November 11-17, 2007
Ljubljana, Slovenia

Proceedings of the

Electroporation based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Edited by:

Peter Kramar
Damijan Miklavčič
Lluis M. Mir

Organised by:

University of Ljubljana
Faculty of Electrical Engineering
and
Institute of Oncology, Ljubljana

Supported and sponsored by:

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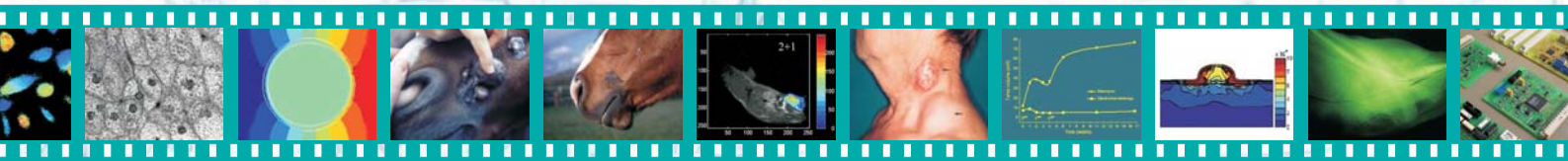

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Welcome note

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. It is now for the third time that we meet. Again it is with great pleasure that we can say: »with participation of many of the world leading experts in the field«. The intended audience are all those interested in applications of electroporation *in vitro*, *in vivo*, and in clinical environment. The most advanced among these applications are electrochemotherapy of tumors, which has already paved its way into clinical environment, and electroporative assisted drug and gene delivery, which is becoming more and more widely used in the experimental environment. The aim of the lectures at this Workshop and Course is to provide the participants with sufficient theoretical background and practical knowledge to allow them to use electroporation effectively in their working environments.

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

This year we celebrate **35 years** since the first publication of controlled electro-release of intracellular compounds[1], **25 years** since the first gene electrotransfer report[2], **20 years** since the first in vitro and in vivo experiments combining electroporation and bleomycin and their publications[3,4], which established grounds for the development of electrochemotherapy and **10 years** since first use of electrochemotherapy in veterinary medicine[5]. I know also that we (Lluis and Damijan) met for the first time in 1992. We have thus more than enough reasons to meet and to celebrate.

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

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

Sincerely Yours,

Damijan Miklavčič and Lluis M. Mir

[1] Neumann, E. and Rosenheck, K., Permeability changes induced by electric impulses in vesicular membranes of chromaffin granules. *J. Membrane Biol.* 10: 279-290, 1972

[2] Neumann, E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H., Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* 1: 841-845, 1982

[3] Okino M., Mohri H., Effects fo a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors, *Jpn J Cancer Res* 78: 1319-1321, 1987

[4] Orlowski S., Belehradek J. Jr, Paoletti C. and Mir L.M. Transient electropermeabilization of cells in culture: increase of the cytotoxicity of anticancer drugs. *Biochemical Pharmacology*, 37: 4727-4733, 1988

[5] Mir L.M., Devauchelle P., Quintin-Colonna F., Delisle F., Doliger S., Fradelizi D., Belehradek J. Jr and Orlowski S.. First clinical trial of electrochemotherapy for the treatment of cat soft tissue sarcomas. *British Journal of Cancer* 76: 1617-1622, 1997

LECTURERS' ABSTRACTS

Biological Cells in Electric Fields

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

THE CELL AND ITS PLASMA MEMBRANE

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

From the electrical point of view, the cell can roughly be described as an electrolyte (the cytoplasm) surrounded by an electrically insulating shell (the plasma membrane), and under physiological conditions, the surroundings of the cell are also an electrolyte. Due to this, when a cell is exposed to an external electric field, practically all of the electric field in its very vicinity concentrates within the membrane, which thus shields the cytoplasm from the exposure. The concentration of the electric field inside the membrane results in a voltage (electric potential difference) on it. As the electric field vanishes, so does the voltage caused by this field.

In the first approximation, one thus treats the membrane as purely dielectric (i.e., having zero electric conductivity), and the cytoplasm and the extracellular space as purely conductive (i.e., having zero dielectric permittivity). This approach becomes an oversimplification for rapidly time-varying electric fields, such as waves with frequencies above 1 MHz, or electric pulses with durations below 1 μs. To analyze the exposures of the cell to such fields, 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. While its inner surface is of course still a spheroid (or an ellipsoid), its outer surface lacks a simple geometrical characterization.[1] Fortunately, this complication does not affect the 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 differs in its shape from the rest. With irregular geometries, the induced voltage cannot be determined analytically, and thus cannot be formulated as a function. This deprives us of some of the insight that is available for spherical or spheroidal cells, but using modern computers and finite-elements methods, the voltage induced on each particular irregular cell can still be determined numerically.

RESTING TRANSMEMBRANE VOLTAGE

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

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

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.

A spherical cell

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 solution of Laplace's equation is a formula often referred to as the static Schwan's equation [3],

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

where $\Delta\Phi_m$ is the induced transmembrane voltage, E is the electric field in the region where the cell is situated, R is the cell radius, and θ is the polar angle measured from the center of the cell with respect to the direction of the field. This formula tells that the maximum voltage is induced at the points where the electric field is perpendicular to the membrane, i.e. at $\theta = 0°$ and $\theta = 180°$, the points we shall refer to as the "poles" of the cell, and varies proportionally to the cosine of the angle in-between these poles (see Fig. 1). Also, the induced voltage is proportional to the applied electric field and to the cell radius.

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

$$\Delta\Phi_m = \frac{3}{2} E R \cos\varphi [1 - \exp(-t/\tau_m)] , \qquad (2)$$

where τ_m is the time constant of the membrane,

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

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, R the cell radius, and d the membrane thickness.

In certain experiments *in vitro*, where the conductivity of the extracellular medium is reduced by several orders of magnitude with respect to the physiological one, the factor 3/2 in (1) and (2) decreases, as described in detail in [5]. But generally, the formulae (2) and (3) are applicable to exposures to sine (AC) electric fields with frequencies below 1 MHz, and to rectangular electric pulses longer than 1 μs.

To determine the voltage induced by even higher field frequencies or even shorter pulses, the dielectric permittivities of the electrolytes also have to be accounted for. This leads to a further generalization of equations (2) and (3) to a second-order model [6-8], and the results it yields will be outlined in the last section of this paper.

A spheroidal or an ellipsoidal cell

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

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

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

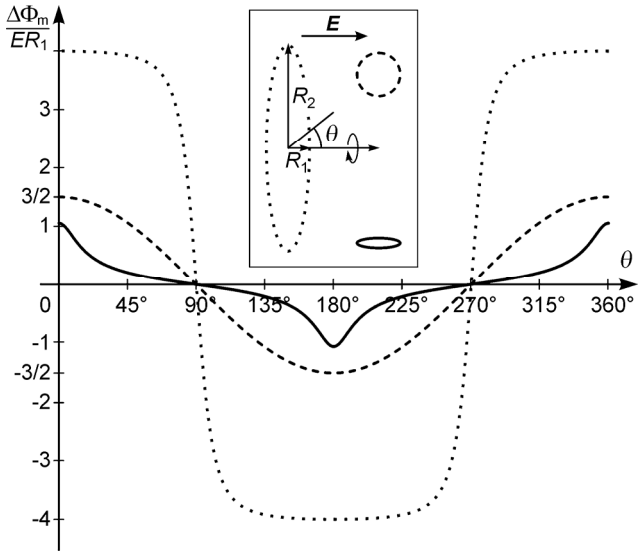

Figure 1: Normalized induced transmembrane voltage for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1$. Dotted: an oblate spheroidal cell with $R_2 = 5 \times R_1$. Adapted from [9] with the permission of the authors.

The second complication of generalizing the cell shape from a sphere to a spheroid or an ellipsoid is that the induced voltage now also becomes dependent on the orientation of the cell with respect to the electric field. To deal with this, one decomposes the field vector into the components parallel to the axes of the spheroid or the ellipsoid, and writes the induced voltage as a corresponding linear combination of the voltages induced for each of the three coaxial orientations [10,11]. Figs. 2 and 3 show the effect of rotation of two different spheroids with respect to the direction of the field.

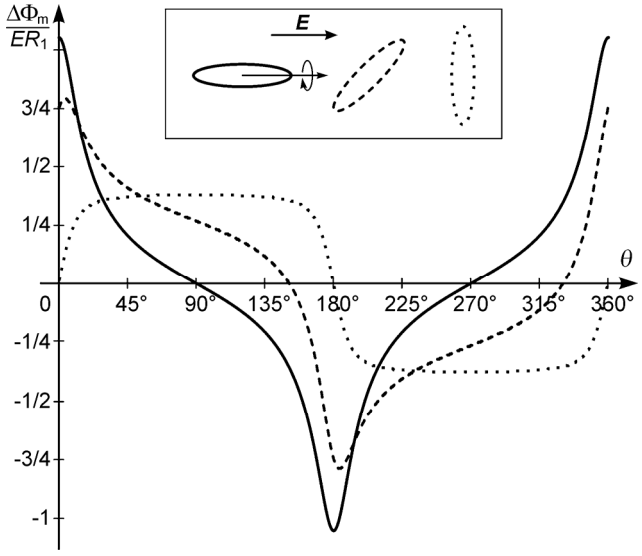

Figure 2: Normalized induced transmembrane voltage for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of the authors.

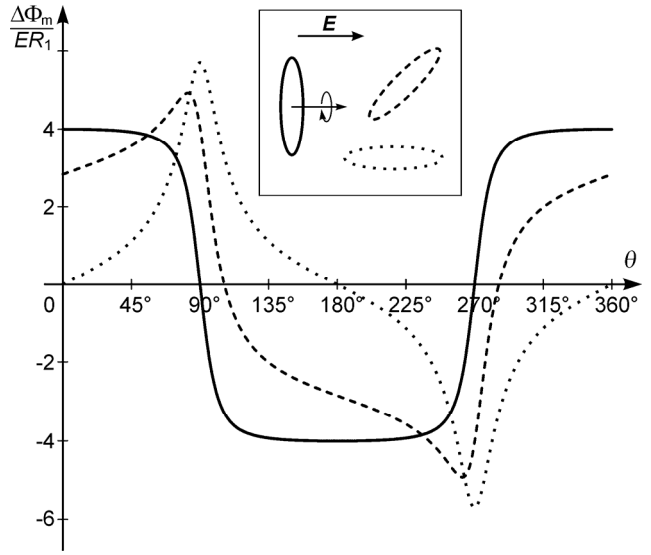

Figure 3: Normalized induced transmembrane voltage for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of the authors.

An irregularly shaped cell

For a cell having an irregular shape, the induced transmembrane voltage cannot be determined exactly, as for such a geometry Laplace's equation is not solvable analytically. Using modern computers and finite-elements tools such as Maxwell or FEMLab, the voltage induced on a given irregular cell can still be determined numerically, as described in detail in [12]. While the results obtained in this manner are quite accurate, they are only applicable to the particular cell shape for which they were computed. Fig. 4 shows examples of two cells growing in a Petri dish and the voltages induced on their membranes.

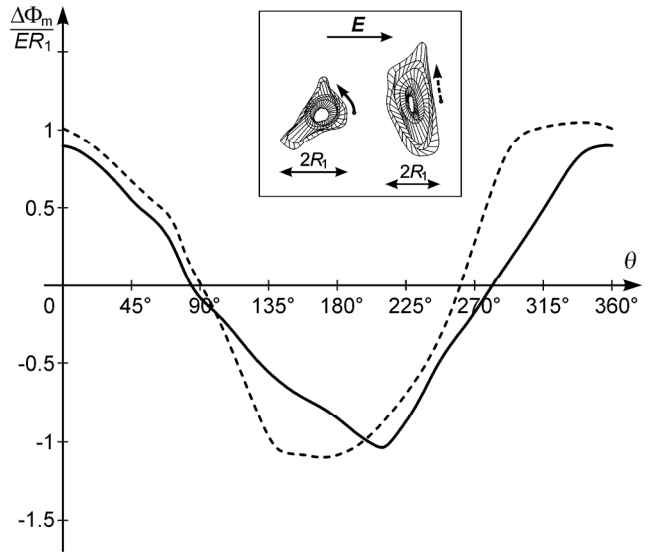

Figure 4: Normalized induced transmembrane voltage for two irregularly shaped cells growing on the flat surface of a Petri dish. Adapted from [12] with the permission of the authors.

Cells in suspension

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. 5). For volume fractions over ten percent, as well as for clusters and lattices of cells, one has to use appropriate numerical or approximate analytical solutions for a reliable analysis of the induced transmembrane voltage [13,14]. Regardless of the volume fraction they occupy, as long as the cells are suspended, they are floating freely, and their arrangement is rather uniform. Asymptotically, this would correspond to a face-centered lattice, and this lattice is also the most appropriate for the analysis of the transmembrane voltage induced on cells in suspension.

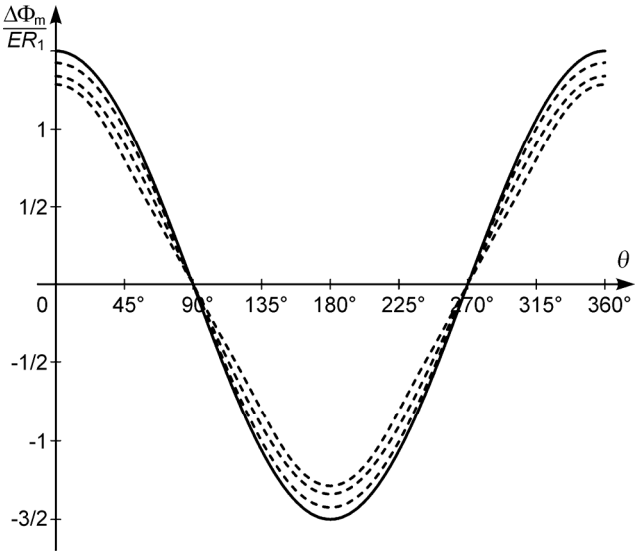

Figure 5: Induced transmembrane voltage normalized to electric field and cell radius. Solid: prediction of Schwan's equation, i.e., of formula (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (from top to bottom) 10%, 30%, and 50% of the total suspension volume. Adapted from [13] with the permission of the authors.

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 the membrane (τ_m) given by equation (3) implies that there is a delay between the external field and the voltage induced by it. As mentioned above, τ_m (and thus the delay) is typically somewhat below a microsecond, but 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 with 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 rectangular 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 [6-8], in which all electric conductivities and dielectric permittivities are accounted for.

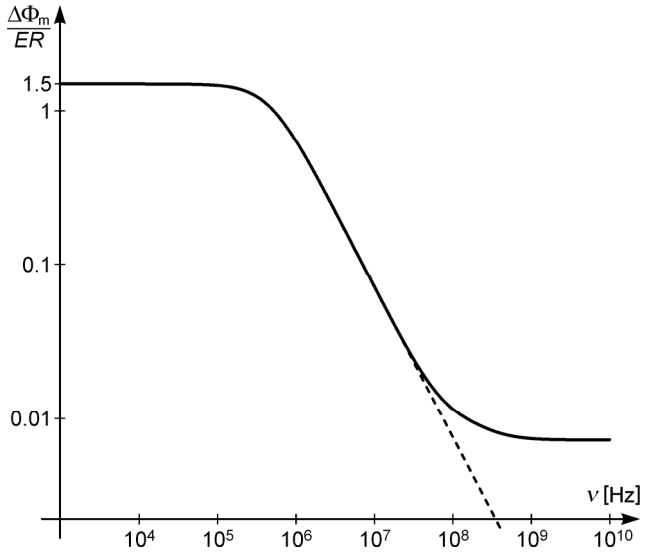

Figure 6: The amplitude of the induced transmembrane voltage, normalized to the amplitude of the field and cell radius, as a function of the frequency of the AC field. The solid curve shows the second-order, and the dashed one the first-order Schwan's equation. Note that both axes are logarithmic. Adapted from [8] with the permission of the authors.

With field frequencies approaching the gigahertz 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 even exceed the voltage induced on the plasma membrane [15]. This could provide a plausible 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 induce selective electroporation of organelle membranes, while the plasma membrane is left intact [16-18].

REFERENCES

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

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

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

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

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

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

[7] 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.

[8] 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.

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

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

[11] 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.

[12] 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.

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

[14] 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.

[15] 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.

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

[17] 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.

[18] 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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Tadej Kotnik is the first author of 17 articles in SCI-ranked journals. In 2001 he received the Galvani Prize of the Bioelectrochemical Society.

NOTES

Electric Properties of Tissues and Their Changes During Electroporation

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

INTRODUCTION

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

BIOLOGICAL MATERIALS IN THE ELECTRIC FIELD

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

If a conductor is placed in an electric field, charges will move within the conductor until the interior field is zero. In the case of an insulator, there are no free charges so net migration of charge does not occur. In polar materials, however, the positive and negative charge centers in the molecules do not coincide. An applied field, E_0, tends to orient the dipoles and produces a field inside the dielectric, E_p, which opposes the applied field. This process is called polarization [1, 3]. Most materials contain a combination of orientable dipoles and relatively free charges so that the electric field is reduced in any material relative to its free-space value. The net field inside the material, E, is then

$$E = E_0 - E_p$$

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

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

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

On a macroscopic level we describe the material as having a permittivity, ε, and a conductivity, σ. The permittivity characterizes the material's ability to trap or store charge or to rotate molecular dipoles whereas the conductivity describes its ability to transport charge. The permittivity also helps to determine the speed of light in a material so that free space has a permittivity ε_0 =8.85 x 10-12 F/m. For other media:

$$\varepsilon = \varepsilon_r \varepsilon_0$$

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

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

Consider a sample of material which has a thickness, d, and cross-sectional area, A. 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 IC = 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 = Ic + Id, hence

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

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

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

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

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

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

We can also denote total current as:

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

We can define a complex-valued, relative permittivity:

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

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

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

We can consider the conductivity of a material as a measure of the ability of its charge to be transported throughout its volume by an applied electric field. Similarly, its permittivity is a measure of the ability of its dipoles to rotate or its charge to be stored by an applied external field. Note that if the permittivity and conductivity of the material are constant, the displacement current will increase with frequency whereas the conduction current does not change. At low frequencies the material will behave like a conductor, but capacitive effects will become more important at higher frequencies. For most materials, however, σ* and ε* are frequency-dependent. Such a variation is called dispersion. Biological tissues exhibit several different dispersions over a wide range of frequencies [1, 3].

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

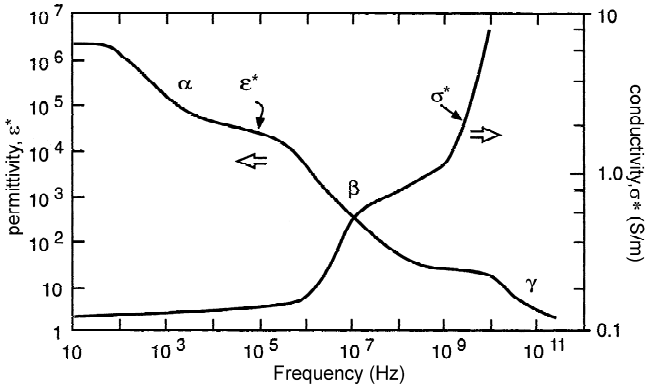

Figure 1: Typical frequency dependence of the complex permittivity and complex conductivity of a heterogeneous material such as biological tissue.

DIELECTRIC MEASUREMENTS OF TISSUES

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

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

Inhomogeneity of tissues

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

Anisotropy of tissues

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

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

Physiological factors and changes of tissue

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

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

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

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

Electrode polarization

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

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

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

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

stabilize. A typical time might be on the order of thirty minutes.

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

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

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

ELECTRICAL RESPONSE OF TISSUE TO ELECTRIC FIELD

Changes in tissue conductivity have been observed in vivo if the tissue is subjected to a high enough electric field. Having said that, we can use the dielectric properties of liver and try to calculate the electrical response to a train of 8 short rectangular voltage pulses having the duration of 100 μs, the rise time of 1 μs and the repetition frequency 1 Hz (typical pulse train used for electrochemotherapy). The complications arise from the facts that i) the pulse parameters (the pulse duration, the rise and the fall time) determine the span of its frequency spectrum and ii) the tissue conductivity and permittivity are frequency dependent. The obtained response for the first pulse is presented in Figure 2. At the onset of voltage pulse, capacitive transient displacement current is observed. As membranes charge, voltage across them rises and the measured current decreases. Soon steady state is reached and current stabilizes through the conductance of extracellular fluid. Since the model describing dielectric dispersions is linear, change of the applied voltage proportionally scales the amplitude of response current.

We can compare this calculated response with the measured response on rat liver *in vivo* for the same pulse train as above and different pulse amplitudes spanning up to electroporative field strengths (Fig. 3) [4]. For the lowest applied voltage we can see good agreement with calculated response. As the field intensity is increased, the electrical response of tissue is no longer linear and increase of conductivity during the pulse is observed. Measuring the passive electrical properties of electroporated tissues could provide real time feedback on the outcome of the treatment [4, 5].

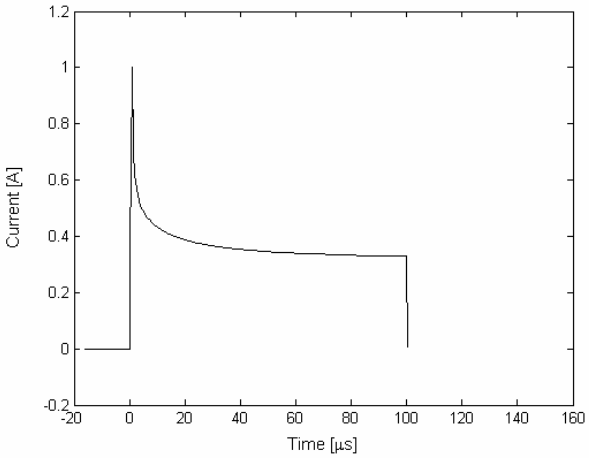

Figure 2: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs having the rise time of 1 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

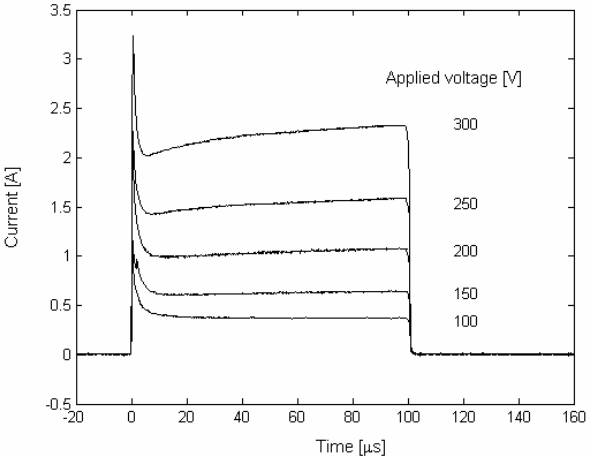

Figure 3: Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [4]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

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 [6, 7].

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 (known as voltage divider) causes the voltage to be distributed between the resistors proportionally to their resistivities. Upon applying electric pulses, almost the entire applied voltage thus rests across the most resistive (least conductive) tissue, in our case skin. That causes a very high electric field in skin tissue, while the electric field in other tissues stays too low for a successful electropermeabilization. If our goal is the electrochemotherapy of the underlying tumor, one might ask the question of how is a successful electrochemotherapy of subcutaneous tumors still possible when external plate electrodes are used. The answer lies in the increase in bulk conductivities of tissues during electroporation, a phenomenon that was also observed in vivo. This conductivity increase leads to a changed electric field distribution, which exposes the tumor to an electric field high enough for a successful permeabilization [8]. To support this hypothesis, we described this process with a numerical model, taking into account the changes of tissue bulk electrical properties during the electropermeabilization. In Figure 4 six steps of the electroporation process in the subcutaneous tumor model for the voltage of 1000 V between the electrodes are shown. The electric field distribution is shown in V/cm. Step 0 denotes the electric field distribution as it was just before the electroporation process started, thus when all the tissues still had their initial conductivities. When the voltage is applied to the electrodes, the electric field is distributed in the tissue according to conductivity ratios of the tissues in the model. The field strength is the highest in the tissues with the lowest conductivity, where the voltage drop is the largest and the voltage gradient the highest. In our case, almost the entire voltage drop occurs in the skin layer which has a conductivity of about 10-100 times lower than the tissues lying underneath.

If we look at the last step of the sequential analysis, step 5, at 1000 V (Figure 4) the tumor is entirely permeabilized, in some areas the electric field is also above the irreversible threshold (800 V/cm).

