by a non-negligible influence of the inductance of a discharge circuit), the pulse should be specified by stating either the amplitude, rise-time and fall time of the pulse or the half width and pulse amplitude (Appendix 2).

The shape of the pulse should be described because it is known to affect the extent of membrane permeabilization (exponential decay, square wave or pseudo-square wave) [48]. Selected information on the voltage pulse rise-time should be included whenever possible because it controls the charging time of the induced transmembrane voltage (Appendix 2, Appendix 3). Information on the pulse polarities in a train should be supplied because they are known to control the biological response [49].

Furthermore, the rise-time of a measurement system T_{rMsys} is given as Tr of the step response of the system (response to an infinite fast-rising pulse). The bandwidth B and rise-time of the measurement system TrMsys are related via the bandwidth-rise-time analogy: $B\,T_{rMsys} = 0.35$ (Gaussian systems). The real rise-time of a pulse T_{rReal} is the vector subtraction of the rise-time of the measurement system TrMsys from the rise-time $T_{rDisplay}$ determined at the screen of the oscilloscope: $T_{rReal} = \mathrm{Sqrt}(T^2_{rDisplay} - T^2_{rMsyS})$ (Fig. 1).

For experiments with nanosecond pulses, it is recommended to specify the measurement system used and to also list either the bandwidth or rise-time of the system. This information becomes important because pulse duration can be shorter than or of the same order as the membrane charging time constant. The authors should also mention either the measured rise-time (as commonly observed in publications if "rise-time" is mentioned) or the real pulse rise-time according to above considerations [2]. The rise-time of a pulse is most often defined as the time between 10% and 90% of the pulse amplitude [17]. Traces of the voltage (and current) should be presented or given as supplementary material whenever possible because it has been demonstrated that pulse shape might have a significant effect on the observed outcome (e.g., cancelation effect) [49,50].

When a train of pulses is delivered, the number of repetitive pulses and the pulse repetition frequency (or delay between pulses) should be reported [51]. Memory effects by the cell are present as the field effect is applied on a different membrane organization when resealing is not fully completed [52]. This effect is known to play a direct role in the electropermeabilization process and to simultaneously support a trivial (but damaging) role by increasing the Joule heating [53].

Complex trains, i.e., combinations of different pulses, are currently popular for gene electrotransfer in preclinical applications such as in the use of bipolar pulses, which are a combination of high voltage and low voltage pulses [54–57]. In these applications, the sequence should be reported with all delays and other parameters (Fig. 2).

Many studies that use commercial devices do not supply the parameters of pulses due to a lack of data from the manufacturer [35,58]. Instead, the authors list the "program" or "sequence" that they have selected on the device, which should be documented elsewhere and referenced in the manuscript, although this practice should be avoided. Whenever possible, actual physical parameters/pulse parameters and electrodes should be described. If this information is not available, the program and catalog number of the device, electrodes, reagents, buffer and program/sequence used should be listed.

4. Statistical analysis

Raw data or average/mean median as a measure of central tendency should be listed depending on the data distribution (normal or otherwise) as well as a measure of data spread such as standard deviation, percentiles, etc. The number of cells, sample volume, and repetitions of the experiment should also be documented. When normalizing data, i.e., reporting results in %, care should be taken to avoid reporting percent of a percent. It should be absolutely clear as to what reference the results were normalized against (Appendix 4). Information on the software package used in statistical calculations and graphical representation should also be listed.

5. Biochemical and biological parameters

Most journals offer complete instructions for authors, and these should be carefully followed (for example: https://www.elsevier.com/journals/bioelectrochemistry/1567-5394/guide-for-authors).

5.1. Chemicals

Manufacturer references are necessary (purity and origin are decisive parameters in many cases). The sizes of the molecules, the vehicle in which the chemicals are dissolved, the concentrations used, whether the solutions were freshly prepared for each experiment and the incubation times before and after the electroporation procedure should all be stated.

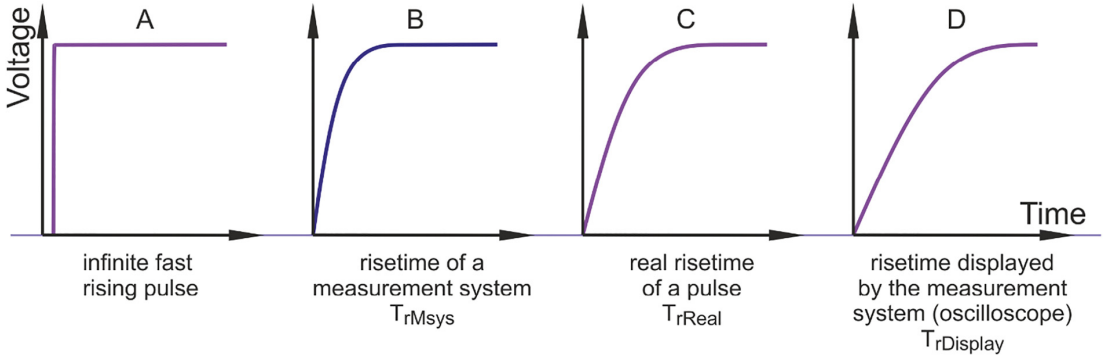

Fig. 1. Influence of the measurement system properties on determination of the pulse rise-time. An infinite fast pulse (A) is displayed by a measurement system of limited bandwidth with a longer rise-time (B). Thus, this measurement system also displays larger values of rise-time (D) when measuring real pulses (C). The rise-time of the measurement system T_{rMsys} (B) is given as T_r of the step response (A). The rise-time $T_{rDisplay}$ observed at the screen of the oscilloscope (D) is the convolution of T_{rMsys} with the real rise-time of a pulse T_{rReal} (C) that is actually delivered on the sample.

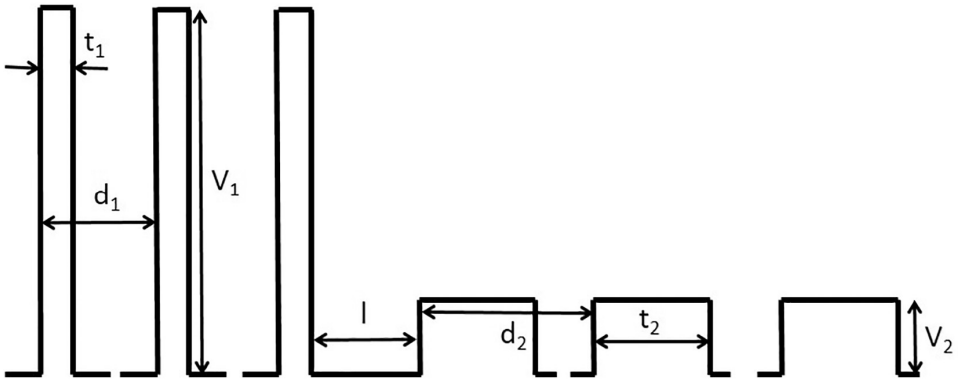

Fig. 2. Example of how to report a complex sequence of pulses. 3 short high voltage pulses are followed by 3 longer low voltage pulses. t_1 and t_2 – pulse duration; d_1 and d_2 – interval (delay) between the pulses; V_1 and V_2 – amplitude of pulses; l - time lag between pulses of different amplitude. The number of every type of pulse should also be stated. As an example of complex pulse sequences, see [59].

In the description of pulsing and post-pulse incubation buffers, in addition to pH, authors should report the osmolarity and conductivity (in S/m), including how and with what (instrument, protocol) these parameters were measured because these factors are known to affect the extent of permeabilization, the response of cells and the contributing effects due to Joule heating. After exposure of cells to electric pulses, post-treatment conditions should also be stated. These conditions include the incubation time, temperature (of the electroporation buffer or medium as well), and addition of fetal bovine serum or other additives (e.g., polymers) that affect cell behavior (viability, survival) as a result of the exposure to pulsed electric fields.

One problem is the use of patented products from companies for which such information is not available, except in the patent protecting the product. In such cases, reference to the patent should be supplied.

5.2. Cells

A complete description of cell systems, as listed below, is required because this information appears to be a decisive parameter in the biophysical response of the cells exposed to electric pulses. The full name of the cells, name of the supplier, catalog number when relevant, confirmation of authentication of the cell line and a statement on mycoplasma testing should also be included. Such reporting on cell lines is increasingly required by most journals in the field of life science. A description of cell cultivation (including the reference of the producer and, if relevant, the size of the plastic dishes), number of passages and whether the cells used in experiments were collected from the exponential growth phase should be presented. Furthermore, preparation of cells for treatment should include the composition of the buffer or medium, the state of the cells (attached vs. suspension), trypsinization or scraping procedure and the cell concentration. Concentration of the cells is highly important because it might affect the sample conductivity during pulse application [23] and the field homogeneity when it is high [60,61]. Additionally, the availability of drug to be introduced into cells can be reduced [29].

5.3. Plasmid DNA (pDNA) and other nucleic acid molecules

When using nucleic acids (including peptide nucleic acid), several parameters should be considered. For plasmids, a reference should be given for the producer if commercially available plasmids are used or to the patent number if the plasmids are proprietary. Otherwise, a map of the plasmid should be supplied stating the size of the plasmid, the promoter used, the therapeutic or reporter gene inserted and the selection marker. The size of the plasmid is particularly important because in the case of smaller plasmids, higher numbers of molecules are present in the suspension if only the concentration (in mg/ml) is stated. Therefore, the molarity of the plasmid should also be included. The preparation procedure should be clearly described, including a statement on endotoxin presence (testing) and verification of the plasmid size and purity. The vehicle in which the plasmid is dissolved should be given. For siRNA, miRNA and other small nucleic acids, including peptide-nucleic acids (PNA), the sequence should be supplied. All information listed for the plasmid DNA, (concentration, molarity, vehicle data) should also be listed [62].