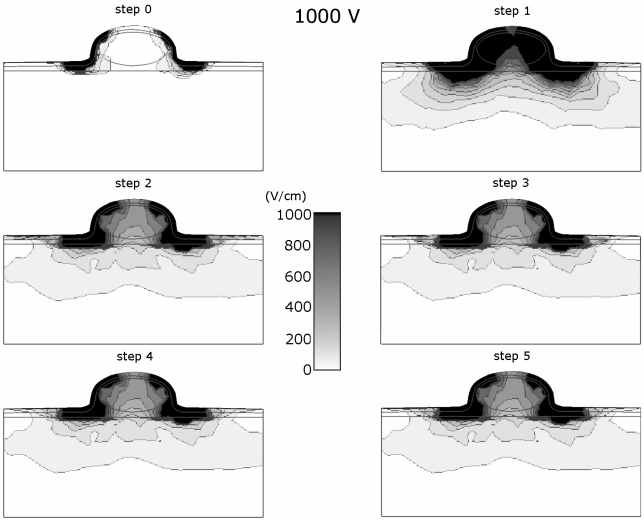

Figure 4: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [8]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact 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 [9]. Skin consists of three main layers: epidermis, dermis and subcutaneous tissue. Skin epidermis is made up of different layers, but the one that defines its electrical properties the most is the outermost layer, the stratum corneum. Although very thin (typically around 20 μm), it contributes a great deal to the electrical properties of skin. Its conductivity is three to four orders of magnitude lower than the conductivities of deeper skin layers. Again, when electric pulses are applied on skin fold through external plate electrodes, almost the entire applied voltage rests across the stratum corneum, which causes a very high electric field in that layer, while the electric field in deeper layers of skin – the layers targeted for gene transfection – stays too low. Similarly as in the case of subcutaneous tumors, the increase in bulk conductivities of skin layers during electroporation exposes the skin layers below stratum corneum to an electric field high enough for a successful permeabilization.

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

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

[4] 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.

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

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

[7] 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.

[8] 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 Transactions on Biomedical Engineering* 52(8):1373-1381, 2005.

[9] 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.

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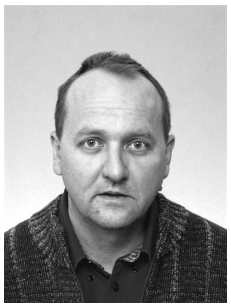

Damijan Miklavčič was born in Ljubljana, Slovenia, in 1963. He received a Masters and a Doctorate in Electrical Engineering from University of Ljubljana in 1991 and 1993, respectively. He is currently Professor and Head of the Laboratory of Biocybernetics, and Head of the Department for Biomedical Engineering at the Faculty of Electrical Engineering, University of Ljubljana.

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

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

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

NOTES

Theory of Membrane Electroporation (Part I) and Mechanisms of Transport Processes (Part II) for Medical Electroporation Treatments

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Abstract: The digression on the physical chemical theory of membrane electroporation (MEP) focuses on the thermodynamics of field pulse effects as well as on the kinetics of the various electroporation processes, including the transport flows, caused by the field induced structural flows. As commonly encountered with field effects on material structures, the primary membrane electroporation process in the applied electric field force is rapid (ns up to ms and seconds) as compared to the very much slower structural pore resealing processes (s, min, h) after the field. Importantly for field effects, the relatively small external fields are geometrically amplified by Maxwell Wagner polarization to yield the large induced membrane fields causing directly rearrangements of membrane components, such lipids and bound water. The concomitant electromechanical Maxwell stress leads to elongations of lipid vesicles and cells. Transiently adsorbed particles facilitate, by curvature effects both, electroporation and material transport, as well as global shape changes -- Together with data from flow analysis, using time-dependent flow coefficients, the thermodynamic and kinetic information may be used not only as a general guideline for the analysis of measured data correlations, but also for the goal-directed design of new experiments and apparatus for the various medical electroporation treatments.

INTRODUCTION

Science is corrective in itself, however, the fundamental formulae descibring laws of nature must be absolutely correct.

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

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

The concept of functional membrane electroporation (MEP) is concomitant with a proposal for a molecular mechanism for the primary effect of the electric field forces [1,5], in directly targeting locally the polar head groups of lipids, finally leading to the formation of (hydrophilic) pores. Translational motions of polar lipids along the curved pore walls, driven by the membrane field, also rationalize the huge acceleration of lipid flip flop and intra-wall motion of phosphoryl inositol from the internal membrane monolayer to the outer monolayer. It is also discussed that electric pulses of already low field intensity, but longer pulse durations, electroporatively facilitate both, endocytotic uptake of external particles and exocytotic release of intracellular cell components, respectively.

Recently, the original features and later advancements of MEP theory of membrane for closed membrane shells have been summarized in a concise digression [5]. In detail, emphasis is on the primary field-induced lipid processes, rationalizing also the observed longevity of the porous structures in terms of the locally defined cooperativity of the lipids in the highly curved pore wall. The theory-based technical developments of the advanced electroporation culminate in the new clinical disciplines of electrochemotherapy and gene electrotherapy (L.Mir, R.Heller, D.Miklavcic, J.Teissie, G.Sersa, J.Gehl, K. Schoenbach, R.Cadossi (IGEA Cliniporator™). Naturally, the following detailed summary of the state of the art, is restricted to a critical appreciation of the data-based physical chemical theory of MEP.

PART I.
PHYSICAL CHEMICAL THEORY OF ELECTROPORATION

The physical chemical theory focuses on the structural features of MEP, as analytically apparent in the conductometric relaxation kinetic data of cells and cell suspensions, as well as in the conductometric and electrooptical data of uni-lamellar lipid vesicles with their closed membrane shells, jugded as a good model for the curved lipid parts of cell membranes. On the other hand, it is noted that most planar lipid bilayers are under torus tension, enhancing the field-induced electromechanical stress, finally leading to bilayer breakdown (rupture). Physical electroporation theories concentrate on the dependence of the electric polarization term, line tension and surface tension, respectively, on the pore radius, and the electric field strength, yet neglecting the chemical free energy term in pore formation and resealing processes.

In summary, the observed *exponential* time courses of electrooptic signal traces and conductometric current traces, analyzed in terms of relaxation normal modes, clearly indicate that there are at least two types of "electropores", in the membranes of lipid vesicles [6] and in the cell membranes of densely packed CHO cells [7], respectively: (I) short-lived pores (of type P_1, after-field life time of 1-5 ms [8], average pore diameter of 1.6 ± 0.2 nm), and (II) a few long-lived larger electropores (of type P_2, after-field life times of 20-40 min, average pore diameter ≥ 2 nm[7]. These P_2 –pores, suggested as "open" and "closed" hydrophilic pore configurations, are the specific candidates to rationalize the structural longevity of the porous/permeable membrane states, leading to the long-lived mass transport after the pulse trains. Since usually the pulse times must be short, mass transport within the field duration is oftenly very small. These structural features of pore longevity are also instrumental for rationalizing the various data obtained with pulse train combination modes of high voltage (HV) pulses and low voltage (LV) pulses and the effects of a time interval between the pulses.

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

Electric field is "acting force" on charge.

In order to rationalize and understand the field effects, also those involved in the medical electroporation treatments, it is essential to realize that the *electric field E* (of a voltage pulse) *acts as a force simultaneously on all the polar (ionic and dipolar) groups of the membrane components.* Maxwell Wagner ionic membrane polarization rationalizes that the field, produced by the surface ions on the two sides of the dielectric membrane, acts also over those molecules which are in interactive (adsorption) contact with the membrane surface, also when adsorbed on the pole caps, where the local external fields are zero. The field forces directly cause the local structural reorganisations (to a higher field-stabilized order), in the lipid parts of the cell membranes, resulting in pores. The pores, subsequently permit, as permeation sites, cross-membrane transport of small ions and large ions (along the field lines) and of both, externally applied (and transiently adsorbed) substances into the inside, and of intracellular components to the outside.

Interactive transport.

Due to the relative smallness of the pores, the migration of substances, including small ions, through the porous parts is *always interactive*, i.e., mainly by electrostatic transient contacts between the transported substance and the structured pore wall. Patch clamp current data suggest that larger molecules like proteins and polyelectrolytes like DNA, are in, usually Ca, Mg–dependent, multiple contacts with the neighbouring pore wall lipids [10]. It is thus essential that detailed theory and analysis of electroporative transport, also incorporates the thermodynamic interactions between the adsorbed molecules with the membrane, and also between the pore wall and the migrating molecules during the transport phase.

Polynucleotide transport

The amplified large membrane field can draw charged particles on to those initially small hydrophilic pores, which are situated near the contact sites. The respective pores apparently percolate to narrow long pores. The lengthwise adsorbed DNA occludes the long overall pore. As a polyanion, DNA electro-migrates in the direction opposite to the direction of the membrane field. So, on one cell hemisphere, a given external field draws the DNA towards and into the membrane and, on the other hemisphere, away from the membrane. Once within a hydrophilic pore structure, the DNA is an interactive part of this local membrane patch. This important feature rationalizes that the small-ion leak currents, measurable in patch clamp configuration with lipid surface-adsorbed DNA, are linearly dependent on the length of the adsorbed parts of long DNA molecules The transient DNA-lipid complexation thus lengthwise occludes the respective "long pore" [10].
Larger globular molecules like proteins, and larger dye and drug molecules, appear to interactively funnel through crater-like bended pore walls, occluding, as transient protein-lipid complexes, an

only apparently large pore. At the end of the pulse, the (drawn-in) "membrane particles" can thermally redistribute. Membrane-adherent DNA after the pulses can, in principle, dissociate from the membrane into the cell interior, or, alternatively, can return into the outside solution. The formerly occluded large pore, freed from the macromolecule, now can reorganize to the closed membrane configuration. The random thermal nature of annealing the pore the structured pore wall, where the respected wall lipids will go through many mismatched lipid-water associations, rationalizes the slowness of the pore resealing process. The indicated coupling of transport to preceding dynamic adsorption, which covers only a small part of the surface, and the relatively small pore density, rationalizes the relatively small efficiency of DNA uptake by single cells in suspension of lower DNA concentration.

Chemical scheme for pore formation

The electroporation data basis suggests that the structural membrane transitions of pore formation and pore resealing, certainly involve a whole cascade of (fluctuating) field sensitive closed membrane states (C) and a sequence of (fluctuating) porous states (P). The initially proposed overall two-state scheme [1]

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

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

$$nL \rightleftharpoons L_n \rightleftharpoons L_n(W).$$

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

Hysteresis

Scheme (1) is also useful to feature another particular aspect of the cyclic electroporation-resealing phenomenon. The data suggest that the cycle may be viewed as a globally reversible, yet intrinsically unidirectional, (i.e., irreversible) hysteresis of pore formation in the field and slower pore resealing after the field, with different structural intermediates on the different branches of the hysteresis loop [9].

Equilibrium distribution constants

The distribution equilibrium constant for the overall Scheme (1) is defined as the state density ratio K= [(P)]/[(C)] = f/(1-f), expressed in terms of the fraction f = [(P)]/([(P)]+[(C)]) of pore states. The data suggest that both, K and f, increase with increasing field, up to a maximum of a few 0.1 percent in biological cells, and a few percent in curved lipid bilayers of vesicles with diameters of several hundred nanometers. It is recalled that the kinetic normal modes of the actual conductometric and electrooptic relaxation spectrometric data [6,7], require the use of reaction schemes of the types:

$$(P^*) = (P_1) \text{ and } (P_1) \rightleftharpoons (P_2) \qquad (2)$$

as a minimum to describe the, at least two, discernible kinetic phases. Here, the equilibrium distribution constant K_2 for the $(P_1) \rightleftharpoons (P_2)$ transition to larger pores of type P_2 is related to the fraction $f_2 = (P_2)/((P_2) + (P_1)$ of pores of type (P_2), in the range $0 \leq f_2 \leq 1$ (and not $f \ll 1$ as in [6,7]), according to $K_2 = f_2/(1-f_2)$. Similar expressions are formulated for the transition $(P^*) = (P_1)$.

Chemical energetics

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

$$RT\,d\ell n \cdot K = (\Delta_r H^0)_{p,E}\,dT + \left(-\Delta_r V^0\right)_{T,E}\,dp + (\Delta_r M^0)_{p,T}\,dE \qquad (3)$$

where $R = k\,N_A$ is the gas constant, k the Boltzmann constant and N_A the Loschmidt-Avogadro constant (mol^{-1}). Note that Eq.(3) refers to the three physical chemical poration phenomena. Electroporation is characterized by the standard value $\Delta_r M° = M(P) - M(C)$ of the reaction dipole moment for the (C) to (P) transition, "sonoporation" by the standard reaction volume $\Delta_r V°$ and "thermoporation", including thermal aspects of laser "opto-poration", by the standard reaction enthalpy $\Delta_r H°$.

Note that $\Delta_r H°$ is the total energy at constant pressure p, at a given temperature T and field strength E. Note that, for field effects, we have to use the reaction enthalpy $\Delta_r \hat{H} = \Delta\hat{G} + T\Delta S$, where the work potential $\Delta\hat{G} = \Delta G - EM$ is the Legendre-

transformed Gibbs reaction energy, ΔG the ordinary Gibbs reaction energy in the field E, M the projection of the total electric moment vector **M** onto the direction of **E**, and ΔS the reaction entropy, all at constant pressure. Similarly, the Legendre-transformed transformed enthalpy is $\Delta\hat{H} = \Delta H - EM$.

Electro-thermodynamic stability criteria

The expression $\Delta_r\hat{H} = \Delta\hat{G} + T\Delta S$ = w_e + q_p , where q_p is the exchangeable heat energy at constant pressure, and w_e the extra work matching the (reversibly) work potential $\Delta\hat{G} = \Delta G - EM$, can be viewed as **the first law of electro-thermodynamics** at constant pressure in a canonical ensemble, i.e., a reactive system neighboured by other molecules. The electro-thermodynamic standard term $\Delta_r G°$ represents the reversible extra work and $q_p = T\Delta_r S°$ refers to the reversible, i.e., exchangeable, heat energy. The Legendre transformation of G transforms dG in such a way that, in $d\hat{G} \le 0$, the dependence on the field variable appears as dE. Hence, the stability criteria at constant p,T, $\Delta_r\hat{G} \le 0$, equality sign for equilibrium, negative for spontaneity, are consistent with general thermodynamic reasoning [11].

Connection between experimental quantity and field

The experimental signal (S), for instance, optical absorbance, current, or conductance, can be cast into a fractional "effect" f = S/S_{max} , which, inturn, is connected to the respective thermodynamic quantity K. When scheme (2) is applied, K refers to K_1 for the transition (P*) = (P_1) and, respectively, K refers to K_2 for the transition (P_1) $\rightleftharpoons$ (P_2). The field dependence of any K = f/(1-f), and thus of the fraction f, is given by:

$$K(E) = K(0) \exp X(E_{loc}) \qquad (4)$$

Here, it is seen that the field dependence of the (experimental) f(E) is described via the field dependence of the electro-thermodynamic quantity K(E). Note that Eq.(4) results from the integration of the appropriate van't Hoff relationship, Eq.(3), and reflects the electrochemical equilibrium stability condition for dipolar molecular organizations according to:

$$\Delta_r\hat{G}_{p,T} = (\Delta_r\hat{G}^0)_{p,T} + RTd\,\ell n \cdot K = 0 .$$

From this, the two familiar chemical thermodynamic relationships result as [11]:

$$RTd\,\ell n \cdot K = -(\Delta_r\hat{G}^0)_{p,T} \quad \text{and}$$

$$K(E) = K(0) \exp\,[-(\Delta_r\hat{G}^0)_{p,T}\ /RT].$$

The respective field effect factor X is specified as

$$X(E_{loc}) = \int \Delta_r\, M dE_{loc}/RT, \qquad (5)$$

where E_{loc} is the local field at the (molecular) site of field action. For instance, if we refer to permanent dipoles, $E_{loc} = E_{dir}$,the directing field, calculated from the induced membrane field E_m , which is given by:

$$E_m = -\Delta\varphi_m / d_m \qquad (6)$$

where d_m is the membrane of thickness, and $\Delta\varphi_m$ = - U_m the potential difference across the membrane and U_m the membrane voltage. In this context it is recalled that the electric current density vector $\mathbf{j_m}$ for the cross membrane by both cation flows and the anion flows, is given by:

$$\mathbf{j_m} = \sigma_m(-\nabla\varphi_m) = \sigma_m \mathbf{E_m} \ , \qquad (7)$$

where σ_m is the specific conductance or conductivity of the membrane, i.e. of all the conductive pores.

Field-induced membrane potential(difference)

For spherical membrane shells, the *stationary value* (ss), after rapid built-up of the Maxwell-Wagner polarization, of the externally induced electric potential difference $\Delta\varphi_m$ (or E-induced membrane potential) under the angle θ to the direction of the homogeneous external field vector **E**, see, Fig. 1, is given by[12]:

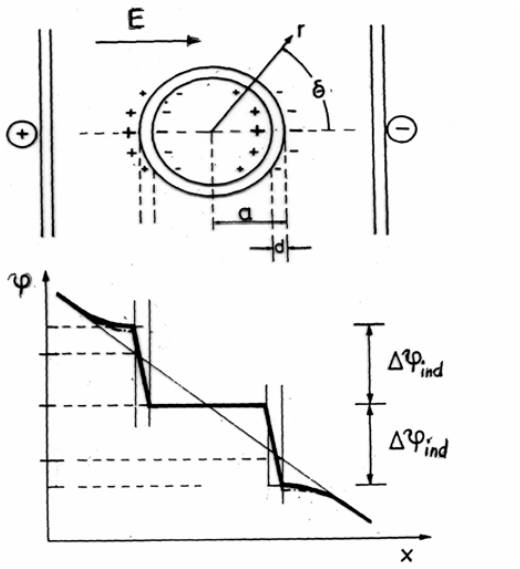
$$\Delta\varphi_m(\theta, E) = -(3/2) \cdot a \cdot E \cdot f_\sigma \left|\cos\theta\right| \qquad (8)$$

where a is the scalar value of the radius vector **r**, f_σ = $1-(1/2)(\sigma_m / \sigma(ext))a/d_m$ is the membrane conductivity factor, for the condition that the inequality $\sigma(int) \gg \sigma(ext)$ holds for conductivities of the cell interior and the outside solution, respectively. Note that Eq.(8) is consistent with the Maxwell definition of the field as the negative gradient $(-\nabla\varphi_m)$ of the potential. Eq.(8) is unrestrictive generally applicable for the description of current flows *in the direction* of the **E** vector, through the two hemispheres of a spherical membrane shell, consistent with the fundamental Eq.(7). The contribution of the natural membrane potential (difference) $\Delta\varphi_{nat}$ is readily incorporated as

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

Ionic current flow direction

Fig. 1 graphically "visualizes" the direction of the **E** vector as the direction of current of the positive ions along and "down" the negative gradient of the potential from left to right, accounting for the minus sign in Eq.(8).



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

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

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

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

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

The voltage U = E/l = $-\Delta\varphi$, where l is the distance between the planar electrodes, refers to the negative value of the potential difference $\Delta\varphi$. On the same line, the form of the membrane voltage U_m = $-\Delta\varphi_m$ refers to the negative value of the potential difference with respect to the positive value of E_m. In previous notations, in Fig. 1 the conductivity factor is given f_λ, here f_σ and the scalar product(E A) of the vectors **E** and **a = r,** here (**E a**).

Note that Eq.(8) reflects the transformation of spherical coordinates into the Cartesian coordinates of the external field vector **E.** If, on the right hand side hemisphere, the radius vector $\mathbf{r}_+$, is in the direction of **E**, then vector algebra dictates that the radius vector of the left hemisphere $\mathbf{r}_- = -\mathbf{r}_+$. Hence the product $r_+ \cos\theta\,(0°,90°) = r_- \cos\theta\,(90°,180°) = a\,|\cos\theta|$ and does thus *not change sign* when going from one hemisphere to the other one. This is the reason for the (necessary) *absolute sign* of the $|\cos\theta|$ term in the (Maxwell-consistent) equation for the externally induced membrane potential given in Eq.(8). Integration over the entire sphere within the boundaries $\theta = 0, \pi$ yields the average membrane potential:

$$\langle \Delta\varphi_m \rangle = -(\sqrt{3}/2)\, E \cdot a \approx -E \cdot a \qquad (9)$$

Eq.(9) is also a practically useful approximation for isolated cells as well as for densely packed cells in cell pellets or tissue. The average membrane field amplification is then given by:

$$\langle E_m \rangle \approx E\, a / d_m \qquad (10)$$

The amplification factor of spherical shell with radius a = 10 μm and d_m = 10 nm, for example, is as large as a/d_m = 10^3. It is this geometrical amplification which rationalizes that comparatively small external fields in the range of 1 kV/cm are amplified to yield the large electric field strength of 10^3 kV/cm, which then has such high (electroporative) power on the membrane structure.

Reaction dipole moment

Finally, substitution of Eq. (4) into f = K/(1+K) and using Eq.(8) results in expressions which permit to determine the reaction dipole moment $\Delta_r M = V_p \Delta_r P$ or the polarization volume V_p (and via $V_p = (4/3)\,\pi r_p^3$, the pore radius r_p, for the cylindrical pore model). Substitution of the respective expressions for K = f /(1-f) yields the relationship for the field-induced fractional change, Δf = f(E) - f(0), relative to the zero-field fluctuation term f(0) as:

$$\Delta f = \left(\frac{K_0 \exp X}{1 + K_0 \exp X} - \frac{K_0}{1 + K_0} \right) = \frac{K_0 (\exp X - 1)}{(1 + K_0 \exp X)} \qquad (11)$$

In the cases, where the overall Scheme (1) is applicable, it is found that $K_o \ll 1$. For this case, Eq.(11) reduces to the expression:

$$f(E)/f_o = \exp(X)/(1 + K_o \exp(X)) \qquad (12)$$

Previously, current relaxations of densely packed cell suspensions have been analyzed, directly using only the initial onset parts of the amplitude values as a function of E, in terms of the simple first order approximation $f(E) = K_0 \exp(X)$, [7], readily derived from Eq.(12).

The reaction parameters are now obtained from the field factor, Eq.(5), rewritten as

$$X(E_{loc}) = \ell n \frac{K(E)}{K(0)} = \frac{\int \langle \Delta_r m \rangle d\, E_{dir}}{kT} = \frac{\langle v_p \rangle \varepsilon_0 (\varepsilon_W - \varepsilon_L)\, E_m^2}{2kT} \quad (13)$$

where $\langle \Delta_r m \rangle / kT = \Delta_r M / RT$ is the average molecular reaction moment for permanent dipole mechanism, ε_o is the dielectric vacuum permittivity, ε_W and ε_L the dielectric constants of water and the lipid phase, respectively [13].

For the case of water entrance, the mean value of the average pore aqueous pore $\langle v_p \rangle$ can be extracted from experimental data using Eq.(13).

Thresholds of detection

In membrane electroporation, like for any other non-linear dependence of the experimental signal on the external force parameter, data correlation uses the term experimental "threshold field strength [14]". The threshold signal S_{thr} is dependent on pulse length, in particular at short pulse times, quantified in the well known strength-duration correlation. Experimental experience shows that E_{th} is a not well defined quantity. Theory shows that E_{th} may serve as a practical term for the range where effects become visible ("visibility" threshold). In physical chemical theory it is not necessary to incorporate the practical detection threshold. Note that structural fluctuations intrinsically would allow for an occasionally occurring (small) pore, even in the absence of an electric field, provided one waits long enough, i.e., f_0 is finite but very small.

Field effects are always finite

In a field, there is *always some finite field effect*, if the reaction dipole moment or reaction susceptibility is finite, no matter how small the acting field force is. The relevant question is, *how large* is the effect, as compared to the randomizing thermal motion. In electroporation theory, the experimental threshold field strength, dependent on pulse length or not, has been used to formulate the energetic balance, of electric polarization and surface tension on the hand and of the (counter-acting) line tension energy on the other hand, getting unreasonably high pore radii at zero field strength.. The deficiencies of purely physical electroporation theories have their origin in leaving out the free energy term covering the very molecular chemical interactions in pore formation and resealing.

PART II.
ELECTROPORATION KINETICS AND TIME - DEPENDENT FLOW COEFFICIENTS

Pore type and size from relaxation kinetics

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

Pore expansion re-defined

The kinetic feature of exponential relaxation modes indicates that it is *the number of pores* of defined size (r_p) which increases with time and field strength [6,7]. Therefore the transport quantities of ionic current, conductance, resistance indicate that the transport cross section for flow is structurally controlled. These kinetic features suggesting average size transport passages, require a re-evaluation of the concept of pore expansion. As a diffusive process, expansion *sensu strictu* is associated with a square root dependence on time. The time dependence in reality is, however, exponential, consistent with defined pore types, increasing in number with time at a given field strength.

Elementary steps and dipole mechanism

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

and by molecular dynamics simulations of the molecular rearrangements of lipid and water molecules involved in electric pore formation.

The technique of cell electroporation has been recently extended to ultra-short pulses with nominally very high external electric field strengths. At these high external field strengths and the short rise times, no Maxwell-Wagner field amplification is necessary. The initial fields are high enough and act internally primarily on the intracellular organelles. The short pulse duration does only marginally affect the slowly responding plasma membrane. Ultra-electroporation is expected to have powerful clinical potential, for instance, for inducing cell apoptosis in malignant tissue (K. Schoenbach, J.C. Weaver).