The use of control plasmids and other control nucleic acids is essential. The control plasmid should be devoid of the therapeutic gene and ideally should have the same size as the therapeutic plasmid but with a scrambled sequence. Specifically, nucleic acids represent foreign DNA to cells (in vitro and in vivo) and can cause several different biological cellular responses with respect to its composition (e.g., the presence of bacterial CpG sequences) [63–65]. This information is required to supply a clear view of the possible biological responses.

6. Microscopy

Permeabilization and reporter gene expression procedures are routinely assayed using a fluorescence approach, and fluorescence microscopy reports a cellular description of the results. A precise description of the microscope type (phase contrast, fluorescence, confocal, biphoton) is required together with a precise description of the different elements (references of the objectives, light source, etc.) (Appendix 6). The size and number of pixels of the camera should be given. The conditions of the experiments should also be reported, as the integration time for the camera, continuous or shuttered illumination. When possible, pictures should be displayed using the same imaging protocols to allow direct comparison. The procedure for image analysis and the software used should also be included [66].

7. Animals in electroporation-based biomedical research

When animals are used in experiments [67], Directive 2010/63/EU and/or U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals guidelines should be followed. The experiments should be conducted according to the following 3R principle: replacement, reduction and refinement of the use of animals. The statement on ethical approval of the study, including the approval number, should be supplied.

A description of the animals (species, strain, sex, and age), the supplier, and the animal housing conditions should be included. In addition, if transgenic animals are used, their characteristics should be listed. The number of animals included in each experimental group and the number of repetitions of the experiments should not be left out (http://www.nih.gov/about/reporting-preclinical-research.htm). In addition,

the paper should state whether and how randomization was performed and whether certain measurements or analyses were applied in a blind fashion (e.g., histological analysis or tumor measurements). If possible, animals of both sexes should be used to avoid gender differences in response (Appendix 7).

7.1. Injection of substances and application of electric pulses in animal experiments

As previously described for in vitro experiments, information on substances injected into laboratory animals should follow specific guidelines. The producer of the chemical, drug, plasmid DNA, etc. should be stated. In cases in which the general name of the product is not sufficiently specific (e.g., lipopolysaccharide or LPS), the catalog number should also be supplied. Information on the route of administration and speed of injection is important. Depending on the route of administration, the dose (in mg/kg for intravenous and intraperitoneal injection) or the concentration (in mg/ml for intra-tissue injection, i.e., intramuscular, intratumoral, intradermal, subcutaneous, etc.) should be stated. The injection volume and speed of injection [68], including the size of the needle, should follow the guidelines for the specific route of administration and specific animal species.

When combining the administration of substances with application of electric pulses, the time interval between these two applications should be specified. The term "immediately" should be avoided because it is prone to different interpretations. Note also that delivery of electric pulses affects blood perfusion [69] and can result in different efficacies of injected substances [70].

Associated with the description of the electrodes, it should also be reported whether the electrodes are invasive or noninvasive. Furthermore, if shaving or depilation is required, the method should be described. For tissue electroporation, is also essential that a detailed description of the electrode placement is included. For example, in muscle gene electrotransfer, due to the shape of muscle cells, the position of the electrodes can play a major role in the effectiveness of transfection. When the electric field is oriented perpendicular to muscle cells, the transfection is higher because a larger area of the membrane of muscle cells is exposed to the electric field above threshold for effective transfection [71]. In addition, if conductive gel is used during the treatment to ensure contact between the tissue/skin and electrodes, this item needs to be listed, and details and abundance of use should be described. The producer/manufacturer of the gel and the product number should be reported, including the electrical conductivity of the gel if possible. If the conductivity is not supplied by the manufacturer, the authors should report how the conductivity was measured/determined [72,73].

7.2. Preparation of animals: anesthesia

Another important aspect is the use of anesthesia and analgesia, which can greatly affect the results from measurement of biological effects. The method of anesthesia and analgesia should be selected according to established procedure and should comply with relevant legislation. A considerable amount of relevant literature in this field is available and should be consulted [74–77].

7.3. Reporting results of animal studies

Depending on the type of study, the data pertinent to the aim of the study should be presented. However, certain general data related to the animals should also be reported, such as data on adverse effects. These data should include bodyweight change as a general index of toxicity and whether specific damage to the tissue used in the experiment occurred. Furthermore, the use of non-invasive imaging techniques such as ultrasound imaging, luminescence and fluorescence imaging, CT, MRI, etc. should be implemented whenever possible to comply with the 3Rs (refinement, reduction, replacement) [71,78].