Dipole moments

Analytically, if the experimental electroporation times are expressed as a function of the respective directing field, (calculated from the external field using the dielectric constant of the polar environment), the entire field strength range, from moderate field strengths and short pulse times up to the very high external fields of the ultra-short – pulses, can be consistently described with one and the same permanent dipole moment. The thermodynamic analysis along the physical chemical theory yields the mean dipole moment of 91.7 ($\pm$ 5.0) 10^{-30} Cm (27.4 $\pm$ 1.4 D) associated with the elementary unit, involved in a defined dipolar rotation process. If we compare this value with the dipole moment of the zwitterionic phosphatidylcholine head group of (70 $\pm$ 5) 10^{-30} Cm (21 $\pm$ 2 D), we may conclude:

1. *The hydrated ionic and dipolar lipid head groups, where the water molecules in the asymmetric hydration shells of the ionic groups contribute to higher dipole moments, are the molecular receptors for the interaction of the local field with the membranes.*
2. *The data and calculations quantify alignment processes of these dipolar field receptors into field-parallel positions in the walls of the hydrophilic(inverted) electropores, as one type of the dominant elementary processes in membrane electroporation.*

Unidirectionality of hysteresis processes

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

Conventional flows

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

Non-linear flow analysis

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

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

$$Y(t) = Y(\max)(1 - e^{(k_0/\tau_R)(1-\exp[-t/\tau_R])}) \quad (14)$$

where Y(max) refers to the maximum possible value for the case of complete equilibration between the intracellular ion concentration and that of the external medium. The lower stationary value at $t = \infty$, $Y(\infty)$, is given by:

$$Y(\infty) = \frac{1}{1 - e^{k_0/\tau_R}} \quad (15)$$

In Eq.(14), k_0 is the flow coefficient at a given field intensisity at the time point t = 0 of switching off the single pulse or the pulse train, and τ_R the (field-independent zero-field) time constant of resealing of

the fractional transporting area f = A(trp)/A_0 or the fraction of conductive pores. The time course of Y(t) indicates that the pore resealing starts with the value f(E) at the given field at the pulse end, denoted here as t = 0. In the simplest case, the zero-field resealing is exponential according to

$$f(t) = f(E) \cdot e^{-t/\tau_R} \qquad (16)$$

This procedure of analysis has, for instance, been successfully applied for the resealing phase of densely-packed CHO cells. As rationalized with Eq.(14), the measured transport curves are therefore exponentials of exponentials. (The actually measured curve types can deceive smeared exponentials of the "Kohlrausch-type"). As seen in this very simple case of exponential decrease in the pore fraction, the after-field kinetics is indicative for mechanistic details of the long-lived electroporative membrane states. This frame has been used to obtain the values of k_0 (E, t_E), dependent on E and on the pulse duration t_E , and the (field-independent) time constant τ_R of the resealing process [7].

Mole flow and mole flux

Any analysis in terms of flow coefficients is suggested to start with the Nernst-Planck *mole flow* equation. For the unidirectional case (1-D), the mole flow is defined as $\partial n_i / \partial t$. where n_i is the amount of substance of species/ion of type i.

If the transport is *orthogonal* across a slap of thickness d_m, the *actually transporting area* is A(trp), within the range $0 \le x \le d_m$ of the flow pathway,

$$\left(\frac{\partial n_i}{\partial t}\right)_x = A(trp) \cdot \left(D_i(-|\nabla c_i|) + u_i c_i(-|\nabla \varphi|\right) \qquad (17)$$

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

f_p = A(trp)/ A_0 relative to the total area A_0 , N_p the number of pores and r_p the mean pore radius of the assumed cylindrical pore volume of $V_p = N_p \pi r_p^2 d_m$ with the pore length equal the membrane thickness d_m .

The *mole flux* (or flow density) **vector $\mathbf{J_i}$** here refers to, and is expressed as, the flow *perpendicular* across the total area vector $\vec{A}_0$, containing the actually transporting fractional area $f_p = A(trp) / A_0$, given as pore fraction f_p.

The respective mole flux is given by the (modified)Nernst-Planck equation:

$$\vec{J}_i = \left(\frac{\partial n_i}{\partial t}\right)_x \frac{1}{\vec{A}_0} = f_p \cdot \left(D_i(-\nabla c_i) + u_i c_i(-\nabla \varphi)\right) \qquad (18)$$

This mole flux equation results from insertion of A_0 and f_0 into the mole flow equation for species/ion i in the local field $\vec{E} = -\vec{\nabla}\varphi$.

Note that for electro-diffusion in free solution f = A/ A_0 =1, with A(trp) = A_0 .

The flux vector, underlying the electric current I , besides the diffusion potentials of ions with different mobities, reducing the actual field in the solution is, solely given by the electric potential term, and is expressed by:

$$\vec{J}_i = \vec{v}_i c_i = u_i c_i \vec{E}, \qquad (19)$$

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

$$u_i = z_i D_i F/RT = z_i D_i e_0/kT, \qquad (20)$$

Note that N_A =R/k = 6.022 10^{23} mol^{-1} and k = 1.380 10^{-23} J/K . In this context, note that the (ionic) Poisson equation for the potential φ is given by:

$$\nabla^2 \varphi = -\nabla \cdot \vec{E} = -\rho/\varepsilon = -(F/\varepsilon) \sum_i z_i c_i , \qquad (21)$$

where $\rho = F \sum_i z_i c_i$ is the ionic charge density, $\varepsilon = \varepsilon_0 \varepsilon_r$ the absolute dielectric permittivity, ε_0 the absolute permittivity and ε_r the dielectric number (or ,conventionally, dielectric constant). The current density vector $\vec{j}$ and the ionic conductivity (= specific ionic conductance), respectively, are "ion-specified" as:

$$\vec{j} = \vec{E}\,\sigma = F\,(\sum_i z_i c_i\, u_i)\,\vec{E} \qquad (22)$$

$$\sigma = F\,(\sum_i z_i c_i\, u_i)\,\vec{E} \qquad (23)$$

where $F=N_A e_0$ =9.6485 10^4 C/mol is the Faraday. The electric current due to ion flows, cations in the

direction of E, anions opposite to cations, where $z_i u_i$ is always positive, is then given by:

$$I = A\,F \sum_i z_i \left|\vec{J}_i\right| = A\,F\,(\sum_i z_i c_i\, u_i)E, \qquad (24)$$

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

$$\nabla\vec{j} = -\,F \sum_i z_i (\partial c_i / \partial t) \qquad (25)$$

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

$$\left(\frac{\partial c_i}{\partial t}\right)_{x,y,z} = -\nabla\vec{J}_i \qquad (26)$$

With Eq.(24), it is readily derived that the drift-diffusion contribution, with cations and anions flowing in the same direction, does not contribute as such to electric current I.

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

General integral flow equations.

Specifically, inspecting Eq.(14), the time courses reflect, in a folded form, the change in the fraction of resealing pores, because the underlying mole flow is proportional to the flow area, i.e., to the increasing or, here decreasing, number of pores. Therefore, proper analysis starts with the mole flow, and not with the mole flow density (mol/s m²) or mole flux (=flow per area), in order to rationalize time-dependent flow coefficients. In addition, the value of the flow coefficient at the time point of the end of the applied pulse, yields the kinetic parameters for the rate limiting, (primary) structural transitions, preceding the (secondary) transport processes. The flow coefficient k summarizes the parameters: (free) diffusion coefficient D, Nernst distribution constant γ for the distribution of the particle in the passage part(pore), relative to a bulk compartment, the thickness of the passage d_m .For instance, $k=(D\gamma/d_m)A(trp)/V_0 = P\, f_p\, A_0/V_0$, with the permeability coefficient $P = D\gamma/d_m$ for flow from volume V_0 through the area A(trp). It is now this area or the fraction of pores which in electroporation systems are time-dependent, e.g., $f(t)= f(0)\exp[-t/\tau_R]$, thus making $k(t) = P\, f(t)\, A_0/V_0$,via f(t), dependent on time [12].

Fractional change

For proper flow analysis, for instance, of the measured signals, S(t) and S(max), they are first expressed as the fraction y(t)=S(t)/S(max). Next, the proper differential equation is selected, formally analogous to the linear form of dy(t)/d(t) as [12]:

$$\frac{d\,y(t)}{dt} = -k(t)\big(y(t) - y(max)\big) \qquad (27)$$

where in some cases y(max) = 1. the integrated form is the integral flow equation according to [12]:

$$y(t) = y(max)\exp[-\int k(t)dt] \qquad (28)$$

Insertion of the respective integrated linear equations for the flow coefficients,

$k(t) = k_0 \exp[-t/\tau]$ or $k(t) = k_0(1 - \exp[-t/\tau])$

yields expressions of the type of Eq.(14). It is stressed, that in each case, it must be carefully checked, which equation can be applied and whether existing equations have to be modified or expanded, as dictated by proper physical chemical reasoning along the fundamental laws of thermodynamics, in particular those of nonequilibrium (or flow) thermodynamics. This applies,too, to the claims of small electromagnetic field (EMF) effects where at a first glance the data appear "unbelievable for physical chemical reasons" [14, 16].

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REFERENCES

[1] Neumann E., Schaefer-Ridder M., Wang Y., Hofschneider P.H. Gene transfer into mouse lyoma cells by electroporaton in high electric fields, *EMBO J*., **1**, 841-845, 1982

[2] Wong T.K, Neumann E, Electric field mediated gene transfer, *Biophys. Biochem. Res. Commun*. **107**, 584-587, 1982.

[3] Neumann E, Rosenheck, K, Permeability changes induced by electric impulses in vesicular membranes. *J. Membr. Biol.* **10**, 279-290,1972; and **14**, 194-196, 1973.

[4] M. Eisenstein A look back: a shock to the system, *Nature Methods*, **3**, 66, 2006

[5] Neumann, E., Systemic electroporation – combining electric pulses with bioactive agents, *Intern. Fed. Med Biol Engin. (IFMBE) Proc.* **16** 40-43, 2007.

[6] Griese, T., Kakorin, S. and Neumann, E., Conductometric and electrooptic relaxation spectrometry of lipid vesicle electroporation at high fields, *Phys. Chem. Chem. Phys.* **4**, 1217 – 1227, 2002.

[7] Schmeer, M., Seipp, T., Pliquett, U., Kakorin, S. and Neumann, E., Mechanism for the conductivity changes caused

by membrane electroporation of CHO cell-pellets, *Phys. Chem. Chem. Phys*., **6**, 5564-5574, 2004.

[8] Pliquett, U., Schmeer, M., Seipp, T., Neumann, E., *Intern. Fed. Med. Biol Engin. (IFMBE) Proc.* **3** 98- 99, 2002.

[9] Neumann, E. The relaxation hysteresis of membrane electroporation, in: *Electroporation and Electrofusion in Cell Biology*. Eds. E. Neumann, A.E. Sowers, and C.A. Jordan, Plenum Press, p. 61-82, 1989.

[10] Hristova, N., Tsoneva, I. and Neumann, E., Sphingosine-mediated electroporative DNA transfer through lipid bilayers, *FEBS Lett*., **415**, 81 – 86, 1997.

[11] Neumann, E. and Kakorin, S., Electroporation of curved lipid membranes in ionic strength gradients, *Biophys. Chem.,* **85**, 249 – 271, 2000.

[12] Neumann, E., Kakorin, S. and Toensing, K., Fundamentals of electroporative delivery of drugs and genes, *Bioelectrochem. Bioenerg.* **48**, 3-16, 1999.

[13] Abidor, I. G., Arakelyan, V. B., Chernomordik, L. V., Chizmadzhev, Y. A., Pastuchenko, V. P., Tarasevich, M. R., Electric breakdown of bilayer lipid membrane. I. The main experimental facts and their theoretical discussion, **6**, 37-52 1979.

[14] Neumann, E., Digresssion on chemical electromagnetic field effects in membrane signal transduction the experimental paradigm of the acetylcholine receptor, *Bioelectrochemistry,* **52**, 43 – 49, 2000.

[15] Neumann, E.,Tönsing, K., Kakorin, S., Budde, P. and Frey, J**.**, Mechanism of electroporative dye uptake by mouse B cells, *Biophys. J.*, **74,** 98 – 108, 1998.

[16] Neumann, E., Electric and magnetic field reception. In: *Encyclopedia of Molecular Cell Biology and Molecular Medicine*, ed. R.A. Meyers, WILEY-VCH, New York (2004), Vol. **4**, pp. 1 – 20.

[17] Okino, M., H. Mori, Effects of a high voltage impulse and an anticancer drug on *in vivo* growing tumors*, Jpn. J. Cancer Res.* **78** 1319-1321, 1987.

[18] Mir, L.L, Tounekti, O., Orlowski, S., Review. Bleomycin: Revival of an old drug. *Gen. Pharamac.* **27**, 745-748, 1996.

[19] Heller, R., Jaroszeski, M., Glass, L., Messina, J., Rapaport, D., DeConti, R., Fenske, N., Gilbert, R., Mir, L., Reintgen, D., Phase I/II trial for treatment of cutaneous and subcutaneous tumors using electrochemotherapy. *Cancer* **77**, 964-971, 1996.

[20] Miklavcic, D., Jarm, T., Cemazar, M., Sersa, G., An, D., Beleradek, Jr., Mir, L., Tumor treatment by direct electric current. Tumor perusion changes. *Bioelectrochem. Bioenerg.* **43**, 253-256, 1997.

[21] Pliquett, U., Gallo, S., Hui, S.W., Gusbeth, Ch. and Neumann , E., Local and transient structural changes in stratum corneum at high electric fields: Contribution of Joule heating, Bioelectrochemistry **67:** 37-46, 2005.

[22] Chizmadzhev, Y. A., Zarnitsin, V. G., Weaver, J. C., Potts, R. O., Mechanism of electro-induced ionic species transport through a multilamellar lipid system. *Biophys*. *J*. 68, 749-756, 1997.

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NOTES

In vitro Cell Electropermeabilization

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

INTRODUCTION

The application of electric field pulses to cells leads to the transient permeabilization of the membrane (electropermeabilization). This phenomenon brings new properties to the cell membrane: it becomes permeabilized, fusogenic and exogenous membrane proteins can be inserted. It has been used to introduce a large variety of molecules into many different cells *in vitro* [1-2].

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

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

A- a biophysical description and a biological validation

A-1 The external field induces membrane potential difference modulation

An external electric field modulates the membrane potential difference as a cell can be considered as a spherical capacitor [3]. The transmembrane potential difference induced by the electric field, $\Delta\Psi_i$ is a complex function g(λ) of the specific conductivities of the membrane (λ_m), the pulsing buffer (λo) and the cytoplasm (λ_i), the membrane thickness and the cell size (r). 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]. In dense systems, self shielding in the cell population affects the local field distribution and reduces the local (effective) field distribution [7]. When the resulting transmembrane potential difference $\Delta\Psi$ (i.e. the sum between the resting value of cell membrane $\Delta\Psi o$ and the electroinduced value $\Delta\Psi i$) reaches locally 250 mV, that part of the membrane becomes permeable for small charged molecules [3, 8].

A-2 parameters affecting electropermeabilization

A-2-1 Electric field parameters

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

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

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

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

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

These theoretical predictions are experimentally directly supported on cell suspension by measuring the leakage of metabolites (ATP) [9] or at the single cell level by digitised fluorescence microscopy [10, 11]. The permeabilized part of the cell surface is a linear function of the reciprocal of the field intensity.

Permeabilization, due to structural alterations of the membrane, remained restricted to a cap on the cell surface. In other words, the cell obeys the physical predictions! The area affected by the electric field depends also on the shape (spheroid) and on the orientation of the cell with the electric field lines [12]. Changing the field orientation between the different pulses increases the fraction of the cell surface which is permeabilized.

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

The field strength controls the geometry of the part of the cell which is permeabilized. 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 [14].

B- Practical aspects of electropermeabilization

B-1 Sieving of electropermeabilization

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

B-2 Associated transmembrane exchange

Molecular transfer of small molecules (< 4 kDa) across the permeabilized area is mostly driven by the concentration gradient across the membrane. Electrophoretic forces during the pulse may contribute [10]. Free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its 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 diference of S across the membrane. Ep depends on r (size). For a given cell, the resealing time (reciprocal of k) is a function of the pulse duration but not of the field intensity as checked by digitised videomicroscopy [9]. A strong control by the temperature is observed. The cytoskeletal integrity should be preserved [16]. Resealing of cell membranes is a complex process which is controlled by the ATP level. Starved cells are fragile.

B-3 Cellular responses

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

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

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

CONCLUSION

All experimental observations on cell electropermeabilization are in conflict with a naive model where it is proposed to result from holes punched in a lipid bilayer (see [20] as a recent review). Structural changes in the membrane organization supporting permeabilization remains poorly characterized. New informations appear provided by coarse grained MD simulations. Nevertheless it is possible by a careful cell dependent choice of the pulsing parameteres to introduce any kind of polar molecules in a mammalian cell while preserving its viability.

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] Kotnik T, Miklavcic D., *Analytical description of transmembrane voltage induced by electric fields on spheroidal cells.* Biophys J. 2000. 79 (2): 670-9

[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] 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.

[16] 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.

[17] 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.

[18] 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.

[19] 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.

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NOTES

Electropermeabilization *in vivo*

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

INTRODUCTION

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

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

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

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

METHODS FOR DETECTING *IN VIVO* CELL ELECTROPORATION

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

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

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

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

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

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

At least the following molecules have been used:

BLEOMYCIN

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

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

^{51}Cr-EDTA

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

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

PROPIDIUM IODIDE

As *in vitro*, Propidium Iodide has also been used to show *in vivo* permeabilisation achievement, based on

the increase of fluorescence of this molecule when it can enter the cells and bind to DNA [11]

(99m)Tc-DTPA

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

ELECTROPORATION OF CELLS IN TISSUES

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

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

MODELS OF TISSUE ELECTROPERMEABILISATION

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

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

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

threshold. Model has also allowed giving instructions to the physicians applying the electrochemotherapy antitumor treatment for the correct use of the various types of available electrodes (plate electrodes or needle electrodes [16]. Indeed the placement of the electrodes with respect to the tissues is very important to get a rather uniform and enough intense local electric field in the tissue: as a general rule, the larger the contact surface between the electrode and the tissue, the better (S. Corovic et al, in preparation).

REFERENCES

[1] D. Miklavcic, D. Šemrov, H. Mekid and 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,* 1519, 73-83, 2000.

[2] O. Tounekti, A. Kenani, N. Foray, S. Orlowski and L.M. Mir. The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway. *Br. J. Cancer,* 84, 1272-1279, 2001.

[3] H. Mekid, O. Tounekti, A. Spatz, M. Cemazar, F.Z. El Kebir and L. M. Mir. *In vivo* evolution of tumor cells after the generation of DNA double strand breaks. *Br. J. Cancer,* 88, 1763-1761, 2003.

[4] B.Poddevin, J.Belehradek Jr and L.M.Mir. Stable [57 Co]-Bleomycin complex with a very high specific radioactivity for use at very low concentrations. *Biochem. Biophys. Res. Comm.* 173, 259-264, 1990.

[5] J. Belehradek Jr, S. Orlowski, L.H. Ramirez, G. Pron, B. Poddevin and L.M. Mir. Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin. *Biochim. Biophys. Acta, Biomembranes,* 1190, 155-163, 1994.

[6] B. Poddevin, S. Orlowski, J. Belehradek Jr and L.M. Mir. Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. *Biochem. Pharmacol.* 42(S), 67-75, 1991.

[7] J. Gehl, T. H. Sorensen, K. Nielsen, P. Raskmark, S.L. Nielsen, T. Skovsgaard, and L.M. Mir. *In vivo* electroporation of skeletal muscle : threshold, efficacy and relation to electric field distribution. *Biochim. Biophys. Acta,* 1428, 233-240, 1999.

[8] S. Šatkauskas, M. F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavcic and L. M. Mir, Mechanisms of *in vivo* DNA electrotransfer : respective contributions of cell electropermeabilization and DNA electrophoresis. *Mol. Ther.,* 5, 133-140, 2002.

[9] J. Gehl and L.M. Mir, Determination of optimal parameters for *in vivo* gene transfer by electroporation, using a rapid *in vivo* test for cell electropermeabilization. *Biochem. Biophys. Res. Comm.* 261, 377-380, 1999.

[10] D. Batiuskaite, D. Cukjati and L. M. Mir. Comparison of in vivo electropermeabilization of normal and malignant tissue using the ^{51}Cr-EDTA uptake test. *Biologija* 2, 45-47, 2003.

[11] M.P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros and J. Teissié. *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nat.Biotechnol.,* 16, 168-171, 1998.

[12] P.E. Engstrom, B.R. Persson, L.G. Salford. Studies of *in vivo* electropermeabilization by gamma camera measurements of (99m)Tc-DTPA. *Biochim. Biophys. Acta,* 1473, 321-328, 1999.

[13] J. Gehl, T. Skovsgaard and L.M. Mir. Vascular reactions to *in vivo* electroporation : Characterization and consequences for drug and gene delivery. *Biochim. Biophys. Acta,* 1569, 51-58, 2002.

[14] G. Sersa, M. Krzic, M. Sentjurc, T. Ivanusa, K. Beravs, V. Kotnik, A. Coer, H.M. Swartz and M. Cemazar. Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. *Br J. Cancer,* 2002 87, 1047-1054, 2002.

[15] D. Sel, D. Cukjati, D. Batiuskaite, T. Slivnik, L. M. Mir and D. Miklavčič. Sequential finite element model of tissue electropermeabilisation *IEEE transactions in Biomedical Engineering*, 52, 816-827, 2005.

[16] 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 Supplement*, 4, 45-51, 2006.

Lluis M. Mir was born in Barcelona, Spain, in 1954. He received a Masters in Biochemistry in 1976 from Ecole Normale Supérieure, Paris, and a Doctorate (D.Sc.) in Cell Biology in 1983. In 1978 he entered CNRS as Attaché de Recherches in the Laboratory of Basic Pharmacology and Toxicology, Toulouse. In 1983 he was promoted to Chargé de Recherches at CNRS, and in 1985 he moved to the Laboratory of Molecular Oncology, Institut Gustave-Roussy. In 1989 he moved to the Laboratory of Molecular Pharmacology, Institut Gustave-Roussy. In 1999, he was promoted to Directeur de Recherches at CNRS.

Lluis M. Mir was one of the pioneers of the research of electropermeabilization (electroporation) and the applications of this technique for antitumor electrochemotherapy and DNA electrotransfer. He is the author of 84 articles in peer-reviewed journals, 8 chapters in books, and over 200 presentations at national and international meetings, invited lectures at international meetings and seminars. He received the Award for the medical applications of electricity of the Institut Electricité Santé in 1994, the Annual Award of Cancerology of the Ligue contre le Cancer (committee Val-de-Marne) in 1996, and the Award of the Research of Rhône-Poulenc-Rorer in 1998. He is an Honorary Senator of the University of Ljubljana (2004).

NOTES

Development of devices and electrodes

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

INTRODUCTION

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

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

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

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

THERAPEUTIC AND TECHNOLOGICAL APPLICATIONS OF ELECTROPORATION:

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

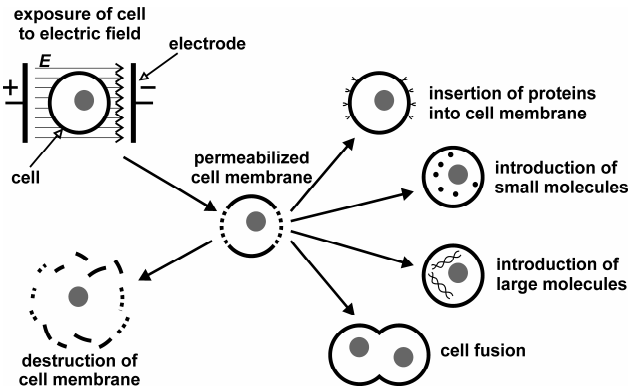

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications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

ELECTROCHEMOTHERAPY

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

GENE ELECTROTRANSFECTION

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

ELECTROINSERTION

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

ELECTROFUSION

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

ELECTROSTERILIZATION

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

TISSUE ABLATION

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

ELECTRIC FIELD DISTRIBUTION *IN VIVO*

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

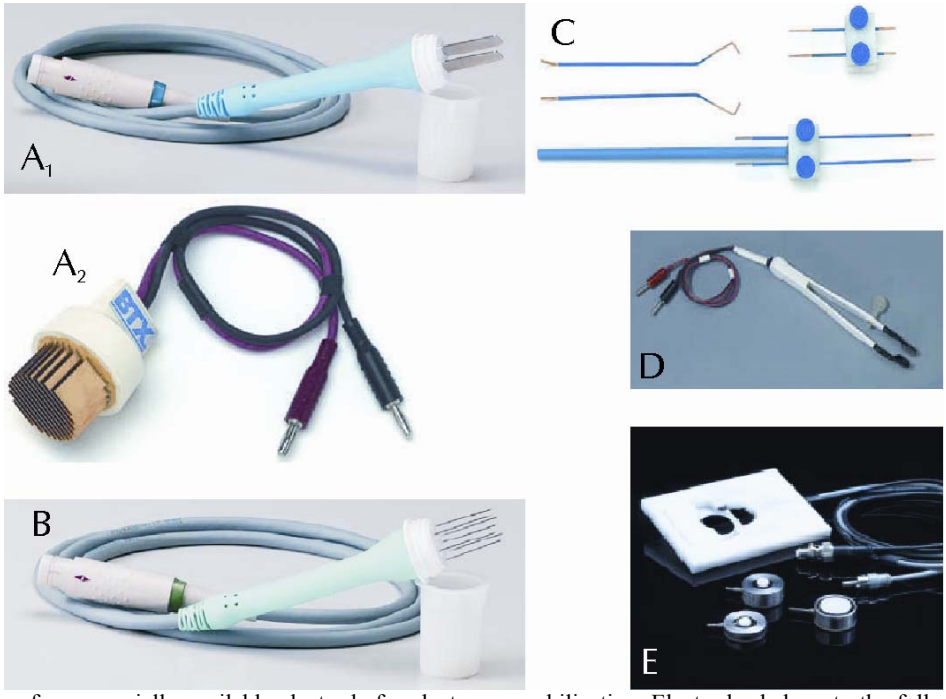

Figure 2: Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: A_1 and A_2 – to parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes and E – coaxial electrodes. Electrodes A_1 and B are produced by IGEA,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

ELECTRODES FOR IN VITRO AND IN VIVO APPLICATIONS

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

Nowadays there are numerous types of electrodes that can be used for electroporation in any of the

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

ELECTROPORATORS – THE NECESSARY PULSE GENERATORS

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

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

This general overview of electrical parameters should however only be considered as a starting point

for a design of experiments or treatments. Optimal values of parameters namely also strongly depend on the cell type used, on the molecule to be introduced, and on specific experimental conditions.

CONCLUSIONS

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

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

FURTHER READING

[1] G. Serša, M. Čemažar, D. Šemrov, D. Miklavčič, Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice, *Bioelectrochem. Bioenerg*., **39**: 61-66, 1996.

[2] R. Gilbert, M.J. Jaroszeski, R. Heller, Novel electrode designs for electrochemotherapy, *Biochim. Biophys. Acta*, **1334**: 9-14, 1997.

[3] 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**: 2152 -2158, 1998.

[4] S. Šatkauskas, M.F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavčič, L.M. Mir, Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis, *Molecular therapy*, **5**: 133-140, 2002.

[5] K. Flisar, M. Puc, T. Kotnik, D. Miklavčič, A system for in vitro cell membrane electropermeabilization with arbitrary pulse waveforms, *IEEE Eng. Med. Biol.*, **22**: 77-81, 2003.

[6] K. Uemura, S. Isobe, Developing a new apparatus for inactivating bacillus subtilis spore in orange juice with a high electric field AC under pressurized conditions, *J. Food Eng.,* **56**: 325-329, 2003.

[7] M. Puc, S. Čorović, K. Flisar, M. Petkovšek, J. Nastran, D. Miklavčič, Techniques of signal generation required in applications of electropermeabilization, *Bioelectrochemistry*, **64**: 113-124, 2004

[8] Pavlin M, Kandušer M, Reberšek M, Pucihar G, Hart FX, Magjarević R, Miklavčič D. Effect of cell electroporation on the conductivity of a cell suspension. *Biophys. J.* **88**: 4378-4390, 2005.

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

[10] Šel D, Cukjati D, Batiuskaite D, Slivnik T, Mir LM, Miklavčič D. Sequential finite element model of tissue electropermeabilization. *IEEE Trans. Biomed. Eng.* **52**: 816-827, 2005.

[11] Tokmakci M. A high-voltage pulse generation instrument for electrochemotherapy method. *J. Med. Syst*. **30**: 145-151, 2006.

[12] Bertacchini C, Margotti PM, Bergamini E, Lodi A, Ronchetti M, Cadossi R. Design of an irreversible electroporation system for clinical use. *Technology in Cancer Research and Treatment* **6**: 313-320, 2007.

[13] Sel D, Macek-Lebar A, Miklavcic D. Feasibility of employing model-based optimization of pulse amplitude and electrode distance for effective tumor electropermeabilization. *IEEE Trans. Biomed. Eng.* **54**: 773-781, 2007.

[14] Miklavcic D, Corovic S, Pucihar G, Pavselj N. Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy. *Eur. J. Cancer Suppl.* **4**: 45-51, 2006.

[15] Lavee J, Onik G, Mikus P, Rubinsky B. A novel nonthermal energy source for surgical epicardial atrial ablation: irreversible electroporation. *The Heart Durgery Forum* **10**: 96-101, 2007.

[16] Rebersek M, Faurie C, Kanduser M, Corovic S, Teissié J, Rols MP, Miklavcic D. Electroporator with automatic change of electric field direction improves gene electrotransfer *in vitro*. *Biomed. Eng. Online* **6**: 25, 2007.

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NOTES

Electroporation in Electrochemotherapy of Tumors

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

INTRODUCTION

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

Chemotherapy, a systemic treatment modality for cancer is effective for drugs that readily pass plasma membrane and are cytotoxic when reaching their intracellular targets. However, among chemotherapeutic drugs that are very cytotoxic exist some having hampered transport through the plasma membrane. These drugs are good candidates for electrochemotherapy. Electrochemotherapy is a local treatment combining chemotherapy and application of electric pulses to the tumor. In electrochemotherapy, the optimal anti-tumor effectiveness is achieved when electric pulses are given at the time of the highest extracellular concentration of hydrophilic chemotherapeutic drug, thereby increasing its transport through plasma membrane towards the intracellular targets [1-3].

PRECLINICAL DATA

In vitro studies

Electroporation proved to be effective to facilitate 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 for only those molecules that are poorly or non-permeant, the suitable candidates for electrochemotherapy are limited to those drugs that are hydrophilic, and lack transport system in the membrane. Several chemotherapeutic drugs were tested *in vitro* for potential application in combination with electroporation of the cells. Among the tested drugs, only two were identified as potential candidates for electrochemotherapy of cancer patients. The first being bleomycin, that is hydrophilic, has very restricted transport through the cell membrane, but its cytotoxicity can be potentiated up to several 1000 times by electroporation of cells. Few hundred internalized molecules of bleomycin are sufficientto kill the cell. The second is cisplatin whose transport through the cell membrane is also hampered. Only 50% of cisplatin is transported through the plasma membrane by the passive diffusion, the rest is by carrier molecules. Electroporation of the plasma membrane enables greater flux and accumulation of the drug in the cells, which results in increase of cisplatinn cytotoxicity by up to 80-fold [1-6]. These promising preclinical data obtained *in vitro* on a number of different cell lines have paved the way for testing these two drugs in electrochemotherapy *in vivo* on fiferent tumor models.

In vivo studies

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

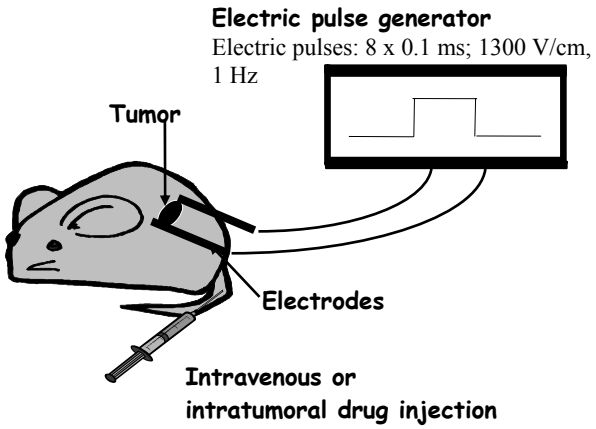

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumorally, at the doses that usually do no exert antitumor effect. After the interval that allows sufficient drug accumulation in the tumors, electric pulses are applied to the tumor either by plate or needle electrodes (1300 V/cm, 100 μs, 1 Hz, 8 pulses). The electrodes are placed in that way that the whole tumor is encompassed between the electrodes, providing good electric field distribution in the tumors for optimal electroporation of cells in the tumors.

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

- The drugs can be given by different *routes of administration*, they can be injected either intravenously or intratumourally. The prerequisite is that, at the time of the application of electric pulses to the tumor, a sufficient amount of drug is present in the tumor. Therefore, after intravenous drug administration into small laboratory animals (4 mg/kg of cisplatin or 0.5 mg/kg bleomycin), only a few minutes' interval is needed to reach the maximal drug concentration in the tumors. After intratumoural administration, this interval is even shorter and the application of electric pulses has to follow the administration of the drug as soon as possible (within a minute) [1-4].
- Good antitumor effectiveness may be achieved by good tissue electroporation. The plasma membrane electroporation 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 geometry of electrodes and tissue. The electric field distribution in the tissue and cell electroporation can be improved by rotating electric field. Surface tumours can be effectively treated by plate electrodes, whereas appropriate electric field distribution in deeper parts of the tumour is assured by using needle electrodes [4-6].

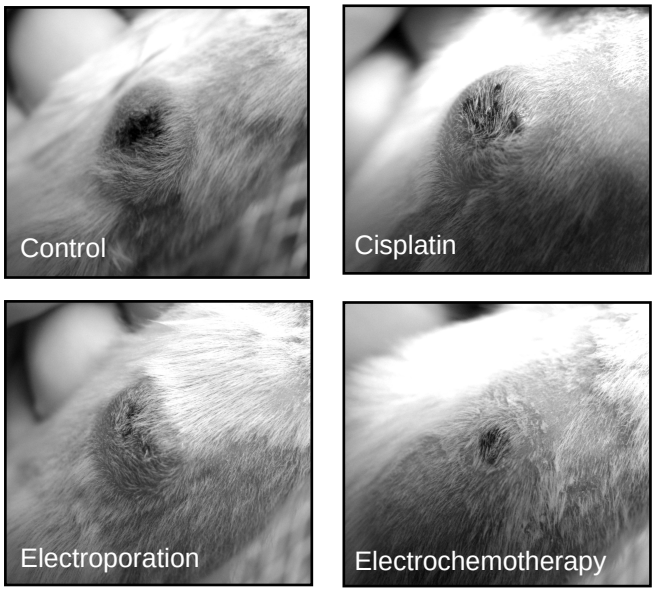

Figure 2: Example of good antitumor effectiveness on SA-1 tumors after electrochemotherapy with cisplatin. Cisplatin vas given intravenously (4 mg/kg), 3 min thereafter 8 electric pulses were applied to the tumor with plate electrodes. Electric pulses were applied in two directions; 4 pulses in one and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

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

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

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

Mechanisms of action

The principal mechanism of electrochemotherapy is *electroporation* of the cells in the tumors, which increases the drug effectiveness by enabling the drugs to reach the intracellular targets. This was demonstrated in the studies that measured the intratumoural drug accumulation and the amount of the drug bound to DNA. Basically, the amounts of bleomycin and cisplatin in the electroporated tumours were up to 2-4 fold higher than in those without application of electric pulses [1-6].

Besides membrane electroporation, which facilitates drug transport and its accumulation in the cell, other mechanisms that are involved in antitumour effectiveness of electrochemotherapy were described. The application of electric pulses to the tissues induces a transient, but reversible *reduction of blood flow* [7]. The restoration of the blood flow in normal tissue is much faster than of that in tumors [8]. The decrease in tumor blood flow induces *drug entrapment* in the tissue, providing more time for the drug to act. Besides, this phenomenon prevents bleeding from the tissue, which is important in clinical situations of haemorrhagic tumors.

The cytotoxic effect of electrochemotherapy is not limited only to tumor cells in the tumors. Electrochemotherapy acts also on stromal cells, including endothelial cells in the lining of tumor blood vessels [9]. This represents yet another mechanism involved in the antitumor effectiveness of electrochemotherapy, i.e. *vascular disrupting effect* [10].

The difference in antitumor effectiveness of electrochemotherapy was observed between immunocompetent and immunodeficient experimental animals, indicating to the involvement of *immune response* in antitumour effectiveness [11]. Due to the massive tumor antigen shedding in the organisms after electrochemotherapy, systemic immunity can be induced, and up-regulated by additional treatment with biological response modifiers like IL-2, GM-CSF and TNF-α [12-14].

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, the tumor drug entrapment, vascular disrupting effect and involvement of immune response were also demonstrated. Based on all these data, electrochemotherapy with bleomycin and cisplatin was promptly evaluated in clinical trials.

PERSPECTIVES

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

The chemotherapeutic drugs that increase effectiveness of radiation therapy are radiosensitizing drugs. Among them are also bleomycin and cisplatin. Since drug delivery induced by electroporation is site-specific, it could be used for tumor-specific delivery of radiosensitizing drugs. By the increased radiosensitizing drug delivery into the tumors and not in the surrounding normal tissue the therapeutic index of tumor irradiation is increased. In our recent studies, we combined electrochemotherapy either with bleomycin or cisplatin with radiotherapy and demonstrated a good potentiation of tumor radiation response: 1.9 fold for electrochemotherapy with bleomycin and 1.6 fold for electrochemotherapy with cisplatin [15-16].

The application of electric pulses was shown to modulate the tumor blood flow. Both, reduced blood flow and lowered partial oxygen pressure (pO_2) in the tumors are consequences of the applied electric pulses [17. The reduced pO_2 can activate bioreductive drugs to exhibit cytotoxic effect on hypoxic cells [18]. In well oxygenated cells, the drug remains inactive. On the other hand, tumor hypoxia induced by application of electric pulses can improve therapeutic conditions for the use of hyperthermia since tumor cells are more sensitive to heat in sub-optimal physiological conditions [19].

Electrochemotherapy with cisplatin or bleomycin was successfully used also in the veterinary medicine. It was used to treat different tumors, such as mammary adenocarcinoma, fibrosarcoma, cutaneous mast cell tumor, hemangioma, hemangiosarcoma, perianal tumors, neurofibroma and sarcoids in dogs,

cats, hamsters, rabbits and horses [20-24]. Recent reports demonstrated a successful treatment of different neoplasms in companion animals and sarcoids in horses [21-24. Hopefully, electrochemotherapy will be broadly used in veterinary medicine for the treatment of different malignancies, both primary and metastatic disease.

Electrochemotherapy is an effective cytoreductive treatment; however, its curative effect is dependent on the permeabilisation of possibly all cells in the tumours. Since permeabilisation of every single cell in the tumour is virtually impossible, electrochemotherapy could be combined with other cytoreductive treatments. Another approach is a combination of electrochemotherapy with electrogene therapy. The first promising reports and data are already available, supporting the effectiveness of this concept [25,26].

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

REFERENCES

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

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

[3] Serša G, Čemažar M, Rudolf Z. Electrochemotherapy:advantages and drawbacks in the treatment of patients. *Cancer Therapy* 2003; 1: 133-42. www.cancer-therapy.org

[4] Serša 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] Miklavčič D, Pucihar G, Pavlovec M, Ribarič S, Mali M, Maček-Lebar A, Petkovšek M, Nastran J, Kranjc S, Čemažar M, Serša 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.

[6] Miklavčič D, Beravs K, Šemrov D, Čemažar M, Demšar F, Serša G. The importance of electric field distribution for effective in vivo electroporation of tissues. *Biophys J* 1998; 74: 2152-8.

[7] 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.

[8] 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.

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

[10] Serša G, Kržič M, Šentjurc M, Ivanuša T, Beravs K, Kotnik V, Coer A, Swartz HM, Čemažar M. Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. *Br J Cancer* 2002; 87:1047-54.

[11] Serša G, Miklavčič D, Čemažar 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.

[12] Serša G, Čemažar 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.

[13] Mir LM, Rath 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.

[14] 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.

[15] Serša G, Kranjc S, Čemažar M. Improvement of combined modality therapy with cisplatin and radiation using electroporation of tumors. *Int J Radiat Oncol Biol Phys* 2000; 46: 1037-41.

[16] Kranjc S, Grošel A, Čemažar M, Šentjurc M, Serša G. Improvement of combined modality therapy with bleomycin and radiation using electroporation of LPB sarcoma cells and tumors in mice. *BMC Cancer* 2005; 5: 115.

[17] Serša G, Kržič M, Šentjurc M, Ivanuša T, Beravs K, Kotnik V, Coer A, Swartz HM, Čemažar M. Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. *Br J Cancer* 2002; 87:1047-54.

[18] Čemažar M, Parkins CS, Holder AL, Kranjc S, Chaplin DJ and Serša G. Cytotoxicity of bioreductive drug tirapazamine is increased by application of electric pulses in SA-1 tumours in mice. *Anticancer Res* 2001; 21: 1151-1156.

[19] Karner KB, Lešničar H, Čemažar M, Serša G. Antitumour effectiveness of hyperthermia is potentiated by local application of electric pulses to LPB tumours in mice. *Anticancer Res* 2004; 24: 2343-8.

[20] Mir LM, Devauchelle P, Quintin_Colonna F, Delisle F, Dolinger S, Fradelizi D, Belehradek JrJ, Orlowski S. First clinical trial of cat soft-tissue carcomas treatment by electrochemotherapy. *Br J Cancer* 1997; 76: 1617-22.

[21] Tozon N, Sersa G, Cemazar M. Electrochemotherapy: Potentiation of local antitumour effectiveness of cisplatin in dogs and cats. *Anticancer Res* 2001; 21: 2483-6.

[22] Rols MP, Tamzali Y, Teissie J. Electrochemotherapy of horses. A preliminary clinical report. *Bioelectrochem* 2002; 55: 101-5.

[23] Spugnini EP, Porrello A. Potentiation of chemotherapy in companion animals with spontaneous large neoplasms by application of biphasic electric pulses. *J Exp Clin Cancer Res* 2003; 22: 571-80.

[24] Tozon N, Kodre V, Serša G, Čemažar M. Effective treatment of perianal tumors in dogs with electrochemotherapy. *Anticancer Res* 2005; 25: 839-46.

[25] Heller L, Pottinger C, Jaroszeski MJ, Gilbert R, Heller R. In vivo electroporation of plasmid encoding GM-CSF or

interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces long-term antitumour immunity. *Melanoma Res* 2000; 10: 577-83.

[26] Matsubara H, Maeda T, Gunji Y, Koide Y, Asano T, Ochiai T, Sakiyama S, Tagawa M. Combinatory anti-tumor effects of electroporation-mediated chemotherapy and wild-type p53 gene transfer to human esophageal cancer cells. *Int J Oncol* 2001; 18: 825-9.

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NOTES

Clinical electrochemotherapy

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

INTRODUCTION

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

CLINICAL STUDIES

The first clinical study on electrochemotherapy was published in 1991, reporting good treatment effectiveness of electrochemotherapy on cutaneous tumor nodules of head and neck tumors [1-3]. The results of this study by the group from the Institute Gustave Roussy, have stimulated other groups to initiate their own clinical studies. The first clinical centres which performed electrochemotherapy were Villejuif and Toulouse in France, the group in Tampa in USA, and our group at the Institute of Oncology Ljubljana in Slovenia. Recently, also new centres reported clinical experience on electrochemotherapy, *e.g.* Copenhagen in Denmark, Mexico City in Mexico, Chicago in USA, Vienna in Austria, Matsumoto and Jamagata in Japan, Sydney in Australia and Cork in Ireland [1-34].

In all clinical studies, 247 patients were included; 202 patients with 655 tumor nodules were treated by electrochemotherapy with bleomycin and 45 patients with 354 tumor nodules were treated by electrochemotherapy with cisplatin. The majority were malignant melanoma patients, and also the patients with metastases in head and neck region, mammary carcinoma, skin cancer, ovarian cancer, Kaposi sarcoma and chondrosarcoma were treated by electrochemotherapy. The results of the studies can be summarized as supporting the assumption that electrochemotherapy has good antitumor effectiveness either using bleomycin or cisplatin, resulting in ~80% objective responses of the treated tumor nodules [1,31,33].

Based on these results, the European project that was aimed at developing and producing electric pulses generator was launched. In the CLINIPORATOR project, this electric pulses generator was developed and is now commercially available for those who would like to perform electrochemotherapy. This generator under the same name as the project - CLINIPORATOR™ (IGEA S.r.l., Carpi, Italy) is certified and is therefore appropriate for clinical use. Along with the development of the electric pulse generator, also plate and needle electrodes were developed.

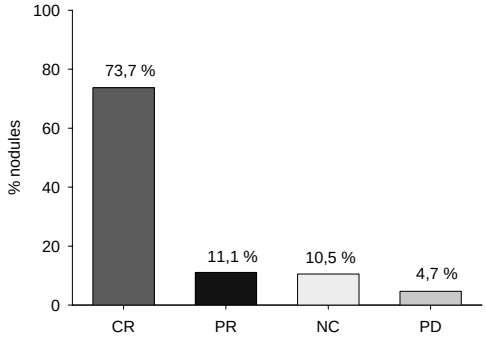

Figure 1: Treatment response of the tumor nodules treated by electrochemotherapy in ESOPE project.

The next step was to gather clinical experience of four cancer centers Villejuif, Copenhagen, Cork and Ljubljana in preparation of Standard operating Procedures (SOP) of electrochemotherapy. This was a prerequisite step to bring electrochemotherapy into standard clinical practice. Now the SOP is prepared and with drug licensing for electrochemotherapy, electrochemotherapy can be used as standard procedure for local tumor treatment [34]. Along with gaining more clinical experience we have gathered a

vast amount of clinical data that were evaluated and confirmed the previously published data that electrochemotherapy is effective in treatment of cutaneous tumor nodules, regardless of the tumor type treated or the drug used (bleomycin, cisplatin) or its route of administration (Figure 1) [33].

TREATMENT PROCEDURES FOR ELECTROCHEMOTHERAPY

Electrochemotherapy is used for the treatment of cutaneous and subcutaneous tumour nodules of different malignancies. The treatment advantages and clinical uses for electrochemotherapy can be summarized:

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

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 tumor histology, but depends on the number and size of the nodules. After drug injection the tumor nodules are exposed to electric pulses. The interval between the intravenous drug injection and application of electric pulses is 8-28 min, and after the intratumoural injection, as soon as possible. Different sets of electrodes are available for application; plate electrodes for smaller tumor nodules and needle electrodes for the treatment of larger (3 cm) and thicker tumor nodules. The treatment can be performed in one-session or can be repeated in case of new emerging nodules or on those nodules that relapsed in some regions not well treated in the first treatment.

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

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

CONCLUSION

Electrochemotherapy is now on the verge of standard treatment in palliative treatment of cutaneous and subcutaneous tumor nodules of different malignancies. However, the development of electrochemotherapy will continue into development of new electrodes that will enable treatment of bigger tumors and tumors in internal organs. Consequently indications for electrochemotherapy will broaden.

REFERENCES

[1] 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.

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

[3] Rudolf Z, Štabuc B, Čemažar M, Miklavčič D, Vodovnik L, Serša G. Electrochemotherapy with bleomycin: The first clinical experience in malignant melanoma patients. *Radiol Oncol* 1995; **29:** 229-35.

[4] Glass LF, Fenske NA, Jaroszeski M, Perrott R, Harvey DT, Reintgen DS, Heller R. Bleomycin-mediated electrochemotherapy of basal cell carcinoma. *J Am Acad Dermatol* 1996; **34:** 82-6.

[5] Glass LF, Pepine ML, Fenske NA, Jaroszeski M, Reintgen DS, Heller R. Bleomycin-mediated electrochemotherapy of metastatic melanoma. *Arch Dermatol* 1996; **132:** 1353-7.

[6] Heller R, Jaroszeski MJ, Glass LF, Messina JL, Rapaport DP, DeConti RC, Fenske NA, Gilbert RA, Mir LM, Reintgen DS. Phase I/II trail for the treatment of cutaneous and subcutaneous tumors using electrochemotherapy. *Cancer* 1996; **77:** 964-71.

[7] Reintgen DS, Jaroszeski MJ, Heller R. Electrochemotherapy, a novel approach to cancer. *The Skin Cancer Foundation Journal* 1996; **14:** 17-9.

[8] Domenge C, Orlowski S, Luboinski B, De Baere T, Belehradek J-Jr, Mir LM (**1996**) Antitumor electrochemotherapy. New advances in the clinical protocol. *Cancer* 1996; **77:** 956-63.

[9] Glass LF, Jaroszeski M, Gilbert R, Reintgen DS, Heller R. Intralesional bleomycin-mediated electrochemotherapy in 20 patients with basal cell carcinoma. *J Am Acad Dermatol* 1997; **37:** 596-9.

[10] Mir LM, Glass LF, Serša G, Teissie J, Domenge C, Miklavčič D, Jaroszeski M-J, Orlowski S, Reintgen DS, Rudolf Z, Belehradek M, Gilbert R, Rols M-P, Belehradek JJr, Bachaud JM, DeConti R, Štabuc B, Čemažar M, Coninx P, Heller R. Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. *Brit J Cancer* 1998; **77:** 2336-42.

[11] Heller R, Jaroszeski MJ, Reintgen DS, Puleo CA, DeConti RC, Gilbert RA, Glass LF. Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. *Cancer* 1998; **83:** 148-57.

[12] Panje WR, Hier MP, Garman GR, Harrel E, Goldman A, Bloch I. Electroporation therapy of head and neck cancer. *Am Otol Rhinol Laryngol* 1998; **107:** 779-85.

[13] Kubota Y, Mir LM, Nakada T, Sasagawa I, Suzuki H, Aoyama N. Successful treatment of metastatic skin lesions with electrochemotherapy. *J Urol* 1998; **160:** 1426.

[14] Serša G, Štabuc B, Čemažar M, Jančar B, Miklavčič D, Rudolf Z. Electrochemotherapy with cisplatin: potentiation of local cisplatin antitumour effectiveness by application of electric pulses in cancer patients. *Eur J Cancer* 1998; **34:** 1213-8.