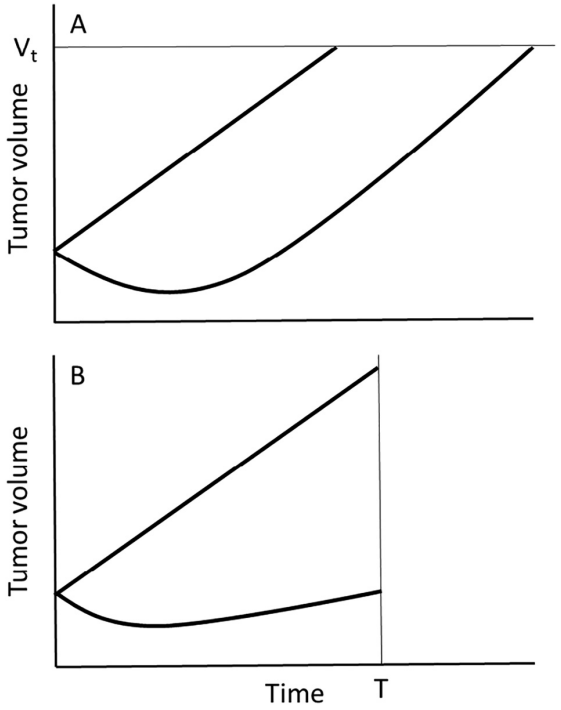

Fig. 3. Schematic representation of growth curves. A growth curves of control and treated tumors. The curves should be drawn to specific volumes (V_t) in all groups, and not only to a specific time point (T) as in B, because the information on possible tumor regrowth in the treated group is therefore missing.

If working with tumor models, the method for measurement and calculation of the tumor volume should be specified. Growth curves, which state the increase in volume or diameter over time, should be followed to the specific target volume V rather than at equal time (Fig. 3A, B). If all animals in the control and treated groups are sacrificed at the same day, then the growth of tumors is followed only to specific time point T. In this case, if the treatment results in regression of the tumor, the data on possible regrowth of the tumors are lost (Fig. 3B).

8. Conclusion

Reporting on applications of electric pulse delivery for electroporation of biological samples in the life sciences requires a description of many factors stemming from the multidisciplinary aspect of electroporation (electropermeabilization). As general guidance in reporting experimental data, a checklist is supplied to aid and guide the authors of research papers in writing and eventually improving the reproducibility of reports.

Conflict of interest

The authors have no relevant financial interest or financial conflict apart from those disclosed.

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CHECKLIST

Recommendations and requirements for reporting electric pulse delivery for electroporation of biological samples

General remarks

The Guidelines for Authors for the journal should always be carefully observed.

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Treatment information:

- Study design, protocol details
- Drug and chemical details (producer, dose, concentration, route of administration)
- Electroporation protocol, time interval between drug and chemical administration and electroporation protocol (number of electric pulses applied)
- Technical details of the electric pulse generator, including type, manufacturer and version of software, if applicable
- Information on the pulsing chamber
- Information on the electrodes (material, size and shape), according to tumor type
- for the nanopulses: report on impedance adaptation and connectors
- Inclusion of a report on electrical parameters (n, T, U, I, f, polarity)*

* Legend: n = number; T = duration of pulses; U = voltage amplitude applied; I = current measured; f = pulse repetition frequency

Culture conditions

- Reference to the cell type and its source.
- Initial inoculum
- Growth medium composition
- Growth temperature
- Incubation time
- Growth phase (exponential or stationary)

Pulsing buffer

- Conductivity and osmolarity of the medium
- Temperature

Recovery conditions

- Time and storage conditions between treatment and plating
- Composition of the recovery medium
- Incubation time
- Incubation temperature

- Reference to the animals used in experiments, strain, breeder, license
- Type of anesthesia for preclinical studies
- Tumor type, site of implantation, number of tumor cells injected, volume of injection, size of the tumor at the beginning of treatment

.....

Treatment outcome assessment:

- Methods of response assessment (viability, biochemical responses)
- Type of endpoint for assessment of effectiveness in vivo

.....

Analysis and interpretation of results:

- Summary of trial endpoints
- Interpretation of results
- Future research directions

Appendix A. Annex

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2018.03.005.