[15] Serša G, Čemažar M, Rudolf Z, Fras AP. Adenocarcinoma skin metastases treated by electrochemotherapy with cisplatin combined with radiation. *Radiol Oncol* 1999; **33:** 291-6.

[16] Gehl J, Geertsen P. Efficient palliation of hemorrhaging malignant melanoma skin metastases by electrochemotherapy. *Melanoma Res* 2000; **10:** 585-9.

[17] Reberšek M, Čufer T, Rudolf Z, Serša G. Electrochemotherapy with cisplatin of breast cancer tumor nodules in a male patient. *Radiol Oncol* 2000; **34:** 357-61.

[18] Serša G, Čufer T, Čemažar M, Reberšek M, Zvonimir R. Electrochemotherapy with bleomycin in the treatment of hypernephroma metastasis: case report and literature review. *Tumori* 2000; **86:**163-5.

[19] 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.

[20] Serša G, Štabuc B, Čemažar M, Miklavčič D, Rudolf Z. Electrochemotherapy with cisplatin: the systemic antitumour effectiveness of cisplatin can be potentiated locally by aplication of electric pulses in the treatment of malignant melanoma skin metastases. *Melanoma Res* 2000; **10:** 381-5.

[21] Serša G, Štabuc B, Čemažar M, Miklavčič D, Rudolf Z. Electrochemotherapy with cisplatin: clinical experience in malignant melanoma patients. *Clin Cancer Res* 2000; **6:** 863-7.

[22] Rodriguez-Cuevas S, Barroso- Bravo S, Almanza- Estrada J, Cristobal- Martinez L, Gonzales-Rodriguez E. Electrochemotherapy in primary and metastatic skin tumors: Phase II trial using intralesional bleomycin. *Arch Med Res* 2001; **32:** 273-6.

[23] Allegretti JP, Panje WR. Electroporation therapy for head and neck cancer including carotid arthery involvement. *Laryngoscope* 2001; **111:** 52-6.

[24] Burian M, Formanek M, Regele H. Electroporation therapy in head and neck cancer. *Acta Otolaryngol* 2003; **123:** 264-8.

[25] Serša G, Čemažar M, Rudolf Z. Electrochemotherapy: advantages and drawbacks in treatment of cancer patients. *Cancer Therapy* 2003; **1:** 133–42.

[26] Shimizu T, Nikaido T, Gomyo H, Yoshimura Y, Horiuchi A, Isobe K, Ebara S, Takaoka K. Electrochemotherapy of digital chondrosarcoma. *J Ortop Sci* 2003; **8:** 248-51.

[27] Gothelf A, Mir LM, Gehl J. Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat Rew* 2003; **29:** 371–87.

[28] Byrne CM, Thompson JF, Johnston H, Hersey P, Quinn MJ, Hughes M, McCarthy WH. Treatment of metastatic melanoma using electroporation therapy with bleomycin (electrochemotherapy). *Melanoma Res* 2004; **15:** 45-51.

[29] Snoj M, Rudolf Z, Čemažar M, Jančar B, Serša G. Successful sphincter-saving treatment of anorectal malignant melanoma with electrochemotherapy, local excision and adjuvant brachytherapy. *Anti-Cancer Drugs* 2005; **16:** 345–8.

[30] Kubota Y, Tomita Y, Tsukigi M, Kurachi H, Motoyama T, Mir LM. A case of perineal malignant melanoma successfully treated with electrochemotherapy. *Melanoma Res* 2005; **15:** 133-4.

[31] Serša G. The state-of-the-art of electrochemotherapy before the ESOPE study: advantages and clinical uses. *Eur J Cancer Suppl* 2006; **4:** 52–9.

[32] Serša G, Miklavčič D, Čemažar M, Rudolf Z, Pucihar G, Snoj M. Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol* 2007; doi:10.1016/j.ejso.2007.05.016

[33] Marty M, Serša 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. *Eur J Cancer Supp*l 2006; 4: 3-13.

[34] Mir LM, Gehl J, Serša 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. *Eur J Cancer Supp*l 2006; 4: 14-25.

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NOTES

Gene transfection *in vitro*: Where do you stand?

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Abstract: Various vectors have been developed in the field of gene transfer. There still exist a number of important unresolved problems associated with secondary toxicity or low gene transfer efficiency of the use of viral as well as non viral vectors. Therefore an efficient and safe method of DNA delivery still needs to be found. DNA electrotransfer is a physical method that consists of the local application of electric pulses after the introduction of DNA into the extra cellular medium. Electrotransfer has proven to be one of the most efficient and simple non viral methods of delivery. It may provide an important alternative technique in the field of cell and gene therapies. The present lecture focuses on questions related to the mechanism of DNA electrotransfer, i.e. the basic processes responsible for the electropermeabilisation of membranes and the current limitations of the method as applied to DNA transfer, in particular its efficiency in achieving gene expression.

INTRODUCTION

Cell membrane acts as a barrier that hinders the free diffusion of molecules between cell cytoplasm and external medium. However, it is known for more than 30 years that the exposure of cells to an electric field induces a position dependent change in their resting transmembrane potential difference that can lead to its transient permeabilization [1]. The field induced potential difference is added to the resting potential. Being dependent on an angular parameter, the field effect is position dependent on the cell surface. Therefore, the side of the cell facing the anode is going to be hyperpolarized while the side of the cell facing the cathode is depolarized [2, 3].

The use of videomicroscopy allowed to visualise the permeabilization phenomenon at the single cell level. Propidium iodide can be used as a probe for small molecules. Its uptake in the cytoplasm is a fast process that can be detected during the seconds following Electro-Pulsation. Exchange across the pulsed cell membrane occurs at the sides of the cells facing the electrodes on an asymmetrical way. It is more pronounced at the anode facing side of the cells than at the cathode one, i.e. in the hyperpolarized area than in the depolarized one, in agreement with theoretical considerations [4].

Therefore, electropermeabilization can be described as a 3-step-process by respect with Electro-Pulsation: before EP: membrane acts as a barrier that prevents the exchange of hydrophilic molecules between cell cytoplasm and external medium. During EP: the transmembrane potential increase induces the formation of local Transient Permeable Structures that allow the exchange of molecules. After EP: resealing is occurring. Membrane permeability to small molecules is present with a lifetime ranging from sec to min. Permeabilization indeed occurs only on the part of the membrane where potential difference has been brought at its critical value.

Theoretical models have been proposed to explain the mechanism of this reversible membrane electropermeabilization. The molecular definition of the "Transient Permeable Structures" is not yet known. Therefore, while the term electroporation is commonly used among biologists, the term electropermeabilization should be preferred in order to prevent any molecular description of the phenomenon.

GENE TRANSFER

The use of electrotropermeabilization to deliver a wide range potentially therapeutic agents including drugs, proteins and nucleic acids in a wide range cells and tissues has been rapidly developed over the last decade [5-8]. This strategy is promising for the systemic secretion of therapeutic proteins. Vaccination and oncology gene therapy are also major fields of application of electrotransfer. But the safe and efficient use of this physical method for clinical purposes requires the knowledge of the mechanism underlying that phenomenon of electropermeabilization. Despite the fact that the puineering work on plasmid DNA electrotransfer in cells was initiated 25 years ago [9], many of the mechanisms underlying DNA electrotransfer remain to be elucidated [see recent reviews [10-12]).

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

plasmids can be used to monitor these different steps of electrotransfection [4].

1) DNA/Membrane interaction

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

New lines of research are now necessary to characterise the membranes domains observed during electrotransfer.

2) DNA expression.

Once the first stage of gene electrotransfection, i.e. migration of the plasmid DNA towards the electropermeabilised plasma membrane and its interaction with it, becomes well understood we will be able to give guidelines to improve this first step in gene electrotransfer protocols. However, successful expression of the plasmid depends on its subsequent migration into the cell. Therefore, the intracellular diffusional properties of plasmid DNA, as well as its metabolic instability and nuclear translocation, represent other cell limiting factors that must be taken into account [13].

The cytoplasm is composed of a network of microfilament and microtubule systems, along with a variety of subcellular organelles present in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration means that there is substantial molecular crowding in the cytoplasm which hinders the diffusion of plasmid DNA. These apparently contradictory results might be reconciled by the possibility of a disassembly of the cytoskeleton network that may occur during electropermeabilisation, and is compatible with the idea that the cytoplasm constitutes an important diffusional barrier to gene transfer. In the conditions induced during electropermeabilisation, the time a plasmid DNA takes to reach the nuclei is significantly longer than the time needed for a small molecule. Therefore, plasmid DNA present in the cytosol after being electrotransferred can be lost before reaching the nucleus, for example because of cell division.

Finally, after the cytoskeleton, the nuclear envelope represents the last, but by no means the least important, obstacle to the expression of the plasmid DNA. The relatively large size of plasmid DNA (2-10 MDa) makes it unlikely that the nuclear entry occurs by passive diffusion.

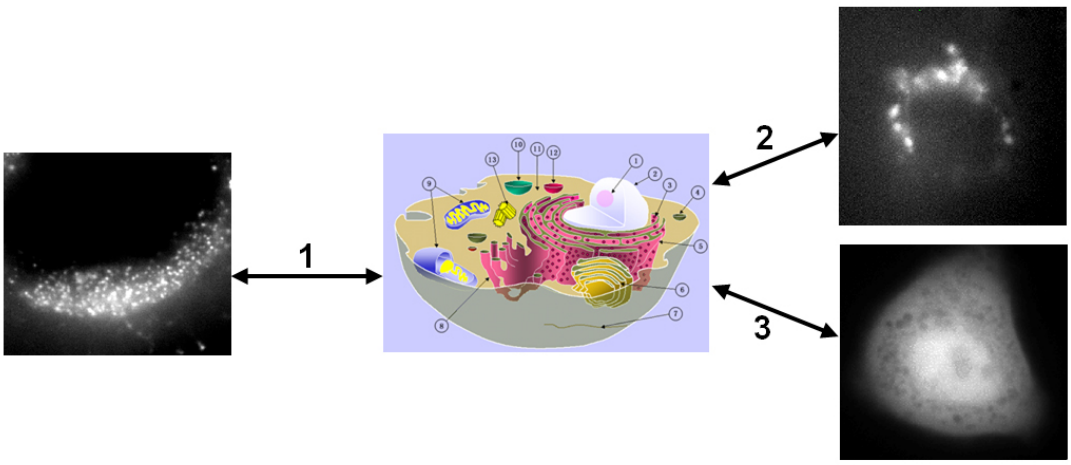

Figure 1: The different steps involved in electrotransfection. 1: during the application of the electric field, DNA molecules migrate towards the electropermeabilised plasma membrane where they interact with the membrane and can become inserted. This interaction proceeds for a few ms to a few min. 2: after the electric field application, transport of DNA in the cytoplasm takes place with a kinetics ranging from min to hours, finally leading to 3) gene expression lasting during several days to weeks.

3) New developments.

Clear limits of efficient gene expression using electric pulses are due to cytoplasmic crowding and transfer through the nuclear envelope. A key challenge for electro-mediated gene therapy is to pinpoint the rate limiting steps in this complex process and to find strategies to overcome these obstacles. As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA. Electrotransferred plasmid DNA, containing specific sequences could then use the microtubule network and its associated motor proteins to move through the cytoplasm to the nucleus [14].

Another alternative, coming from new technologies, concerns nanosecond pulsed electric fields. Recent studies indicate that very short (10-300 ns) but high pulses (up to 300 kV/cm) induce effects that primarily affect intracellular structures and functions. As the pulse duration is decreased, below the plasma membrane charging time constant, plasma membrane effects decrease and intracellular effects predominate [15.16]. A possible idea, to improve transfection success, is to perform classical membrane permeabilisation allowing plasmid DNA electrotransfer, and then after, when DNA has reached the nuclear envelope, to specifically permeabilise it using these short strong nanopulses. Thus, when used in conjunction with classical electropermeabilisation, nanopulses could be used to increase gene expression yields. In this way, it becomes possible not only to simply electropermeabilise cells to transfer DNA into the cytosol, but to fully electromanipulate cells by allowing DNA to be efficiently transferred into the nuclei.

CONCLUSIONS

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

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

REFERENCES

[1] Neuman, E., A.E. Sowers, and C.A. Jordan. *Electroporation and electrofusion in cell biology*. New York: Plenum., 1989.

[2] Bernhardt, J. and H. Pauly. On the generation of potential differences across the membranes of ellipsoidal cells in an alternating electrical field*, Biophysik* 10(3): 89-98, 1973.

[3] Kotnik, T. and D. Miklavcic. Analytical description of transmembrane voltage induced by electric fields on spheroidal cells*, Biophys J* 79(2): 670-9, 2000.Weaver, J.C. Electroporation theory. Concepts and mechanisms*. Methods Mol Biol*. 55: 3-28, 1995.

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

[5] Gothelf, A., L. M. Mir, and J. Gehl. Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation. *Cancer Treat Rev* 29:371-387, 2003.

[6] Mir, L. M., P. H. Moller, F. Andre, and J. Gehl. Electric pulse-mediated gene delivery to various animal tissues. *Adv Genet* 54:83-11,. 2005.

[7] Trollet C, Bloquel C, Scherman D, Bigey PElectrotransfer into skeletal muscle for protein expression. Curr Gene Ther. 6(5):561-78, 2006

[8] Heller, L. C., and R. Heller. In vivo electroporation for gene therapy. *Hum Gene Ther* 17:890-897, 2006.

[9] Neumann, E., M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *Embo J* 1:841-845, 1982.

[10] Cemazar, M., M. Golzio, G. Sersa, M. P. Rols, and J. Teissie. Electrically-assisted nucleic acids delivery to tissues in vivo: where do we stand? *Curr Pharm Des* 12:3817-3825, 2006.

[11] Favard, C., D. S. Dean, and M. P. Rols. Electrotransfer as a non viral method of gene delivery. *Curr Gene Ther* 7:67-77, 2007.

[12] Escoffre, J. M., D. S. Dean, M. Hubert, M. P. Rols, and C. Favard. Membrane perturbation by an external electric field: a mechanism to permit molecular uptake. *Eur Biophys J*., 2007.

[13] Lechardeur, D., and G. L. Lukacs. Nucleocytoplasmic Transport of Plasmid DNA: A Perilous Journey from the Cytoplasm to the Nucleus, 2006.

[14] Vaughan, E. E., and D. A. Dean. Intracellular trafficking of plasmids during transfection is mediated by microtubules. *Mol Ther* 13:422-428, 2006.

[15] Beebe, S. J., J. White, P. F. Blackmore, Y. Deng, K. Somers, and K. H. Schoenbach. Diverse effects of nanosecond pulsed electric fields on cells and tissues. *DNA Cell Biol* 22:785-796, 2003.

[16] Vernier, P. T., Y. Sun, and M. A. Gundersen. Nanoelectropulse-driven membrane perturbation and small molecule permeabilization. *BMC Cell Biol* 7:37, 2006.

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Marie-Pierre Gorsse Rols was born in Decazeville, the "gueules noires" city of the Duc Decazes, France, in 1962. She received a Masters in Biochemistry, a Ph.D. in Cell Biophysics and the Habilitation à Diriger les Recherches from the Paul Sabatier University of Toulouse in 1984, 1989 and 1995, respectively. She is currently Director of Research at the IPBS-CNRS laboratory in Toulouse and President of the GEIMM French group on molecule-membrane interactions. Her main research interests lie in the fields of membrane electropermeabilization *in vitro* and *in vivo*. In the last years, she has focused on the mechanism of DNA electrotransfer. Marie-Pierre Rols is the author of 60 articles in peer-reviewed journals. In 1993 she received the Galvani Prize of the Bioelectrochemical Society, in 2006 a joined prize of the Midi-Pyrénées region.

NOTES

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

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Abstract: At the end of the 90's, several publications from various laboratories reported efficient in vivo electrotransfer of plasmids coding for several reporter genes. This was the natural evolution resulting from the finding that Eberhard Neumann and colleagues published in 1982. In 2007, we thus commemorate the 25 years of this first pioneering publication [1] that reported successful DNA electrotransfer into living cells in vitro by means of electric pulses.

INTRODUCTION

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

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

DNA ELECTROTRANSFER IN SKELETAL MUSCLE

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

DNA ELECTROTRANSFER MECHANISMS ANALYSED IN MUSCLE

The mechanisms of DNA electrotransfer have been analysed in the skeletal muscle using combinations of high voltage short duration pulses (HV; 100 µs and voltage such as the ratio of applied voltage to electrodes distance is comprised between 800 and 1300 V/cm, as a function of the tissue treated and of the electrodes used) and of low voltage long duration pulses (LV; 50 to 400 ms and several tens of V/cm, that is of a strength below the electropermeabilisation threshold of the tissue) [11]. It has been shown that, as expected, the electric pulses must "permeabilize" the targeted cells. This can be obtained even with a single HV pulse, that does not result in a very high level of muscle fiber permeabilisation [12] as measured using the ^{51}Cr EDTA uptake test [13]. The electric pulses have a second role: to electrophoretically move the DNA towards or across the "electropermeabilized" membrane. Moreover, DNA does not need to be present at the time of the cell electropermeabilisation [11] but it is mandatory to inject it before the electrophoretic LV pulse [4, 11]. Actually, the LV component is the real responsible for plasmid transfer to muscle fibers since it efficiently pulls the DNA towards the membrane still altered after the delivery of the electroporating pulse. It is not possible to say towards the "electroporated" membrane because high levels of permeabilisation, under the same

experimental conditions, only last for 300 seconds (5 minutes)[11]. The precise structure of the membrane during this period of time (between 5 and 50 minutes after the delivery of the HV) is not known but this kind of observations might argue in favor of the electropermeabilisation theory. Nevertheless, it is possible to conclude that target cell electropermeabilisation is mandatory, but that electrotransfer efficacy is determined by the EP electrophoretic component [11, 14].

DNA ELECTROTRANSFER IN LIVER

DNA transfer in liver, using short pulses, was described in 1996 [15] (this was the second paper relating DNA electrotransfer in vivo, after the article by Titomirov et al in 1991 [16], in which exogenous myc and ras genes were expressed in a few of the skin cells exposed in vivo to the DNA and the electric pulses). However much care is necessary in experiments dealing with gene transfer in liver. Indeed, hepatocytes in vivo are easily transfected by simple hydrostatic pressure [17]. Recent data indicates that using long LVs (for example 4 LV of 100 ms) at field strengths even rather low (for example as low as 20 V/cm), there is no need for an HV. The exact reasons for such a behaviour are not yet understood (F. André et al, submitted).

DNA ELECTROTRANSFER IN TUMORS

The first tissue to which DNA was transferred by means of long electric pulses were tumors transplanted in the flank of mice (M. P. Rols, 1998) [18]. A clear increase in the efficacy of DNA transfer was shown. DNA has been transferred to various types of tumors. However, the results are much less reproducible than in the case of plasmid DNA transfer to the skeletal muscle. The main reason for such variability lies on the structure of the tumors themselves: tumors are heterogeneous tissues, not limited by a physiological physical barrier (like the fascia in muscle or the capsule in liver). Injection is more or less easy, reproducible and complete depending on the consistence of the tumor (for example, experimental melanomas like the B16 melanoma are soft, inflatable tissue while fibrosarcoma is a hard, breakable one). Injection often results in a very heterogeneous distribution of the fluid and thus of the DNA. Nevertheless DNA transfer has been achieved both using trains of similar 20 ms squave wave pulses (but the voltage was adapted to obtain a ratio of the voltage applied to the electrodes distance of 600 V/cm) [19] or using combinations of HV and LV (F. André et al, submitted).

PERSPECTIVES

DNA electrotransfer to non accessible targets

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

DNA electrotransfer combined to ECT

DNA electrotransfer uses electric pulses, like the electrochemotherapy. Some attempts have been performed to deliver genes and drugs either simultaneously or successively. When using bleomycin in electrochemotherapy, most of the published work has been performed by the group of R. and L. Heller. The DNA electrotransferred coded for either the IL-2 or the GM-CSF. To obtain an increase of the ECT efficacy due to an appropriate stimulation of the immune system, GM-CSF gene must be transferred to the tumor cells the day before the ECT, while IL-2 gene must be transferred to the dying tumor cells (and most probably to the stromal and surrounding normal cells) the day after the ECT [20]. No beneficial effect of the combination was found if bleomycin and these genes were transferred simultaneously.

Interesting studies have been performed on horses affected by sarcoids, a skin tumor. ECT using cisplatin has been combined with the electrotransfert of IL-12 genes to the tissues around the treated tumors. Because of the immune response mediated by the IL-12, the authors of this study (Y. Tamzali, J Teissié, M.P Rols and B. Couderc) have termed this approach electro-chemo-immuno-gene-therapy (ECIGT).

Painless approaches or methods to control the sensations

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

The Standard Operating Procedures for the Electrochemotherapy of cutaneous and subcutaneous tumor nodules provide the physicians with the rules to avoid pain during ECT application [21]. The same procedures have been applied before delivering genes in humans using HV + LV combinations. Pain was prevented, which means that the same procedures seem valid for Electrogenetherapy (G. Sersa and J. Gehl, personal communication).

Clinical perspectives

Currently 5 clinical trials are ongoing, with different genes, using trains of identical long pulses in muscles, trains of identical short pulses in melanoma [22], and one using the HV+LV combinations in tumors of any origin (J. Gehl, G. Sersa, L.M. Mir et al, unpublished data).

REFERENCES

[1] E. Neumann, M. Schaefer-Ridder, Y. Wang and P. H. Hofschneider, Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.,* vol. 1, pp. 841-845, 1982.

[2] L.M. Mir, S. Orlowski, J. Belehradek Jr and C. Paoletti, Electrochemotherapy : Potentiation of antitumor effect of bleomycin by local electric pulses. *European Journal of Cancer*, vol. 27, pp. 68-72, 1991.

[3] L.M. Mir, F.L. Glass, G.Šersa, J.Teissié, C.Domenge, D. Miklavcic, M.J. Jaroszeski, S. Orlowski, D.S. Reintgen, Z. Rudolf, M. Belehradek, R. Gilbert, M.P. Rols, J. Belehradek Jr, J.M. Bachaud, R. DeConti, B. Stabuc, P. Coninx, M. Cemazar and R.Heller, Effective treatment of cutaneous and subcutaneous malignant tumors by electrochemotherapy. *British Journal of Cancer,* vol. 77, pp. 2336-2342, 1998.

[4] L.M. Mir, M.F. Bureau, J. Gehl, R. Rangara, D. Rouy, J.M. Caillaud, P. Delaere, D. Branellec, B. Schwartz and D. Scherman, High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. U.S.A*., vol. 96, pp. 4262-4267, 1999.

[5] L. Grossin, C. Cournil-Henrionnet, L.M. Mir, B. Liagre, D. Dumas, S. Etienne, C. Guingamp, P. Netter and P. Gillet. Direct gene transfer into rat patellar chondrocytes by *in vivo* electroporation. *FASEB J*, vol. 17, pp. 829-35, 2003.

[6] L.M. Mir, M.F. Bureau, R. Rangara, B. Schwartz and D. Scherman, Long-term, high level *in vivo* gene expression after electric pulses-mediated gene transfer into skeletal muscle. *Compte rendus de l'Académie des Sciences, sér III*, vol. 321, pp. 893-899, 1998.

[7] Mathiesen, Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther.* vol. 6, pp. 508-514, 1999.

[8] H. Aihara and J. Miyazaki, Gene transfer into muscle by electroporation in vivo. *Nat. Biotechnol.* vol. 16, pp. 867-870, 1998.

[9] D. Martel-Renoir, V. Trochon-Joseph, A. Galaup, C. Bouquet, F. Griscelli, P. Opolon, D. Opolon, E. Connault, L. M. Mir and M. Perricaudet, Coelectrotransfer to skeletal muscle of three plasmids coding for antiangiogenic factors and regulatory factors of the tetracycline-inducible system: tightly regulated expression, inhibition of transplanted tumor growth, and antimetastatic effect. *Molecular Therapy*, vol. 8, pp. 425-433, 2003.

[10] Rubenstruck et al., Transcriptional activation of the metallothionein I gene by electric pulses in vivo: basis for the development of a new gene switch system. *The Journal of Gene Medicine*, vol. 5, pp. 773-783, 2003.S. Šatkauskas, M. F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavcic and L. M. Mir, Mechanisms of in vivo DNA electrotransfer : respective contributions of cell electropermeabilization and DNA electrophoresis. *Molecular Therapy*, vol. 5, pp. 133-140, 2002.

[12] M. F. Bureau, J. Gehl, V. Deleuze, L. M. Mir and D. Scherman. Importance of association between permeabilization and electrophoretic forces for DNA electrotransfer. *Biochim Biophys Acta*, vol. 1474, pp. 353-359, 2000.

[13] J. Gehl and L.M. Mir, Determination of optimal parameters for in vivo gene transfer by electroporation, using a rapid in vivo test for cell electropermeabilization. *Biochem. Biophys. Res. Comm*., vol. 261, pp. 377-380, 1999.

[14] S. Šatkauskas, F. André, M. F. Bureau, D. Scherman, D. Miklavčič and L. M. Mir. The electrophoretic component of the electric pulses determines the efficacy of in vivo DNA electrotransfer. *Human Gene Ther.*, vol. 16, pp. 1194-1201, 2005

[15] R. Heller, M.J. 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 ,pp. 225-228, 1996

[16] A.V. Titomirov, S. Sukharev, E. Kistanova. In vivo electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. *Biochim Biophys Acta*. vol. 1088, pp. 131-134, 1991.