References

[1] B. Mali, T. Jarm, M. Snoj, G. Serša, D. Miklavčič, Antitumor effectiveness of electrochemo-therapy: a systematic review and meta-analysis, Eur. J. Surg. Oncol. 39 (2013) 4–16.
[2] T. Batista Napotnik, M. Reberšek, P.T. Vernier, B. Mali, D. Miklavčič, Effects of high voltage nanosecond electric pulses on eukaryotic cells (*in vitro*): a systematic review, Bioelectrochemistry 110 (2016) 1–12.
[3] M. Borenstein, L.V. Hedges, J.P.T. Higgins, H.R. Rothstein, Introduction to Meta-Analysis, Wiley, West Sussex, UK, 2009.
[4] J.P.T. Higgins, S. Green (Eds.), Cochrane Handbook for Systematic Reviews of Interventions, 1st EditionWiley-Blackwell, Chichester, England, Hoboken, NJ, 2008.
[5] A.B. Haidich, Meta-analysis in medical research, Hippokratia 14 (Suppl. 1) (2010) 29–37.
[6] N.S. Blow, The key to good science: increased transparency, BioTechniques 61 (2016) 221.
[7] F.S. Collins, L.A. Tabak, NIH plans to enhance reproducibility, Nature 505 (2014) 613.
[8] B. Pulverer, Reproducibility blues, EMBO J. 34 (2015) 2721–2724, https://doi.org/10.15252/embj.
[9] M. Baker, 1,500 scientists lift the lid on reproducibility, Nature 533 (7604) (2016) 452–454, https://doi.org/10.1038/533452a.
[10] N.S. Blow, A simple question of reproducibility, BioTechniques 56 (2014) 8, https://doi.org/10.2144/000114117.
[11] J.C. Lopez, Raising standards, Nat. Med. 19 (2013) 508, https://doi.org/10.1038/nm0513-508.
[12] E. Dolgin, Publication checklist proposed to boost rigor of pilot trials, Nat. Med. 19 (2013) 795–796.
[13] L. Campana, A.J.P. Clover, S. Valpione, P. Quaglino, J. Gehl, C. Kunte, M. Snoj, M. Cemazar, C.R. Rossi, D. Miklavcic, G. Sersa, Recommendations for improving the quality of reporting clinical electrochemotherapy studies based on qualitative systematic review, Radiol. Oncol. 50 (2016) 1–12.
[14] J. Raso, W. Frey, G. Ferrari, G. Pataro, D. Knorr, J. Teissie, D. Miklavcic, Recommendations guidelines of information that should be reported in studies on microbial inactivation and improving mass transfer by pulsed electric fields, Innovative Food Sci. Emerg. Technol. (2016)https://doi.org/10.1016/j.ifset.2016.08.003 (accepted).
[15] D. Miklavčič, Network for development of electroporation-based technologies and treatments, J. Membr. Biol. 245 (2012) 591–598.
[16] R. Korenstein, D. Somjen, H. Fischler, I. Binderman, Capacitative pulsed electric stimulation of bone cells. Induction of cyclic-AMP changes and DNA synthesis, Biochim. Biophys. Acta 803 (4) (1984) 302–307.
[17] M. Reberšek, D. Miklavčič, C. Bertacchini, M. Sack, Cell membrane electroporation – part 3: the equipment, IEEE Electr. Insul. Mag. 30 (3) (2014) 8–18.
[18] V. Novickij, J. Dermol, A. Grainys, M. Kranjc, D. Miklavčič, Membrane permeabilization of mammalian cells using bursts of high magnetic field pulses, PeerJ 5 (2017), e3267.
[19] A. Golberg, M. Sack, J. Teissie, G. Pataro, U. Pliquett, G. Saulis, S. Töpfl, D. Miklavčič, E. Vorobiev, W. Frey, Energy-efficient biomass processing with pulsed electric fields for bioeconomy and sustainable development, Biotechnol. Biofuels 9 (94) (2016).
[20] S. Haberl, D. Miklavčič, G. Serša, W. Frey, B. Rubinsky, Cell membrane electroporation – part 2: the applications, IEEE Electr. Insul. Mag. 29 (1) (2013) 29–37.
[21] T. Kotnik, W. Frey, M. Sack, S. Haberl Meglič, M. Peterka, D. Miklavčič, Electroporation-based applications in biotechnology, Trends Biotechnol. 33 (2015) 480–488.
[22] L. Rems, D. Miklavčič, Tutorial: electroporation of cells in complex materials and tissue, J. Appl. Phys. 119 (2016), 201101.