[17] F. Liu, Y. Song, D. Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. vol. 6, pp. 1258-1266, 1999.

[18] M.P. Rols, C. Delteil, M. Golzio, P. Dumond, S. Cros and J. Teissié. In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat.Biotechnol.,* vol. 16, pp. 168-171, 1998.

[19] M. Bettan, M.-A. Ivanov, L. M. Mir, F. Boissière, P. Delaere, and D. Scherman, Efficient DNA electrotransfer into tumors. *Bioelectrochem*., vol. 52, pp. 83-90, 2000.

[20] 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, pp. 577-583, 2000.

[21] L.M. Mir, J. Gehl, G. Sersa, C. Collins, JR Garbay, V. Billard, P. Geertsen, Z. Rudolf, G. O'Sullivan, M. Marty. Standard Operating Procedures of the Electrochemo-therapy: 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". *Eur. J. of Cancer Supplements*, vol. 4, pp. 14-25, 2006.

[22] L.C. Heller and R. Heller. In vivo electroporation for gene therapy. *Hum. Gene Ther.* vol. 17, pp 890-897, 2006.

NOTES

Gene expression regulation by siRNA electrotransfer

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

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

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

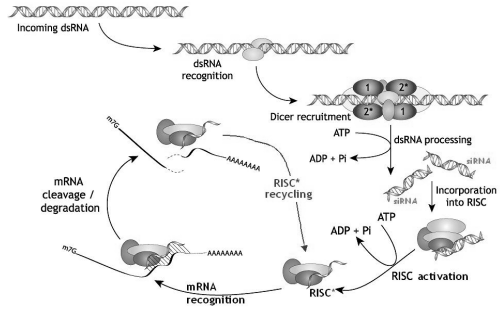

Figure 1: Current model for RNAi-induced mRNA degradation

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

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

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

SiRNA gene silencing could be obtained *in vivo* on reporter as well as endogenous genes. This remains a critical issue for the development of siRNA as an effective therapeutic.

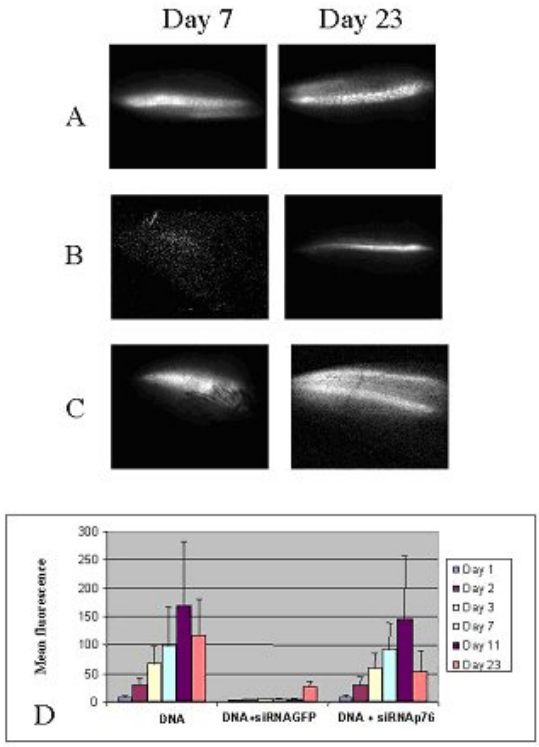

Figure 2: RNA interference in 9 weeks old C57Bl/6 mouse leg muscle. Representative images of the GFP fluorescence of the mouse leg are shown. (Each image is 1 cm wide).
A- GFP expression resulting from plasmid alone electrotransfer as observed on days 7 and 23 in the same leg.
B- GFP expression silencing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

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

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

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

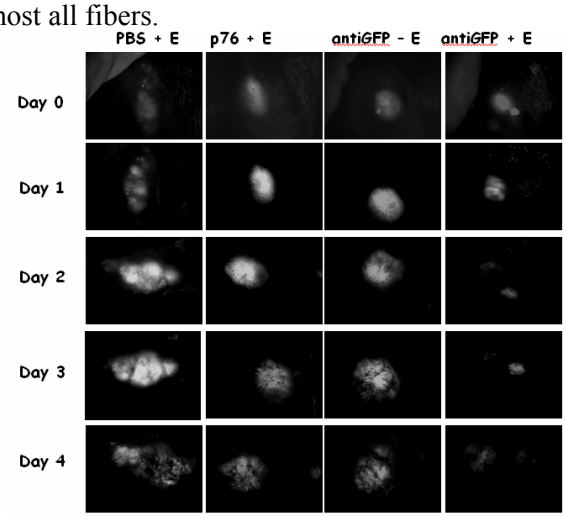

Figure 3: Time dependence of mean fluorescence intensity of the B16F10 GFP tumors in tumors treated with; electric field : PBS + E, unrelated siRNA : p76 + EP, 12 µg of egfp22 siRNA alone : AntiGFP - E and egfp22 siRNA electro-transferred : AntiGFP + E.

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

More and more studies investigated the effectiveness of electropulsation for the localized

delivery of siRNA in adult mice (muscle, tumor, testis …)[29-34].

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

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REFERENCES

[1] Fire A *et al.* Potent and specific genetic interference by double-stranded RNA in aenorhabditis elegans. *Nature* 1998; **391:** 806-811.

[2] Denli AM, Hannon GJ. RNAi: an ever-growing puzzle. *Trends Biochem Sci* 2003; **28:** 196 01.

[3] Hutvagner G, Zamore PD. RNAi: nature abhors a double-strand. *Curr Opin Genet Dev* 2002; **12:** 225-232.

[4] Hannon GJ. RNA interference. *Nature* 2002; **418:** 244-251.

[5] McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* 2002; **3:** 737-747.

[6] Sioud M. Therapeutic siRNAs. *Trends Pharmacol Sci* 2004; **25:** 22-28.

[7] Leung RK, Whittaker PA. RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol Ther* 2005; **107:** 222-239.

[8] Ryther RC, Flynt AS, Phillips JA, Patton JG. siRNA therapeutics: big potential from small RNAs. *Gene Ther* 2005; **12:** 5-11.

[9] Xu Z, Xia XG. RNAi therapy: Dominant disease gene gets silenced. *Gene Ther* 2005.

[10] Behlke MA. Progress towards in vivo use of siRNAs. *Mol Ther* 2006; **13:** 644-670.

[11] Herweijer H, Wolff JA. Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther* 2003; **10:** 453-458

[12] Xie FY, Woodle MC, Lu PY. Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. *Drug Discov Today* 2006; **11:** 67-73.

[13] Rossi JJ. RNAi therapeutics: SNALPing siRNAs in vivo. *Gene Ther* 2005.

[14] Schiffelers RM *et al.* Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res* 2004; **32:** e149.

[15] Song E *et al.* Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* 2005; **23:** 709-717.

[16] Sorensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J Mol Biol* 2003; **327:** 761-766.

[17] Sioud M, Sorensen DR. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun* 2003; **312:** 1220-1225.

[18] Mir LM *et al.* Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. *Br J Cancer* 1998; **77:** 2336-2342.

[19] Rols MP *et al.* In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat Biotechnol* 1998; **16:** 168-171.

[20] Potts RO, Chizmadzhev YA. Opening doors for exogenous agents. *Nat Biotechnol* 1998; **16:** 135.

[21] Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer in vivo. *Gene Ther* 1999; **6:** 508-514.

[22] Faria M *et al.* Phosphoramidate oligonucleotides as potent antisense molecules in cells and in vivo. *Nat Biotechnol* 2001; **19:** 40-44.

[23] Heller R *et al.* In vivo gene electroinjection and expression in rat liver. *FEBS Lett* 1996; **389:** 225-228.

[24] Glasspool-Malone J, Somiari S, Drabick JJ, Malone RW. Efficient nonviral cutaneous transfection. *Mol Ther* 2000; **2:** 140-146.

[25] Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol* 1998; **16:** 867-870.

[26] Harrison RL, Byrne BJ, Tung L. Electroporation-mediated gene transfer in cardiac tissue. *FEBS Lett* 1998; **435:** 1-5.

[27] Rizzuto G *et al.* Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. *Proc Natl Acad Sci U S A* 1999; **96:** 6417-6422.

[28] Yang M *et al.* Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. *Proc Natl Acad Sci U S A* 2001; **98:** 2616-2621.

[29] Takahashi, Y., et al., Gene silencing in primary and metastatic tumors by small interfering RNA delivery in mice: quantitative analysis using melanoma cells expressing firefly and sea pansy luciferases. J Control Release, 2005. **105**(3): p. 332-43.

[30] Akaneya, Y., B. Jiang, and T. Tsumoto, RNAi-induced gene silencing by local electroporation in targeting brain region. J Neurophysiol, 2005. **93**(1): p. 594-602.

[31] Golzio, M., et al., Inhibition of gene expression in mice muscle by in vivo electrically mediated siRNA delivery. Gene Ther, 2005. **12**(3): p. 246-51.

[32] Shoji, M., et al., RNA interference during spermatogenesis in mice. Dev Biol, 2005. **282**(2): p. 524-34.

[33] Tao, J., et al., Inhibiting the growth of malignant melanoma by blocking the expression of vascular endothelial growth factor using an RNA interference approach. Br J Dermatol, 2005. **153**(4): p. 715-24.

[34] Golzio Met al., In vivo gene silencing in solid tumors by targeted electrically mediated siRNA delivery. Gene Ther. 2007.**14**(9):752-9.

[35] *35 Nakai et al.,* Therapeutic RNA interference of malignant melanoma by electrotransfer of small interfering RNA targeting Mitf. Gene Ther. 2007. **14**(4):357-65.

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NOTES

Skin and Transdermal drug delivery by electroporation

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STRUCTURE OF THE SKIN

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

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

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

TRANSDERMAL AND TOPICAL DRUG DELIVERY

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

TRANSDERMAL DRUG DELIVERY BY ELECTROPORATION

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

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

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

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

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

TOPICAL DRUG DELIVERY BY ELECTROPORATION

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

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

REFERENCES

References on skin structure

[1] http://en.wikipedia.org/wiki/Skin

References on transdermal and topical drug delivery

[2] Schuetz YB, Naik A, Guy RH, Kalia YN. Emerging strategies for the transdermal delivery of peptide and protein drugs. Expert Opin Drug Deliv. 2005):533-48.

[3] Nanda A, Nanda S, Ghilzai NM. Current developments using emerging transdermal technologies in physical enhancement methods. Curr Drug Deliv. 2006 3:233-42.

[4] Hadgraft J, Lane ME. Skin permeation: the years of enlightenment. Int J Pharm. 2005 305:2-12

References on transdermal drug delivery by electroporation

[5] Denet A.R., Vanbever R. and Préat V., Transdermal drug delivery by electroporation, Advanced drug delivery reviews, 2003, in press

[6] Jadoul A., Bouwstra J., and Préat V., Effects of iontophoresis and electroporation on the stratum corneum – Review of the biophysical studies, Advanced drug delivery reviews, 35, 1999, 89-105

[7] Prausnitz M.R., A practical assessment of transdermal drug delivery by skin elecroporation, Advanced drug delivery reviews, 35, 1999, 61-76

[8] Vanbever R. and Préat V., *In vivo* efficacy and safety of skin electroporation, Advanced drug delivery reviews, 35, 1999, 77-88

NOTES

Electroporation in skin gene delivery

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SKIN GENE DELIVERY

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

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

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

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

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

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

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

ELECTROPORATION IN SKIN GENE DELIVERY

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

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

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

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

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

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

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

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

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

NOTES

REFERENCES

References on skin gene delivery

[1] Spirito F, Meneguzzi G, Danos O et al. Cutaneous gene transfer and therapy: the present and the future. J Gene Med 2001; 3(1):21-31.

[2] Hengge UR. Gene therapy progress and prospects: the skin--easily accessible, but still far away. Gene Ther 2006; 13(22):1555-1563.

[3] Branski LK, Pereira CT, Herndon DN et al. Gene therapy in wound healing: present status and future directions. Gene Ther 2007; 14(1):1-10.

References on electroporation on skin gene delivery

[4] Byrnes CK, Malone RW, Akhter N et al. Electroporation enhances transfection efficiency in murine cutaneous wounds. Wound Repair Regen 2004; 12(4):397-403.

[5] Drabick JJ, Glasspool-Malone J, King A et al. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electropermeabilization. Mol Ther 2001; 3(2):249-255.

[6] Dujardin N, Staes E, Kalia Y et al. In vivo assessment of skin electroporation using square wave pulses. J Control Release 2002; 79(1-3):219-227.

[7] Dujardin N, Van Der Smissen P., Preat V. Topical gene transfer into rat skin using electroporation. Pharm Res 2001; 18(1):61-66.

[8] Glasspool-Malone J, Somiari S, Drabick JJ et al. Efficient nonviral cutaneous transfection. Mol Ther 2000; 2(2):140-146.

[9] Heller LC, Jaroszeski MJ, Coppola D et al. Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. Gene Ther 2007; 14(3):275-280.

[10] Heller R, Schultz J, Lucas ML et al. Intradermal delivery of interleukin-12 plasmid DNA by in vivo electroporation. DNA Cell Biol 2001; 20(1):21-26.

[11] Maruyama H, Ataka K, Higuchi N et al. Skin-targeted gene transfer using in vivo electroporation. Gene Ther 2001; 8(23):1808-1812.

[12] Medi BM, Hoselton S, Marepalli RB et al. Skin targeted DNA vaccine delivery using electroporation in rabbits. I: efficacy. Int J Pharm 2005; 294(1-2):53-63.

[13] Medi BM, Singh J. Skin targeted DNA vaccine delivery using electroporation in rabbits II. Safety. Int J Pharm 2006; 308(1-2):61-68.

[14] Pavselj N, Préat V. DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse. J Control Release 2005; 106(3):407-415.

[15] Vandermeulen G, Staes E., Vanderhaeghen ML et al. Optimisation of intradermal DNA electrtransfer for immunisation. J.Contrlled release, 2007 epub Aug 19.

[16] Zhang L, Widera G, Rabussay D. Enhancement of the effectiveness of electroporation-augmented cutaneous DNA vaccination by a particulate adjuvant. Bioelectrochemistry 2004; 63(1-2):369-373.

Other Physical and Mechanical Methods in Skin Gene Delivery

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INTRODUCTION

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

MICRONEEDLES

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

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

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

SONOPORATION

Sonoporation is the ultrasound-mediated enhancing of cell permeability.

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

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

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

MICROCHANNELS

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

LASER IRRADIATION

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

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

REFERENCES

[1] Birchall J, Coulman S, Anstey A et al. Cutaneous gene expression of plasmid DNA in excised human skin following delivery via microchannels created by radio frequency ablation. Int J Pharm 2006; 312(1-2):15-23.

[2] Lentacker I, De Geest BG, Vandenbroucke RE et al. Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. Langmuir 2006; 22(17):7273-7278.

[3] Mitragotri S, Kost J. Low-frequency sonophoresis: a review. Adv Drug Deliv Rev 2004; 56(5):589-601.

[4] Ogura M, Sato S, Nakanishi K et al. In vivo targeted gene transfer in skin by the use of laser-induced stress waves. Lasers Surg Med 2004; 34(3):242-248.

[5] Paliwal S, Mitragotri S.Ultrasound-induced cavitation: applications in drug and gene delivery. Expert Opin Drug Deliv. 2006 (6):713-26.

[6] Pearton M, Allender C, Brain K, Anstey A, Gateley C, Wilke N, Morrissey A, Birchall J. Gene Delivery to the Epidermal Cells of Human Skin Explants Using Microfabricated Microneedles and Hydrogel Formulations. Pharm Res. 2007 Aug 2; [Epub ahead of print]

[7] Sintov AC, Krymberk I, Daniel D et al. Radiofrequency-driven skin microchanneling as a new way for electrically assisted transdermal delivery of hydrophilic drugs. J Control Release 2003; 89(2):311-320.

[8] Tao W, Wilkinson J, Stanbridge EJ et al. Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane. Proc Natl Acad Sci U S A 1987; 84(12):4180-4184.

[9] Yang L, Shirakata Y, Tamai K et al. Microbubble-enhanced ultrasound for gene transfer into living skin equivalents. J Dermatol Sci 2005; 40(2):105-114.

[10] Zeira E, Manevitch A, Khatchatouriants A et al. Femtosecond infrared laser-an efficient and safe in vivo gene delivery system for prolonged expression. Mol Ther 2003; 8(2):342-350.

[11] Zeira E, Manevitch A, Manevitch Z, et al. Femtosecond laser: a new intradermal DNA delivery method for efficient, long-term gene expression and genetic immunization. FASEB J. 2007 Jun 15; [Epub ahead of print]

[12] Denet A.R., Vanbever R. and Préat V., Transdermal drug delivery by electroporation, Advanced drug delivery reviews, 2003, in press

[13] Jadoul A., Bouwstra J., and Préat V., Effects of iontophoresis and electroporation on the stratum corneum – Review of the biophysical studies, Advanced drug delivery reviews, 35, 1999, 89-105

[14] Prausnitz M.R., A practical assessment of transdermal drug delivery by skin elecroporation, Advanced drug delivery reviews, 35, 1999, 61-76

[15] Vanbever R. and Préat V., *In vivo* efficacy and safety of skin electroporation, Advanced drug delivery reviews, 35, 1999, 77-88

NOTES

Biotechnological developments of electropulsation

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Abstract: Electropulsation is known as a very efficient tool for obtaining gene transfer in many species to produce genetically modified organisms (GMO). This is routinely used for industrial purposes to transfer exogenous activities in bacteria, yeasts and plants. Electropulsation associated membrane alterations can be irreversible. The pulsed species can not recover after the treatment. Their viablity is strongly affected. This appears as a very promising technology for the eradication of pathogenic microorganisms. Recent developments are proposed for sterilization purposes. New flow technologies of field generation allow the treatment of large volumes of solution. Several examples for the treatment of domestic water and in the food industry are under development. Walled microorganisms are affected at the membrane and wall level. This brings a controlled leakage of the cytoplasmic soluble proteins. Large dimeric proteins such as β-galactosidases can be extracted at a high yield. High volumes can be treated by using a flow process.

INTRODUCTION

Electrotransformation (electrically mediated gene transfer) is routinely used at the bench to obtain genetically modified organisms (GMO) [1-3]. This was described in previous lectures and is not the topic of the present one

For many years biotechnological applications remain focused on small scale experiments. Getting a limited number of transformed microorganisms is enough to prepare the availability of GMO for the market. The selected microorganisms can be grown and expanded under selective pressure.

New developments of Electropulsation in Biotechnology are obtained when large volumes can be treated. Metabolites can be extracted or introduced as a result of Electropermeabilization. They can be small sized but cytoplasmic proteins can be the target by using suitable electrical parameters [4]. Microorganisms can be eradicated when stringent pulse conditions are used, which bring an irreversible electropermeabilization [5].

Theory

When applied on a cell suspension, an external field induces a time and position dependent membrane potential difference modification ΔV

The resulting membrane potential difference is the sum of the resting membrane potential difference (assumed to be independent of the external field) and of the field dependent modulation. Electropermeabilization is triggered as soon as locally the resulting membrane potential difference reaches a critical value (between 200 and 300 mV, i.e. for an applied field larger than a thereshold Ep).

The conclusion is that for long pulses with a field intensity E (E> Ep), a cap on the cell surface is in the permeabilized state and its surface is

$$A_{perm} = 2\pi r^2 \left(1 - \frac{E_P}{E}\right) \quad (1).$$

The density of local defects supporting the permeabilization is increased with pulse duration and number of successive pulses but not with the delay between pulses if delay is larger than one millisecond and shorter than 10 s.

Technological problems linked to large volume treatment

Working on large volumes can be obtained by an up-sizing of the present laboratory scale processes. Batch technology is always limited by the amount of energy which can be delivered by the power generators. The volume Vol which can be treated with a pulse of duration T at a field E in a buffer with a conductance Λ requires an available energy:

$$W = E^2 \cdot \Lambda \cdot Vol \cdot T \quad (2)$$

i.e. 15 kJ and high currents are needed to pulse 1 liter of phosphate buffer saline (PBS) at 1 kV/cm during 1 ms.

Other methodologies are clearly needed. Flow processes appear to be a suitable approach [6].

Flow Electropulsation

The basic concept is to apply calibrated pulses as in batch process but at a delivery frequency which is linked to the flow rate (Fig. 1). The relationship between frequency and flow is such that the desired numbers of pulses are actually delivered on each cell during its residency in the pulsing chamber. The geometry of the chamber is choosen to give a homogeneous field distribution and a uniform flow rate. Therefore, the residency time T_{res} of a given cell in the chamber is:

$$T_{res} = \frac{Vol}{Q} \quad (3)$$

where Vol is the volume of the pulsing flow chamber and Q is the average flow rate. The number of pulses delivered per cell is:

$$N = T_{res} \cdot F \quad (4)$$

F being the frequency of the pulses. Due to heterogeneities in flow velocities in chamber, a better treatment is obtained by a train of pulsing chambers within mixing chambers in between.

A major problem is that due to repetitive pulses, electrochemistry at the surface of the electrodes becomes a serious problem with bubbles formation and toxic species generation.

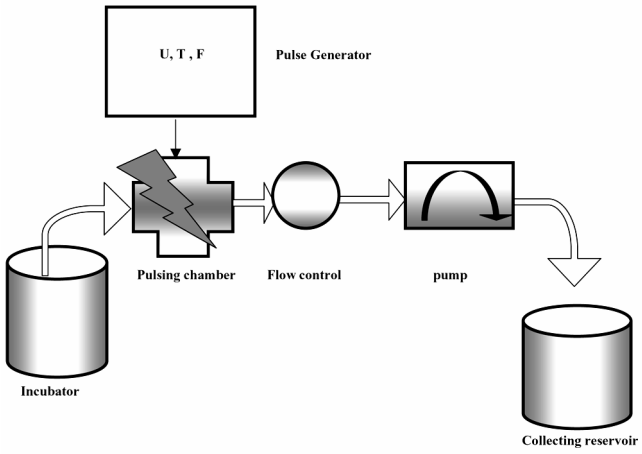

Figure 1: Flow electropulsation; A- Cells are taken from the incubator where they are growing. B- They flow through the pulsing chamber where a controlled number of calibrated pulses is applied. The pulsing chamber is connected to the high power pulse electropulsator where the voltage U, the pulse duration T and the pulse frequency F are under control. C- The flow Q is controlled by a pump. D- Pulsed cells are collected and processed in a collecting reservoir

Protein extraction

Yeasts (*Sacharomyces, Kluyveromyces, Picha*) are a well establish cell factory for the production of endogenous proteins. Their electrotransformation to produce exogenous proteins follows an easy to perform protocol on intact systems [7].

A technological bottle neck is the extraction of proteins from the cytoplasm under conditions where the protein integrity (i.e. activity) is preserved. Many approaches are proposed and already used in the Biotech industry. As the cell wall must be degradated, drastic mechanical, chemical or enzymatic methods are used. A critical drawback is presently due to the non specificity of these methods: the vacuoles are destroyed allowing the proteases to have a free access to the cytoplasmic enzymes. These methods are energy consuming because the treatment is most of the time operated at high temperature.

A simple procedure is obtained with electropulsation [8]. Yeasts cells are washed and suspended in pure water, a low conductance medium. A limited number of pulses is applied with pulse duration in the ms time range. Field intensities are less than 4 kV/cm. Pulsed cells are then incubated in 0.105 M salt solution (PBS and glycerol as osmotic protector) at room temperature. A slow release of cytoplasmic proteins is obtained, but up to 90% of the cell content can be recovered within 6-8 hours (100% being assumed to be obtained by the bead mill process or the enzyme lysis procedures). A key feature is that the specific activity of the recovered proteins is higher by a factor of 1.5-2 than with the mechanical extraction. This was explaind by the size specificity of the method leaving unaffected the subcellular organelles such as the vacuole where most proteases are present. Electrophoretic characterization of the extracted proteins does not indicate a size limit in the recovered proteins.

The electric conditions which are requested are easily obtained due to the low current intensity which is needed as the experiments are run on a suspension in pure water.

Optimization of the extraction procedure can be obtained by playing on the electrical parameters (field intensity, pulse duration, number of pulses) in such a way as to obtain a high flow rate. The cellular load can be high (up to 20% dry w/vol).

Leakages of species with molecular weights larger than 200 kDa were evidences that defects were present in the yeast wall. The outflow was slow (several hours) suggesting that no large defects were present. This was confirmed by electron microscopy studies [5]. The creation of these defects as a result of membrane electropermeabilization remained unexplained.

Most results were obtained on the yeast system, but we were able to obtain analogous results with mammalian cells and other walled systems may be targets (plant cells, molds)[9]. While proteins are products with a high added value, the electro-assisted extraction is valid for small metabolites.

Pathogen eradication

Electropulsation is known for many years to cause irreversible membrane permeabilization when drastic electrical conditions are used (Fig. 2). This offers a new physical approach for the elimination of microorganisms.

1-Food Industry

Cold sterilization is supposed to eliminate the microorganisms in the food (milk, fruit juices) while

preserving the "real" taste of the product [5]. The idea is that the field is able to disrupt the cell enveloppe but is too weak to inactivate enzymes [9]. Electrical parameters are always using strong electric pulses (more than 20 kV/cm) with microsecond pulse duration with a capacitor discharge technology.

2-Amoeba downstream of power plants

The presence of pathogens such as amoebae (Naegleria fowleri) is detected at increasing level in the closed looped cooling systems of power plants which use water for cooling. This is due to the facilitated growth of protozoa above 40°C. A continuous treatment system of the cooling water at the system drain appears necessary.

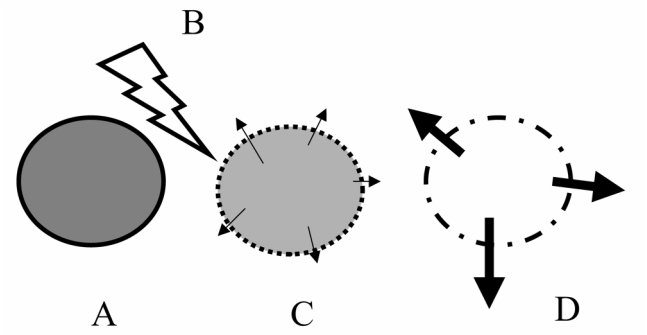

Figure 2: Irreversible Electropermeabilization; A- Intact cells. Their cytoplasm content is pictured in dark grey. B- Electropulsation. C- Cell membranes are permeabilized. The cytoplasm content leaks out as shown by the light grey colour and the small arrows. D-The cell membrane is irreversibly permeabilized and can not be repaired. All the cytoplasmic content leaks out.

Eradication can be obtained under low field long pulse duration conditions by inducing an irreversible permeabilization. Industrial developments required to reduce the cost of the treatment. Short pulses with high field intensity (microseconds, more than 10 kV/cm) were the most cost effective for eradication [10]. A pilot set up was recently tested on a power plant [11]. Results are encouraging. A 2 log eradication was obtained for less than 1 kW when treating 1 dm3/s (2) with one single pulsing chamber.

Most of the results are explained by an irreversible permeabilization. But it cannot explain microorganism death under these very short pulse conditions. Other physical factors are present when a field pulse is applied on a vesicle [12, 13]. Electrical fields induce mechanical forces. As it is a field effect on a field induced dipole, the general expression of the force F is given by

$$F = U(t) \cdot E^2 \qquad (4)$$

where the U(t) parameter is dependent on the frequency of the field and on the membrane state [14]. The final result is that a time dependent strain is applied on a cell with a time dependent membrane organization. Electromechanical stretching appears as a driving force in the irreversible damage.

More recently, the use of nanosecond high intensity pulses was introduced by the group of Schoenbach [15]. Under these procedures, the field effect was targeted on small sized vesicles such as organelles inside the cytoplasm or bacteria.

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REFERENCES

[1] N. Eynard J. Teissié J. "Electrotransformation of Bacteria", 2000, Springer

[2] J.A. Nickoloff "Electroporation protocols for Microorganisms", *Meth. Mol. Biol.* 47, 1995, Humana press

[3] J.A. Nickoloff. "Plant Cell Electroporation and Electrofusion protocols", *Meth. Mol. Biol.* 55, 1995, Humana press

[4] M.P. Rols , J. Teissié . Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. *Biophys. J.* 75, 1998, 1415-1423

[5] G.V. Barbosa-Canovas et al. "Preservation of Foods with Pulsed Electric Fields", 1999, Academic Press

[6] J. Teissié. Electropulsation de cultures cellulaires. *Biofutur 45,* 1986, 64-67

[7] E. Meilhoc, J. Teissié J., J.M. Masson. High Efficiency transformation of intact yeast cells by electric field pulses. *Bio/technology* 8, 1990, 223-227

[8] V. Ganeva, B. Galutzov B., N. Eynard, J. Teissié. Electroinduced extraction of b-galactosidase from kluyveromyces lactis. *Appl. Microbiol. Biotech.* 56, 2001, 411-

[9] D. Knorr, novel approaches in food processing technology: new technologies for preserving foods and modifying function. *Curr. Op. Biotechnol* 10, 1999, 485-91

[10] C.N. Haas, D. Aturaliye. Semi-quantitative Characterization of Electroporation-assisted Disinfection Processes for Inactivation of Giardia and Cryptosporidium. *Journal of Applied Microbiology* 86, 1999, 899-905.

[11] P. Baraton, P.A. Cabanes, M.C. Vernhes. Eradication of Naegleria lovaniensis amoebae by pulsed electric fields. Validation of the effectiveness of a high-flow process, *Proceedings 9th international meeting on the biology and pathology of free living amoebae,* 2001, 323-329, J. Libbey Eurotext

[12] E. Neumann, S. Kakorin, K. Toensing . Membrane electroporation and electromechanical deformation of vesicles and cells. *Faraday Discuss.* 111, 1998, 111-25

[13] N. Asgharian, Z.A. Schelly . Electric field-induced transient birefringence and light scattering of synthetic liposomes. *Biochim Biophys Acta.* 1418, 1999 , 295-306

[14] H. Mussauer, V.L. Sukhorukov , A. Haase, U. Zimmermann. Resistivity of red blood cells against high-intensity, short-duration electric field pulses induced by chelating agents. *J Membr Biol.* 170, 1999, 121-33.

[15] J. Deng, K.H. Schoenbach, E.S. Buescher, P.S. Hair, P.M. Fox , S.J. Beebe. The effects of intense submicrosecond electrical pulses on cells. *Biophys J*. 84, 2003, 2709-14.

NOTES

INVITED LECTURERS

Nanoscale Electroporation — Dipoles and Dielectrics, Nanoseconds and Nanometers

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INTRODUCTION

Recent investigations in the new field of nanosecond, megavolt-per-meter bioelectrics — the study of the effects of high-intensity but short duration (therefore low energy) electric pulses on biological systems — have shown that ultra-short (≤ 100 ns), high-field (≥ 1 MV/m) electrical signals have unique and profound effects on cells and tissues, confirming predictive hypotheses based on fundamental electromagnetics. These nanoscale stimuli reach biomolecular structures in the cell interior, unlike slower (≥ 1 μs) and lower-field (≤ 100 kV/m) pulses, which exert their effects primarily at the cell perimeter through the accumulation of mobile charges at the membrane. Nanosecond electric pulses do perturb the plasma membrane, but in addition they stimulate the release of calcium from compartments of the endoplasmic reticulum, permeabilize intracellular vesicles, produce changes in the organization of nuclear material, activate cardiac and neuroendocrine responses, and induce apoptosis (programmed cell death) in cancer cells. Potential applications of nanoscale bioelectric effects include noninvasive therapeutics for the removal of cancer and other diseased tissue, and remote delivery of stimuli for triggering muscular and nervous activity. The mechanisms for all of these effects remain essentially unknown.

BIOLOGICAL RESPONSES TO NANOSECOND ELECTRICAL PULSES

Nanosecond, megavolt-per-meter pulsed electric fields nondestructively perturb the intracellular environment, causing calcium bursts [1, 2], eosinophil sparklers [3], vacuole permeabilization [4], nuclear chromatin rearrangement [5], activation of excitable cells (cardiac myocytes and adrenal chromaffin cells) [6], and the appearance of apoptotic indicators such as release of cytochrome c into the cytoplasm [7], loss of mitochondrial membrane potential, and caspase activation [8, 9]. Nanoelectropulse-induced killing of cancer cells and shrinking of tumors has been demonstrated in vitro and in vivo [10,11].

In addition to these responses in the cell interior, nanoelectropulse exposure also induces phosphatidylserine (PS) externalization (Figure 1) — translocation of PS from the cytoplasmic face of the plasma membrane to the cell exterior — a normal event in platelet activation and blood coagulation [12], a diagnostic feature of apoptotic cells which serves as a physiological semaphore for their phagocytic removal [13], and a means of intramembrane signal transduction in lymphocytes [14]. The ability to activate this signal remotely, with non-ionizing, non-thermal (high power, but low total energy), non-invasive electric pulses may be useful in both research and clinical settings.

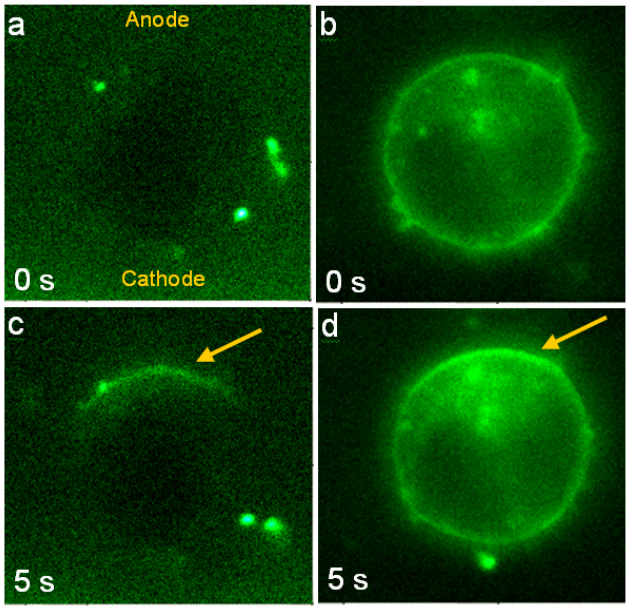

Figure 1: PS externalization in RPMI 8226 human multiple myeloma cells after exposure to 4 ns, 8 MV/m pulses. Separate cells stained with annexin V-FITC (a) and FM1-43 (b) are shown before and 5 s after pulse delivery (c and d). The anode (positive terminal) is at the top of each image; the cathode is at the bottom. Fluorescence indicating PS translocation appears at the anode-facing pole of the cell. Note correspondence of the regions of pulse-induced annexin V binding and FM1-43 fluorescence intensification. From [n].

All of these effects are mediated by the generation of perturbative potentials on cellular structures for periods shorter than the charging time constant of the plasma membrane (tens to hundreds of nanoseconds for mammalian cells of various sizes and shapes) [3,15]. Although the electrical power associated with these pulse exposures is high (megawatts), nanosecond pulse durations limit the total energy delivered to nanojoules per cell, and there is no immediately apparent physical damage at the cellular level. Despite these low energies, the initial effects are

abrupt, well-defined, and apparent using fluorescence microscopy within milliseconds.

DOSE RESPONSE AND PULSE PARAMETERS

The unique electrophysical properties of the biological cell, which can be modeled in its simplest form as a dielectric shell enclosing and suspended in conductive media and containing within its bounded volume smaller dielectric shells (membrane-bound intracellular structures), provide a basis for understanding these responses [7,9,16], but more extensive experimental studies are needed to refine the models, to systematically define the sensitivities of different cell types, and to identify and characterize the mechanisms underlying the response patterns that have already been observed.

It is of particular importance to chart the minima of the pulse parameters — electric field, pulse rise time and duration, pulse count and repetition rate — required to produce these biological responses. Large pulse doses are likely to produce multiple effects, which may be useful for cancer therapy or other applications where cell killing is the overriding desired outcome, but in order to draw out and delineate the molecular disequilibrations caused by nanoelectropulse exposure it will be necessary to minimize the perturbations to the cellular machinery instead of delivering an overwhelming dose.

Previous studies indicate that nanosecond pulse effects may be absent when the electric field amplitude is less than about 1 MV/m [17], but a careful review of these observations is essential in order to confirm this threshold for the various cellular responses mentioned above, and to investigate whether the failure to detect a response in each case is a consequence of a true threshold or whether it results from the relative insensitivity of the assay or the instrumentation. Other variables may affect the level of the observed threshold (for example, electropermeabilization with 4 ns pulses is observed only at high pulse repetition rates [18]), and the effects of different combinations of pulse parameters on the final response is undoubtedly complex.

RESPONSES VARY WITH CELL TYPE

A wide variety of cellular reactions to pulses with widths from 30 ns down to 3 ns have been characterized to a limited extent, including membrane permeabilization and phosphatidylserine externalization [17,19,20], intracellular calcium release [1], chromatin changes, and diagnostic indicators of apoptosis like caspase activation, PARP cleavage, nuclear condensation, and loss of mitochondrial membrane potential [21]. These phenomena have been observed in our laboratories in living cells during pulse exposures in microfabricated electrode chambers [22] and in electroporation cuvettes. Some of the outlines of dose-response curves and the kinetics of the responses have been obtained, but many combinations of pulse parameters, exposure conditions, and cell types remain to be investigated. Experiments performed at Cedars-Sinai Medical Institute in collaboration with clinical oncologists concentrated on cell killing after pulse exposures in electroporation cuvettes and on the responses of tumor implants in nude athymic mice. A wide range of sensitivities among cell types was observed, and we are encouraged by the clinically complete responses of pancreatic tumor implants in mice [11].

The nanoelectropulse responses of the two types of electrically active cells we have studied — cardiomyocytes and adrenal chromaffin cells — are very different, as one might expect, from those of the tumor cells. A single nanosecond pulse causes depolarization, calcium waves, and contraction of cardiomyocyte fibers [6]. Adrenal chromaffin cells also respond to a single pulse as short as 3 ns with elevated intracellular calcium levels and catecholamine release. Preliminary data from experiments with calcium channel blockers indicates that L-type calcium channels participate in this response [23].

PHYSICAL AND ELECTRICAL MODELS OF BIONANOSYSTEMS

Direct observation of nanosecond events in living biological systems is challenging and for the most part beyond the practical reach of instrumentation and methods available today. A notable exception to this is the effort at Old Dominion University to monitor membrane potential changes in real time during nanoelectropulse exposure using potential-sensitive fluorescent dyes [24]. Molecular dynamics (MD) and micro- and macroscale modeling with field solvers, electrophysical analytic approaches, and electrical circuit representations can be used profitably to create simulations for consistency checks and verification of proposed nanoelectropulse perturbation mechanisms, and to generate hypotheses for testing with available laboratory resources. In order to identify the molecular mechanisms operative on a nanosecond time scale during electroporation of phospholipid bilayers, for example, MD simulations of phospholipid bilayers in high electric fields can suggest details of electroporation kinetics and dynamics not directly accessible by experiment [19,25].

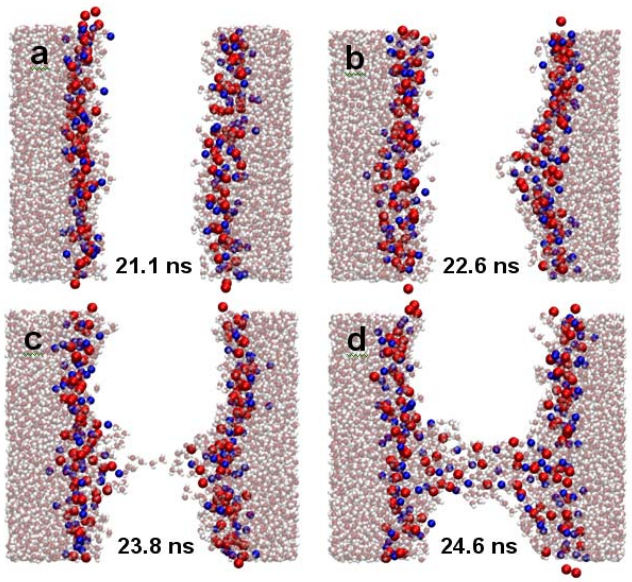

Figure 2: Snapshots from a POPC bilayer electroporation simulation. (a) Membrane before poration. (b) Pre-pore intrusion of water and lipid head groups into bilayer interior (lipid tails not shown). (c) Hydrophobic pore. (d) Hydrophilic pore. Electric field is directed normal to the bilayer, along the z-axis from left to right. P – blue; N – red; H_2O – pale red (O) and white (H); other phospholipid atoms not shown.

Continuum [26] and transport lattice circuit models [27] based on empirical membrane properties and stochastic interpretations of the measured conductance and capacitance of bilayers in electric fields led to predictions by Weaver [28] that nanosecond, megavolt-per-meter pulses cause the formation of numerous, nanometer-diameter pores in all cell membranes — external and internal — in a system, unlike longer, low-field pulses, which produce the fewer, larger pores associated with conventional electroporation. Persistent experimental efforts to demonstrate this "supra-electroporation", initially yielding only negative results, finally succeeded, with appropriate fluorescent dyes and high electric fields at high pulse repetition rates, in detecting the nanoelectropulse-driven electropermeabilization of cell membranes [18].

MOLECULAR DYNAMICS SIMULATIONS OF NANOELECTROPORATION

MD simulations of phospholipid bilayers in supraphysiological electric fields show a tight association between PS externalization and membrane pore formation on a nanosecond time scale that is consistent with experimental evidence for electropermeabilization and anode-directed PS translocation after nanosecond electric pulse exposure, suggesting a molecular mechanism for nanoelectroporation and nanosecond PS externalization: electrophoretic migration of the negatively charged PS head group along the surface of nanometer-diameter electropores initiated by field-driven alignment of water dipoles at the membrane interface [19].

Pore formation in these simulations of homogeneous lipid bilayers is a molecular process, an extension of water defects into hydrophobic and then hydrophilic, nanometer-diameter pores which takes place in a few nanoseconds (Figure 2). The occurrence of electroporation even in octane "membranes" suggests that the headgroups play a secondary role. Mechanical forces on the membrane (flexure, tension, compression) are not required for poration in these simulations, beyond the electrostatic torques, repulsions, and attractions expressed at the molecular level, nor are large statistical fluctuations involving the entire transmembrane region required for the nanometer scale electroporation observed in our systems. Water defect propagation into the bilayer interior is enhanced by an energetically unstable alignment of interfacial water dipoles in the applied electric field, which lowers the barrier for this pore-propagating configuration. The extent and direction of the water dipole ordering is influenced by the length and degree of saturation of the lipid hydrocarbon tails and is relatively insensitive to the head group dipole moment and charge. Once a hydrophilic pore has formed, however, the head group dipoles readily rotate and align in the relatively large electric field component tangential to the plane of the pore wall (Figure 3), and this may be important for the longer-term size and stability of the pore.

A stabilizing role for the field-aligned head group P→N dipole, which lies nearly flat in the plane of a hydrated, fluid-phase phospholipid bilayer, was proposed in early studies of electroporation [29]. More recently it has been suggested on the basis of coarse-grained models [25,30] that poration defects are initiated by substantial changes in the tilt of the head group dipole relative to the bilayer plane, but in all-atom MD simulations electroporation is observed in a water-octane system [31] and in "chargeless" POPC (palmitoyloleoylphosphatidylcholine) bilayers, indicating that lipid head group electrostatic interactions are not an essential component of the poration mechanism. Water dipole rearrangements at the membrane interface in a porating electric field are striking, however, and may be a driving factor in defect initiation in at least some systems [32,33,34].

An electric field normal to a phospholipid bilayer exerts a torque on the P→N dipole, tilting the N closer to the bilayer interior in one leaflet and farther away in the other, countered by intra- and intermolecular bonding forces and steric factors. When we consider only lipid molecules directly involved in pore

formation we find that a minimum porating electric field normal to POPC membrane produces a mean head group dipole tilt of less than 5° from the zero-field value everywhere in the bilayer before pore initiation. Although it is possible that this modified conformation facilitates water intrusion into the membrane interior, we observe that even after the formation of a pre-pore defect the angle of the head group dipoles for the lipids that will line the pore a nanosecond or two later remains relatively constant. During pore formation, as the head groups migrate into the bilayer interior, the P→N dipoles rotate in the plane of the pore wall to align with the now-tangential electric field.

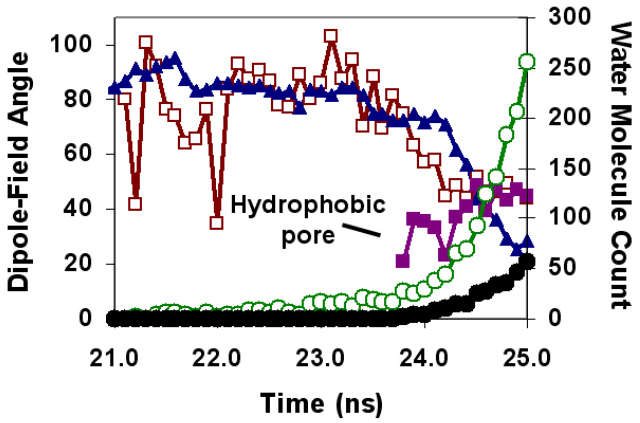

Figure 3: Dipole-electric field alignment during pore formation in a POPC bilayer. Head group dipoles (▲) of pore-forming lipids remain in the unporated bilayer plane (nearly perpendicular to the field) until a pre-pore defect of lipids and penetrating (within 1 nm of the bilayer center) waters (□) appears. P→N dipoles on the developing pore surface rotate in the new pore wall plane to align with the electric field, shown by the decrease in the head group dipole-field angle. Deep interior (within 0.25 nm of bilayer center) water then appears for the first time, forms a transient, hydrophobic pore, and solvates head groups in the pore wall (■). Open and closed circles are the numbers of shallow and deep penetrating water molecules, respectively.

In contrast to the small tilt of the P→N vector relative to the bilayer surface, we observe that head group dipoles rotate readily in the plane of the bilayer, following a tangential field both in the pore wall and on the unporated membrane surface (Figure 4). Reversing the field direction causes a rapid (within 1 ns) tracking rotation of the dipole vectors, and removing the field, or setting the charges on the head group atoms to zero, results in rapid (within 200 ps) thermalization of the mean P→N vector angle.

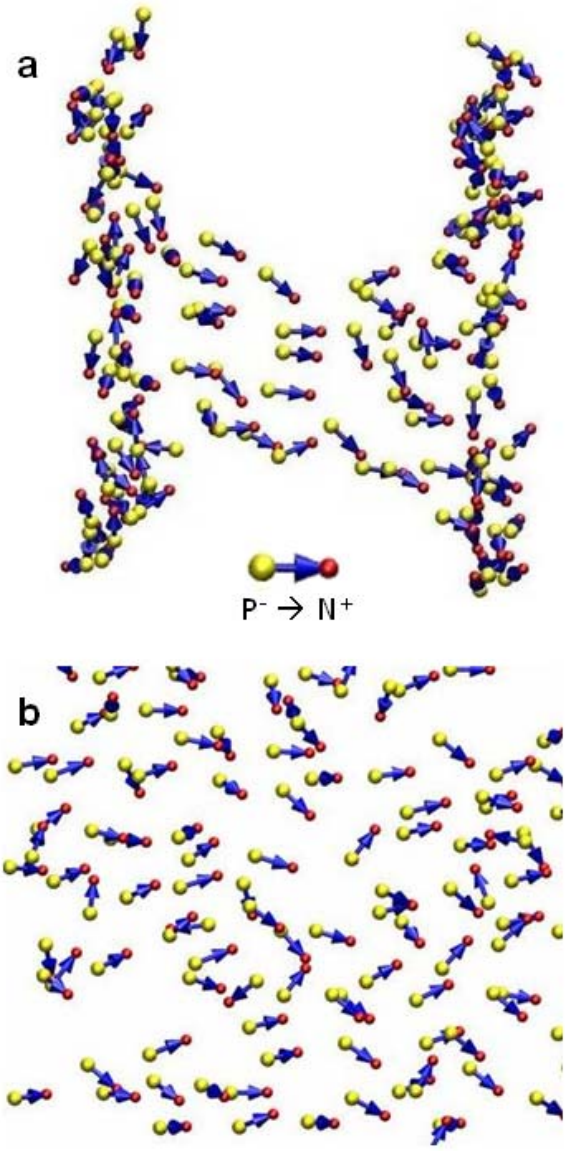

Figure 4: Field-driven head group dipole alignment. (a) Projection of P→N dipoles in a porated membrane on a plane parallel with a 320 mV/nm electric field directed along the pore axis (left to right). (b) Projection of P→N dipoles in an unporated membrane on a plane parallel with the bilayer surface with a 140 mV/nm electric field applied in the same plane (left to right).

All of our MD simulations to date indicate that water dipoles oriented by the electric field and by entropic and enthalpic considerations at the membrane interface are the primary transducers for converting applied pulsed electric field energy into membrane pores. The mechanism of pore formation represented in these simulations — extension of stochastic water defects into bilayer-spanning water channels which are lined and stabilized by the diffusive migration of phospholipid head groups along the incipient nanometer-diameter pore walls within nanoseconds — is consistent with observations of artificial membranes and living cells in electric fields, and with continuum

physical and electrostatic models. The key electric field-sensitive determinant of poration is the ordering of membrane-associated water dipoles, the result of competitive and cooperative interactions of water dipole rotation, hydrogen bond formation, and lipid solvation interactions in different regions of the membrane interface.

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REFERENCES

[1] P. T. Vernier, Y. Sun, L. Marcu, S. Salemi, C. M. Craft, and M. A. Gundersen, "Calcium bursts induced by nanosecond electric pulses," *Biochemical and Biophysical Research Communications,* vol. 310, pp. 286-95, Oct 17 2003.

[2] J. A. White, P. F. Blackmore, K. H. Schoenbach, and S. J. Beebe, "Stimulation of capacitative calcium entry in HL-60 cells by nanosecond pulsed electric fields," *J Biol Chem,* vol. 279, pp. 22964-22972, 2004.