[23] M. Pavlin, M. Kandušer, M. Reberšek, G. Pucihar, F.X. Hart, R. Magjarević, D. Miklavčič, Effect of cell electroporation on the conductivity of a cell suspension, Biophys. J. 88 (6) (2005) 4378–4390.
[24] T.Y. Tsong, Electroporation of cell membranes, Biophys. J. 60 (2) (1991) 297–306.
[25] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon, Biophys. J. 58 (5) (1990) 1089–1098.
[26] J. Gehl, T.H. Sorensen, K. Nielsen, P. Raskmark, S.L. Nielsen, T. Skovsgaard, L.M. Mir, *In vivo* electroporation of skeletal muscle: threshold, efficacy and relation to electric field distribution, Biochim. Biophys. Acta 1428 (2–3) (1999) 233–240.
[27] D. Miklavčič, K. Beravs, D. Šemrov, M. Čemažar, F. Demšar, G. Serša, The importance of electric field distribution for effective in vivo electroporation of tissues, Biophys. J. 74 (1998) 2152–2158.
[28] M. Pavlin, N. Pavselj, D. Miklavcic, Dependence of induced transmembrane potential on cell density, arrangement, and cell position inside a cell system, IEEE Trans. Biomed. Eng. 49 (6) (2002) 605–612.
[29] G. Pucihar, T. Kotnik, J. Teissié, D. Miklavcic, Electropermeabilization of dense cell suspensions, Eur. Biophys. J. 36 (3) (2007) 173–185.
[30] J. Langus, M. Kranjc, B. Kos, M. Šuštar, D. Miklavčič, Dynamic finite-element model for efficient modelling of electric currents in electroporated tissue, Sci. Rep. 6 (26409) (2016) 2016.
[31] S. Corovic, I. Lackovic, P. Sustaric, T. Sustar, T. Rodic, D. Miklavcic, Modeling of electric field distribution in tissues during electroporation, Biomed. Eng. Online 12 (2013) 16.
[32] D. Šel, D. Cukjati, D. Batiuskaite, T. Slivnik, L.M. Mir, D. Miklavčič, Sequential finite element model of tissue electropermeabilization, IEEE Trans. Biomed. Eng. 52 (2005) 816–827.
[33] H. Akiyama, S. Katsuki, L. Redondo, M. Akiyama, A.J.M. Pemen, T. Huiskamp, F.R.J.C.M. Beckers, E.J.M. van Heesch, G.J.J. Winands, S.J. Voeten, L. Zhen, J.W.M. van Bree, S. Xiao, R. Petrella, in: H. Akiyama, R. Heller (Eds.), Pulsed Power Technology, Springer, Japan, 2017 (Bioelectrics, Chap 2, doi 10.1007/978-4-431-56095-1_2).
[34] L.M.S. Redondo, Basic concepts of high-voltage pulse generation, in: D. Miklavcic (Ed.), Handbook of Electroporation, Springer 2017, pp. 1–21, https://doi.org/10.1007/978-3-319-26779-1_209-1.
[35] E. Pirc, M. Reberšek, D. Miklavčič, Dosimetry in electroporation-based technologies and treatments, in: M.S. Markov (Ed.), Dosimetry in Bioelectromagnetic, CRC Press, Boca Raton 2017, pp. 233–268.
[36] M. Reberšek, in: D. Miklavcic (Ed.), Beyond Electroporation Pulse Parameters: From Application to Evaluation Handbook of Electroporation, Springer 2017, pp. 1–21, https://doi.org/10.1007/978-3-319-26779-1_222-1.
[37] D. Miklavčič, D. Šemrov, H. Mekid, L.M. Mir, A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy, Biochim. Biophys. Acta 1523 (2000) 73–83.
[38] F. Maglietti, S. Michinski, N. Olaiz, M. Castro, C. Suárez, G. Marshall, The role of pH fronts in tissue electroporation based treatments, PLoS One 8 (11) (2013), e80167https://doi.org/10.1371/journal.pone.0080167.
[39] G. Pataro, M. Falcone, G. Donsì, G. a Ferrari, Metal release from stainless steel electrodes of a PEF treatment chamber: effects of electrical parameters and food composition, Innovative Food Sci. Emerg. Technol. 21 (2014) 58–65.
[40] N. Pavšelj, D. Miklavčič, Numerical modeling in electroporation-based biomedical applications, Radiol. Oncol. 42 (2008) 159–168.
[41] L.G. Campana, B. Mali, G. Serša, S. Valpione, C.A. Giorgi, P. Strojan, D. Miklavčič, C.R. Rossi, Electrochemotherapy in non-melanoma head and neck cancers: a retrospective analysis of the treated cases, Br. J. Oral Maxillofac. Surg. 52 (2014) 957–964.
[42] O.N. Pakhomova, B.W. Gregory, V.A. Khorokhorina, A.M. Bowman, S. Xiao, A.G. Pakhomov, Electroporation-induced electrosensitization, PLoS One 6 (2) (2011), e17100https://doi.org/10.1371/journal.pone.0017100 (Launikonis B, editor).
[43] O.N. Pakhomova, B.W. Gregory, A.G. Pakhomov, Facilitation of electroporative drug uptake and cell killing by electrosensitization, J. Cell. Mol. Med. 17 (2013) 154–159.
[44] B. Zorec, S. Becker, M. Reberšek, D. Miklavčič, N. Pavšelj, Skin electroporation for transdermal drug delivery: the influence of the order of different square wave electric pulses, Int. J. Pharm. 457 (2013) 214–223.
[45] T. Blagus, B. Markelc, M. Cemazar, T. Kosjek, V. Preat, D. Miklavcic, G. Sersa, In vivo real-time monitoring system of electroporation mediated control of transdermal and topical drug delivery, J. Control. Release 172 (3) (2013) 862–871, https://doi.org/10.1016/j.jconrel.2013.09.030.
[46] A. Donate, D. Coppola, Y. Cruz, R. Heller, Evaluation of a novel non-penetrating electrode for use in DNA vaccination, PLoS One 6 (4) (2011), e19181.
[47] F. Lin, X. Shen, G. Kichaev, J.M. Mendoza, M. Yang, P. Armendi, J. Yan, G.P. Kobinger, A.J. Bello, A. Khan, K.E. Broderick, N.Y. Sardesai, Optimization of electroporation-enhanced intradermal delivery of DNA vaccine using a minimally invasive surface device, Hum. Gene Ther. 23 (3) (2012) 157–168, https://doi.org/10.1089/hgtb.2011.209.
[48] T. Kotnik, G. Pucihar, M. Rebersek, D. Miklavcic, L.M. Mir, Role of pulse shape in cell membrane electropermeabilization, Biochim. Biophys. Acta 1614 (2) (2003) 193–200.
[49] A.G. Pakhomov, I. Semenov, S. Xiao, O.N. Pakhomova, B. Gregory, K.H. Schoenbach, J.C. Ullery, H.T. Beier, S.R. Rajulapati, B.L. Ibey, Cancellation of cellular responses to nanoelectroporation by reversing the stimulus polarity, Cell. Mol. Life Sci. 71 (22) (2014) 4431–4441, https://doi.org/10.1007/s00018-014-1626-z.
[50] C.M. Valdez, R.A. Barnes Jr., C.C. Roth, E.K. Moen, G.A. Throckmorton, B.L. Ibey, Asymmetrical bipolar nanosecond electric pulse widths modify bipolar cancellation, Sci. Rep. 7 (1) (2017) 16372, https://doi.org/10.1038/s41598-017-16142-6.
[51] C. Muratori, M. Casciola, O. Pakhomova, in: D. Miklavcic (Ed.), Electric Pulse Repetition Rate: Sensitization and Desensitization Handbook of Electroporation, Springer 2017, pp. 1–16, https://doi.org/10.1007/978-3-319-26779-1_23-1.
[52] G. Pucihar, L.M. Mir, D. Miklavcic, The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy, Bioelectrochemistry 57 (2) (2002) 167–172.
[53] P.A. Garcia, R.V. Davalos, D. Miklavcic, A numerical investigation of the electric and thermal cell kill distributions in electroporation-based therapies in tissue, PLoS One 9 (8) (2014), e103083https://doi.org/10.1371/journal.pone.0103083.
[54] C. Faurie, E. Phez, M. Golzio, C. Vossen, J.C. Lesbordes, C. Delteil, J. Teissié, M.P. Rols, Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells, Biochim. Biophys. Acta 1665 (1–2) (2004) 92–100.
[55] P. Hojman, H. Gissel, F.M. Andre, C. Cournil-Henrionnet, J. Eriksen, J. Gehl, L.M. Mir, Physiological effects of high- and low-voltage pulse combinations for gene electrotransfer in muscle, Hum. Gene Ther. 19 (11) (2008) 1249–1260, https://doi.org/10.1089/hum.2008.059.
[56] Q. Hu, R.P. Joshi, Comparative evaluation of transmembrane ion transport due to monopolar and bipolar nanosecond, high-intensity electroporation pulses based on full three-dimensional analyses, J. Appl. Phys. 122 (034701) (2017)https://doi.org/10.1063/1.4994310.

[57] V. Todorović, U. Kamenšek, G. Serša, M. Čemažar, Changing electrode orientation, but not pulse polarity, increases the efficacy of gene electrotransfer to tumors *in vivo*, Bioelectrochemistry (2014) 1567–5394, https://doi.org/10.1016/j.bioelechem.2013.12.002 ([Print ed.], str. [1-9]).
[58] L. Chicaybam, A.L. Sodre, B.A. Curzio, M.H. Bonamino, An efficient low cost method for gene transfer to T lymphocytes, PLoS One 8 (3) (2013), e60298https://doi.org/10.1371/journal.pone.0060298.
[59] D.C. Sweeney, M. Reberšek, J. Dermol, L. Rems, D. Miklavčič, R.V. Davalos, Quantification of cell membrane permeability induced by monopolar and high-frequency bipolar bursts of electrical pulses, Biochim. Biophys. Acta 1858 (11) (2016) 2689–2698, https://doi.org/10.1016/j.bbamem.2016.06.024.
[60] M. Essone Mezeme, G. Pucihar, M. Pavlin, C. Brosseau, D. Miklavčič, A numerical analysis of multicellular environment for modeling tissue electroporation, Appl. Phys. Lett. 100 (2012), 143701.
[61] R. Susil, D. Šemrov, D. Miklavčič, Electric field induced transmembrane potential depends on cell density and organization, Electro- Magnetobiol. 17 (1998) 391–399.
[62] T. Dolinsek, G. Sersa, L. Prosen, M. Bosnjak, M. Stimac, U. Razborsek, M. Cemazar, Electrotransfer of plasmid DNA encoding an anti-mouse endoglin (CD105) shRNA to B16 melanoma tumors with low and high metastatic potential results in pronounced anti-tumor effects, Cancers (Basel) 8 (1) (2015)https://doi.org/10.3390/cancers8010003 (pii: E3).
[63] L. Heller, V. Todorovic, M. Cemazar, Electrotransfer of single-stranded or double-stranded DNA induces complete regression of palpable B16.F10 mouse melanomas, Cancer Gene Ther. 20 (12) (2013) 695–700, https://doi.org/10.1038/cgt.2013.71.
[64] C.J. Mann, X.M. Anguela, J. Montane, M. Obach, C. Roca, A. Ruzo, P. Otaegui, F. Mir LM Bosch, Molecular signature of the immune and tissue response to non-coding plasmid DNA in skeletal muscle after electrotransfer, Gene Ther. 19 (2012) 1177–1186, https://doi.org/10.1038/gt.2011.198.
[65] K. Znidar, M. Bosnjak, M. Cemazar, L.C. Heller, Cytosolic DNA sensor upregulation accompanies DNA electrotransfer in B16.F10 melanoma cells, Mol. Ther.–Nucleic Acids 5 (2016)https://doi.org/10.1038/mtna.2016.34 (ISSN 2162-2531, http://www.nature.com/mtna/journal/v5/n6/full/mtna201634a.html).
[66] E. Bellard, B. Markelc, S. Pelofy, F. Le Guerroué, G. Sersa, J. Teissié, M. Cemazar, M. Golzio, Intravital microscopy at the single vessel level brings new insights of vascular modification mechanisms induced by electropermeabilization, J. Control. Release 163 (3) (2012) 396–403, https://doi.org/10.1016/j.jconrel.2012.09.010.
[67] M. De Robertis, U. Lampreht Tratar, M. Cemazar, E. Signori, in: D. Miklavcic (Ed.), Predictable Animal Models for Translational Electroporation-Based Cancer Immunotherapy Studies Handbook of Electroporation, Springer 2016, pp. 1–21, https://doi.org/10.1007/978-3-319-26779-1_50-1.
[68] F.M. André, C. Cournil-Henrionnet, D. Vernerey, P. Opolon, L.M. Mir, Variability of naked DNA expression after direct local injection: the influence of the injection speed, Gene Ther. 13 (23) (2006) 1619–1627.
[69] T. Jarm, M. Cemazar, D. Miklavcic, G. Sersa, Blood flow-modifying and vascular-disrupting effects of electroporation and electrochemotherapy – implications in treatment of melanoma metastases, Expert. Rev. Anticancer. Ther. 10 (5) (2010) 729–746.
[70] M. Čemažar, R. Milačič, D. Miklavčič, V. Dolžan, G. Serša, Intratumoral cisplatin administration in electrochemotherapy: antitumor effectiveness, sequence dependence and platinum content, Anti-Cancer Drugs 9 (1998) 525–530.
[71] S. Corović, A. Zupanic, S. Kranjc, B. Al Sakere, A. Leroy-Willig, L.M. Mir, D. Miklavcic, The influence of skeletal muscle anisotropy on electroporation: in vivo study and numerical modeling, Med. Biol. Eng. Comput. 48 (7) (2010) 637–648.
[72] S. Corovic, B. Al Sakere, V. Haddad, D. Miklavcic, L.M. Mir, Importance of contact surface between electrodes and treated tissue in electrochemotherapy, Technol. Cancer Res. Treat. 7 (5) (2008) 393–400.
[73] A. Ivorra, B. Al-Sakere, B. Rubinsky, L.M. Mir, In vivo electrical conductivity measurements during and after tumor electroporation: conductivity changes reflect the treatment outcome, Phys. Med. Biol. 54 (19) (2009) 5949–5963, https://doi.org/10.1088/0031-9155/54/19/019.
[74] N. Abou-Madi, Anesthesia and analgesia of small mammals, in: R.D. Gleed, J.W. Ludders (Eds.), Recent Advances in Anesthesia and Analgesia: Companion Animals, International Veterinary Information Service, Ithaca, NY, 2006 (online).
[75] D.F. Kohn, S.K. Wixson, W.J. White, G.J. Benson, Anesthesia and Analgesia in Laboratory Animals, Academic Press, New York, 1997.
[76] R.I. Mazze, A.J. Wilson, S.A. Rice, J.M. Baden, Fetal development in mice exposed to isoflurane, Teratology 32 (1985) 339–345.
[77] Y. Miwa, K.K. Sladky, Small Mammals: Common Surgical Procedures of Rodents, Ferrets, Hedgehogs, and Sugar Gliders, Vet. Clin. North Am. Exot. Anim. Pract. 19 (1) (2016) 205–244, https://doi.org/10.1016/j.cvex.2015.09.001.
[78] D. Cressey, News Nature, 2011https://doi.org/10.1038/news.2011.391 (Published online 29 June 2011).

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