[3] K. H. Schoenbach, S. J. Beebe, and E. S. Buescher, "Intracellular effect of ultrashort electrical pulses," *Bioelectromagnetics,* vol. 22, pp. 440-8, 2001.

[4] E. Tekle, H. Oubrahim, S. M. Dzekunov, J. F. Kolb, K. H. Schoenbach, and P. B. Chock, "Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses," *Biophys J,* vol. 89, pp. 274-84, 2005.

[5] Y. Sun, P. T. Vernier, M. Behrend, J. Wang, M. M. Thu, M. Gundersen, and L. Marcu, "Fluorescence microscopy imaging of electroperturbation in mammalian cells," *J Biomed Opt,* vol. 11, p. 24010, 2006.

[6] P. T. Vernier, Y. Sun, J. Wang, M. M. Thu, E. B. Garon, M. Valderrabano, L. Marcu, H. P. Koeffler, and M. A. Gundersen, "Nanoelectropulse intracellular perturbation and electropermeabilization technology: phospholipid translocation, calcium bursts, chromatin rearrangement, cardiomyocyte activation, and tumor cell sensitivity," in *27th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, Shanghai, 2005.

[7] S. J. Beebe, P. M. Fox, L. J. Rec, K. Somers, R. H. Stark, and K. H. Schoenbach, "Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: Apoptosis induction and tumor growth inhibition," *IEEE Transactions on Plasma Science,* vol. 30, pp. 286-292, 2002.

[8] S. J. Beebe, P. M. Fox, L. J. Rec, E. L. Willis, and K. H. Schoenbach, "Nanosecond, high-intensity pulsed electric fields induce apoptosis in human cells," *FASEB J,* vol. 17, pp. 1493-5, 2003.

[9] P. T. Vernier, A. M. Li, L. Marcu, C. M. Craft, and M. A. Gundersen, "Ultrashort pulsed electric fields induce membrane phospholipid translocation and caspase activation: Differential sensitivities of Jurkat T lymphoblasts and rat glioma C6 cells," *IEEE Transactions on Dielectrics and Electrical Insulation,* vol. 10, pp. 795-809, 2003.

[10] R. Nuccitelli, U. Pliquett, X. Chen, W. Ford, R. James Swanson, S. J. Beebe, J. F. Kolb, and K. H. Schoenbach, "Nanosecond pulsed electric fields cause melanomas to self-destruct," *Biochem Biophys Res Commun,* vol. 343, pp. 351-60, 2006.

[11] E. B. Garon, D. Sawcer, P. T. Vernier, T. Tang, Y. Sun, L. Marcu, M. A. Gundersen, and H. P. Koeffler, "In vitro and in vivo evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies," *Int J Cancer,* vol. 121, pp. 675-82, 2007.

[12] K. Balasubramanian and A. J. Schroit, "Aminophospholipid asymmetry: A matter of life and death," *Annu Rev Physiol,* vol. 65, pp. 701-34, 2003.

[13] V. A. Fadok, A. de Cathelineau, D. L. Daleke, P. M. Henson, and D. L. Bratton, "Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts," *J Biol Chem,* vol. 276, pp. 1071-7, 2001.

[14] J. I. Elliott, A. Surprenant, F. M. Marelli-Berg, J. C. Cooper, R. L. Cassady-Cain, C. Wooding, K. Linton, D. R. Alexander, and C. F. Higgins, "Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes," *Nat Cell Biol,* vol. 7, pp. 808-16, 2005.

[15] L. D. Sher, E. Kresch, and H. P. Schwan, "On the possibility of nonthermal biological effects of pulsed electromagnetic radiation," *Biophys J,* vol. 10, pp. 970-9, 1970.

[16] R. Plonsey and K. W. Altman, "Electrical stimulation of excitable cells - a model approach," *Proceedings of the IEEE,* vol. 76, pp. 1122-1129, 1988.

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

[18] P. T. Vernier, Y. Sun, and M. A. Gundersen, "Nanoelectropulse-driven membrane perturbation and small molecule permeabilization," *BMC Cell Biology,* vol. 7, p. 37, 2006.

[19] 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, pp. 6288-6289, 2006.

[20] P. T. Vernier, Y. Sun, L. Marcu, C. M. Craft, and M. A. Gundersen, "Nanosecond pulsed electric fields perturb membrane phospholipids in T lymphoblasts," *FEBS Lett,* vol. 572, pp. 103-8, 2004.

[21] M. Behrend, A. Kuthi, X. Y. Gu, P. T. Vernier, L. Marcu, C. M. Craft, and M. A. Gundersen, "Pulse generators for pulsed electric field exposure of biological cells and tissues," *IEEE Transactions on Dielectrics and Electrical Insulation,* vol. 10, pp. 820-825, 2003.

[22] Y. Sun, P. T. Vernier, M. Behrend, L. Marcu, and M. A. Gundersen, "Electrode microchamber for noninvasive perturbation of mammalian cells with nanosecond pulsed electric fields," *IEEE Transactions on Nanobioscience,* vol. 4, pp. 277-283, 2005.

[23] G. L. Craviso, Y. Sun, M.-T. Chen, M. A. Gundersen, and P. T. Vernier, "Single, nanosecond electric pulse elevates intracellular calcium in bovine adrenal chromaffin cells," *Bioelectromagnetics Society 28th Annual Meeting*, Cancun, 2006.

[24] W. Frey, J. A. White, R. O. Price, P. F. Blackmore, R. P. Joshi, R. Nuccitelli, S. J. Beebe, K. H. Schoenbach, and J. F. Kolb, "Plasma membrane voltage changes during nanosecond

pulsed electric field exposure," *Biophys J,* vol. 90, pp. 3608-15, 2006.

[25] 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 Nonlin Soft Matter Phys,* vol. 71, p. 031914, 2005.

[26] K. A. DeBruin and W. Krassowska, "Modeling electroporation in a single cell. I. Effects of field strength and rest potential," *Biophys J,* vol. 77, pp. 1213-24, 1999.

[27] D. A. Stewart, I. R. Gowrishankar, and J. C. Weaver, "Transport lattice approach to describing cell electroporation: Use of a local asymptotic model," *IEEE Transactions on Plasma Science,* vol. 32, pp. 1696-1708, 2004.

[28] T. R. Gowrishankar, A. T. Esser, Z. Vasilkoski, K. C. Smith, and J. C. Weaver, "Microdosimetry for conventional and supra-electroporation in cells with organelles," *Biochem Biophys Res Commun,* vol. 341, pp. 1266-76, 2006.

[29] E. Neumann, M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider, "Gene transfer into mouse lyoma cells by electroporation in high electric fields," *EMBO J,* vol. 1, pp. 841-5, 1982.

[30] M. Kotulska, K. Kubica, S. Koronkiewicz, and S. Kalinowski, "Modeling the induction of lipid membrane electropermeabilization," *Bioelectrochemistry,* vol. 70, pp. 64-70, 2007.

[31] 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, pp. 6382-3, 2003.

[32] D. P. Tieleman, "The molecular basis of electroporation," *BMC Biochem,* vol. 5, p. 10, 2004.

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

[34] S. K. Kandasamy and R. G. Larson, "Cation and anion transport through hydrophilic pores in lipid bilayers," *Journal of Chemical Physics,* vol. 125, p. 74901, 2006.

NOTES

Electro-deformation, -poration and –fusion of Membranes Visualized on Giant Vesicles

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Abstract: This lecture is dedicated to effects of electric fields on giant unilamellar vesicles, a model membrane system of cell-size scale. We summarize various types of behavior observed when vesicles are subjected either to weak AC fields at various frequency, or to strong DC pulses. Different processes such as electro-deformation, -poration and -fusion of giant vesicles are considered. Some recent developments are introduced, which allowed us to detect the dynamics of the vesicle response with a resolution below milliseconds for all of these processes. The dynamics of deformation is discussed in view of the properties inherent to lipid membranes.

INTRODUCTION

Giant lipid vesicles provide biomembrane models suitable for systematic measurements of the interaction between electric fields and lipid bilayers. The membrane response to various external factors can be directly visualized under the microscope [1]. AC fields induce stationary deformation of the vesicles, while the response to short DC pulses is very dynamic and difficult to resolve with standard video recording. Recently, using a fast imaging digital camera, we were able to capture the immediate response of giant lipid vesicles to electric fields [2-5]. The vesicle response and relaxation dynamics was recorded with a high temporal resolution using phase contrast microscopy.

This report summarizes our observations on electro-deformation, -poration and -fusion of giant vesicles. We first introduce some basic relations describing the interaction between electric fields and membranes. Then, we consider the shape transitions observed when vesicles are subjected to AC fields for different frequencies and/or media conductivities. The response of vesicles to DC pulses is considered in terms of vesicle deformation and poration.

SOME EQUATIONS

Lipid membranes are essentially impermeable to ions. Thus, in the presence of an electric field, charges accumulate on both sides of the bilayer. For a spherical vesicle of radius R and tilt angle θ between the electric field and the surface normal, the charge accumulation gives rise to the transmembrane potential [6]

$$V_m = 1.5 \cdot |\cos\theta| \cdot E \cdot R \left[1 - \exp\left(-t / \tau_{ch\,\mathrm{arg}} \right) \right] \quad (1)$$

as a function of time t. Here E is the amplitude of the applied electric field and τ_{charg} is the membrane charging time given by [6]:

$$\tau_{ch\,\mathrm{arg}} = R \cdot C_m \cdot \left[\frac{1}{\lambda_{in}} + \frac{1}{2\lambda_{ex}} \right] \quad (2)$$

where C_m is the membrane capacitance, of the order of 1 μF/cm^2 for fluid lipid membranes [7], and λ_{in} and λ_{ex} are the conductivities of the internal and external vesicle solutions, respectively. Above some electroporation threshold, the transmembrane potential V_m cannot be further increased, the membrane porates, thus becoming conductive and permeable.

The electroporation phenomenon can also be understood in terms of a stress in the bilayer created by the electric field. The transmembrane potential, V_m, induces an effective electrical tension σ_{el}, as defined by the Maxwell stress tensor [3, 7, 8] This tension is given by

$$\sigma_{el} = \frac{\varepsilon \varepsilon_0 h V_m^2}{2 h_e^2} \quad (3)$$

where ε is the dielectric constant of the aqueous solution, ε_0 the vacuum permittivity, h is the total bilayer thickness, (~ 4 nm), and h_e the dielectric thickness (~ 2.8 nm for lecithin bilayers [9]). For vesicles with some initial tension σ_0, the total tension reached during the pulse is

$$\sigma = \sigma_0 + \sigma_{el} \quad (4)$$

The total membrane tension cannot exceed the tension of rupture. For lipid membranes, the tension of rupture is in the range 5 – 10 dyn/cm also known as lysis tension, σ_{lys}; see e.g. references [7] and [10]. The lysis tension can be reached either by applying an overall mechanical tension to the vesicle, for example using micropipettes or osmotic pressure, and/or by locally building up an electric tension. Thus, electroporation occurs when the membrane tension

reaches the lysis tension. This corresponds to building up a certain critical transmembrane potential, $V_m = V_c$, which for cell membranes is $V_c \approx 1V$. Similarly, for tension-free vesicles, the critical potential $V_c \sim 1.1$ V. The value of the critical poration potential decreases when the initial membrane tension increases.

In the next section we first consider the response of the vesicle membrane when subjected to "mild" AC fields before considering the cases of membrane deformation and poration induced by DC pulses.

VESICLE DEFORMATION IN AC FIELDS

The shape deformation of vesicles in AC fields has been previously studied, but the dependence of the vesicle morphology on both field frequency and media conductivity has not been fully characterized. At low field frequencies (few kHz), vesicles in water deform into prolates with the longer axis oriented along the field direction [11]. At intermediate frequencies (several kHz), again for vesicles in water medium, prolate-oblate transitions were observed [12,13] as theoretically predicted earlier [14,15]. This behavior was observed also when the conductivity of the external vesicle solution, λ_{ex} was higher than the internal conductivity λ_{in}. Interestingly, when the conductivity of the internal solution is raised so that it exceeds the external conductivity, i.e. at $\lambda_{in} > \lambda_{ex}$, the prolate-oblate transition is suppressed. With increasing field frequency, the vesicles undergo only the prolate-to-sphere transition. Two examples for the shape evolution of vesicles at different conductivity conditions are provided in Fig. 1.

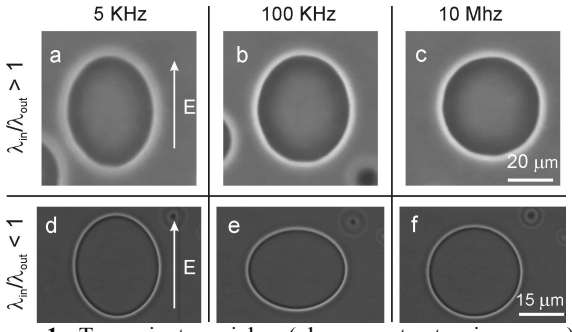

Figure 1: Two giant vesicles (phase contrast microscopy) in different conductivity conditions subjected to AC field of 0.2 kV/cm and various field frequencies as indicated above the images. The field direction is indicated with an arrow in (a, d). The conductivity conditions are $\lambda_{in} > \lambda_{ex}$ for (a-c) where only prolate-to-sphere transformation is observed, and $\lambda_{in} < \lambda_{ex}$ for (d-f) where prolate-to-oblate and oblate-to-sphere transitions are detected. Reproduced from [16].

It is worth mentioning that non-spherical cells have been observed to *orient* parallel or perpendicular to the field [17], whereas, in a shape-analogous way, vesicles in water solutions *deform* with their largest cross section also being parallel or perpendicular to the field. The cell behavior was also found to depend on the medium conductivity conditions and field frequency [18,19].

DYNAMICS OF THE VESICLE RESPONSE TO DC PULSES

While the discussion of vesicles exposed to AC fields was limited to stationary shapes, DC pulses induce short-lived shape deformations. Because the application of both AC fields and DC pulses creates a transmembrane potential, vesicle deformations of similar nature are to be expected in both cases. However, in the presence of salt in the vesicle exterior (e.g. NaCl solution with concentration above 0.1 mM), unusual shape changes were observed [3]. The vesicles assume cylindrical shapes during the pulse; see Fig. 2. These deformations are short-lived (their lifetime is about 1 ms) and occur only in the presence of salt outside the vesicles, irrespective of their inner content. The formation of these shapes is not well understood. One possible hypothesis is related to electrophoretic forces exerted by the ions in the external solution; for details see reference 3. Another interpretation might be an electrohydrodynamic instability caused by electric fields interacting with flat membranes, which was predicted to increase the membrane roughness [20].

Vesicle deformation induced by DC pulses has been studied theoretically [21,22]. Several experimental studies have been pursued but mainly small vesicles (hundreds of nanometers in size) have been used [23-26]. Because of the small size of the vesicles, direct observation of the deformation and poration is not feasible. In addition, membrane tension and curvature may play a significant role. The vesicle response has been detected using turbidity, absorbance, and conductivity measurements where microsecond resolution can be achieved.

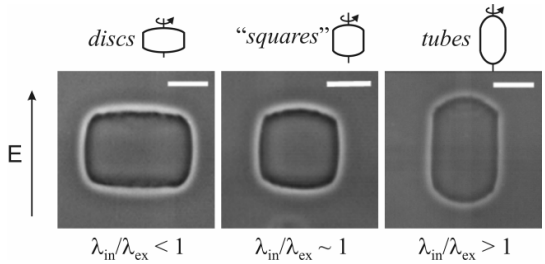

Figure 2: DC-pulse induced deformation of vesicles containing salt in the vesicle exterior at different conductivity conditions. Schematic illustrations of the cross sections of the vesicles are given above every snapshot. The field direction is indicated with an arrow on the left. The presence of salt in the vesicle exterior causes flattening of the vesicle membrane into disc-like, "square"-like, and tube-like shapes, whereby the overall vesicle shape depends on the conductivity ratio. The scale bars correspond to 15 μm. Reproduced from [16].

Experiments on giant vesicles are of special relevance because their size is comparable to cells and, in addition, it allows for direct observation using optical microscopy. However, microscopy observation of effects caused by electric pulses on giant vesicles is difficult because of the short duration of the pulse. A typical video frequency of 30 frames per second (fps) would provide a snapshot every 33 millisecond. Having in mind that typical pulse duration of 100 μs is about three orders of magnitude shorter, the immediate dynamics of the vesicle response would be indiscernible at such acquisition speed. To deal with this time resolution challenge, recently in our lab, we have used a fast imaging digital camera to record the giant vesicle response with a high temporal resolution, up to 30 000 fps, i.e. acquiring 1 image every 33 μs [2-5]. Thus, some characteristic features of the response and relaxation of the deformation of vesicles when subjected to square wave pulses were revealed.

In the absence of salt, spherical vesicles subjected to electric pulses assume ellipsoidal shapes, which relax back to the initial vesicle shapes after the end of the pulse. The degree of deformation of an ellipsoidal vesicle can be characterized by the aspect ratio of the two principal radii, *a* and *b*. For $a/b = 1$, the vesicle is a sphere, for $a/b > 1$ the vesicle is a prolate (like in Fig. 1b) with the long axis *a* oriented in the direction of the field. The relaxation dynamics of this aspect ratio depends on whether the vesicle has been porated or not.

The typical decay time for the relaxation of non-porated vesicles, τ_1, is on the order of 100 μs [2]. It is defined by the relaxation of the total membrane tension achieved at the end of the pulse, which is the sum of the electrotension σ_{el} and the initial tension σ_0; see Eq. 4. Thus, τ_1 relates mainly to the relaxation of membrane stretching: $\tau_1 \sim \eta_m/\sigma$, where η_m is the surface viscosity of the membrane, $\eta_m \approx 3.5\times10^{-4}$ dyn s/cm (the surface viscosity of a membrane has units [bulk viscosity]×[bilayer thickness]). For membrane tensions of the order of 5 dyn/cm (which should be around the maximum tension before the membrane ruptures) one obtains $\tau_1 \sim \eta_m/\sigma \sim 100$ μs. The latter corresponds to the value experimentally measured.

The relaxation dynamics of porated vesicles is significantly different from the one of non-porated vesicles [2]. Indeed, two different types of dynamics can be distinguished for porated vesicles. The relaxation of vesicles with no excess area is described by a single exponential decay, while vesicles with excess area exhibit two characteristic decay times. These two cases for vesicles above the poration limit are illustrated in Fig. 3. Naturally, vesicles with excess area deform much more than those without (compare the two curves in Fig. 3). The relaxation of porated vesicles completes over a much longer time than that of nonporated ones. For vesicles with no excess area, the relaxation time is $\tau_2 \cong 7 \pm 3$ ms. When the vesicles have some excess area, the relaxation proceeds in two steps, fast relaxation characterized by τ_2, and a second, longer, relaxation with decay time, τ_3: $0.5 \text{ s} < \tau_3 < 3$ s.

The relaxation process associated with τ_2, takes place during the time interval when pores are present (see shaded region in Fig. 3). Thus, τ_2 is determined by the closing of the pores: $\tau_2 \sim \eta_d r_{pore}/(2\gamma)$. Here r_{pore} is the pore radius and γ is the line energy per unit length, $\gamma \cong 10^{-6}$ dyn [27]. For a typical pore radius of 1 μm one obtains $\tau_2 \sim 10$ ms.

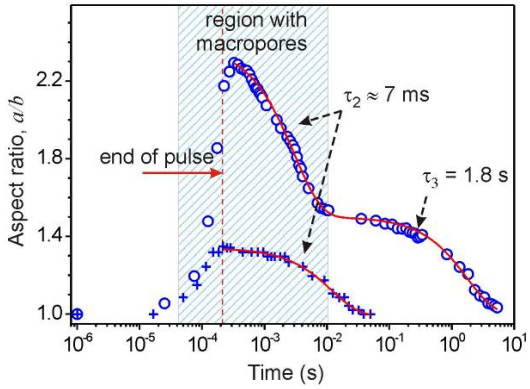

Figure 3: Data from the response and relaxation of two vesicles, in which macropores were observed. One of the vesicles (crosses) did not have excess area ($v = 1$) and the relaxation is described by a single exponential fit (solid curve) with a decay time τ_2. The other vesicle (open circles) had excess area ($v < 1$) and its relaxation is described by a double exponential fit (solid curve) with decay times τ_2 and τ_3 as described in the text. In both cases, the pulse strength was E = 2 kV/cm and the pulse duration was $t_p = 200$ μs (the end of the pulse is indicated with a vertical dashed line). The time $t = 0$ was set as the beginning of the pulse. The shaded area indicates the time interval when macropores were optically detected. The radii of the vesicles were around 10 μm. Reproduced from [16].

The relaxation time τ_3 is related to the presence of some excess area available for shape changes. The latter can be characterized by a dimensionless volume-to-area ratio $v = (3V/4\pi)(4\pi/A)^{3/2}$ where V and A are the vesicle volume and area respectively. This reduced volume v is 1 when the vesicle is a sphere and smaller than 1 in the rest of the cases. The relaxation described by τ_3 is associated with the process of pushing away the volume of fluid involved in the ellipsoidal vesicle deformation compared to a relaxed spherical shape. The restoring force is related to the bending elasticity of the lipid bilayer. Then, the decay time can be presented as

$\tau_3 \sim (4\pi\eta R^3/3\kappa)(1/v - 1)$, where η is the bulk viscosity of media, and κ is the bending elasticity modulus of the membrane, $\kappa \approx 10^{-12}$ erg [28]. Thus, for typical values of v between 0.99 and 0.94 one obtains τ_3 between approximately 0.5 s and 3 s which corresponds excellently to the measured data.

CONCLUDING REMARKS

Giant vesicles as cell-size systems provide a very useful model for resolving the effect of electric fields on lipid membranes. They allow for direct optical microscopy observation of membrane deformations in the micrometer range. We examined the behavior of giant vesicles subjected to AC fields of various frequencies. The solution conductivity appeared to be a major factor determining the overall deformation of the vesicles. It remains to be seen whether similar behavior is found for cells. In many cases, the cell deformation will be mainly determined by the cytoskeleton flexibility rather than the membrane stiffness. However, for cells like leukocytes, it would be interesting to explore the influence of solution conductivity and field frequency on the cell shape.

Until recently, the temporal limit of optical microscopy observations with analogue video technology was in the range of milliseconds. Using fast digital imaging revealed the dynamics of electrodeformation and electroporation of vesicles subjected to DC pulses, as well as electrofusion events with microsecond resolution [1-5,16]. Due to this high temporal resolution, new shape deformations, such as cylindrical ones with square cross section have been detected [3]. In addition, the dynamics of vesicle fusion and, in particular, the opening of the fusion neck became accessible. The observations revealed the presence of two stages of the fusion process [4,5,16]. The expansion of the fusion neck is extremely fast in the beginning and is characterized by rates on the order of a couple of cm/s. For membranes whose thickness is only several nanometers, such a velocity is tremendous. The later stage of fusion is a few orders of magnitude slower and is governed mainly by hydrodynamics. Finally, electrofusion of vesicles with different composition has been demonstrated to be a feasible method for creating multidomain vesicles and provides new opportunities for studying the dynamics of domain formation and stability [5,16].

REFERENCES

[1] R. Dimova, S. Aranda, N. Bezlyepkina, V. Nikolov, K. A Riske and R. Lipowsky, "A practical guide to giant vesicles. Probing the membrane nanoregime via optical microscopy," *J. Phys.: Condens. Matter*, vol. 18, pp. S1151-S1176, 2006.

[2] K. A. Riske and R. Dimova, "Electro-deformation and -poration of giant vesicles viewed with high temporal resolution," *Biophys. J.*, vol. 88, pp. 1143-1155, 2005.

[3] K. A. Riske and R. Dimova, "Electric pulses induce cylindrical deformations on giant vesicles in salt solutions," *Biophys. J.*, vol. 91, pp. 1778-1786, 2006.

[4] C. K. Haluska, K.A. Riske, V. Marchi-Artzner, J.-M. Lehn, R. Lipowsky and R. Dimova, "Timescales of membrane fusion revealed by direct imaging of vesicle fusion with high temporal resolution," *Proc. Natl. Acad. Sci. USA.*, vol. 103, pp. 15841–15846, 2006.

[5] K. A. Riske, N. Bezlyepkina, R. Lipowsky and R. Dimova, "Electrofusion of model lipid membranes viewed with high temporal resolution," *Biophys. Rev. Lett.*, vol. 4, pp. 387-400, 2006.

[6] K. Kinosita Jr., I. Ashikawa, N. Saita, H. Yoshimura, H. Itoh, K. Nagayama and A. Ikegami, "Electroporation of cell membrane visualized under a pulsed-laser fluorescence microscope," *Biophys. J.*, vol. 53, pp. 1015-1019, 1988.

[7] D. Needham and R. M. Hochmuth, "Electro-mechanical permeabilization of lipid vesicles. Role of membrane tension and compressibility." *Biophys. J.*, vol. 55, pp. 1001-1009, 1989.

[8] I. G. Abidor, V.B. Arakelyan, L.V. Chernomordik, Y.A. Chizmadzhev, V.F. Pastushenko and M.R. Tarasevich, "Electric breakdown of bilayer lipid membranes. I. The main experimental facts and their qualitative discussion." *J. Electroanal. Chem.* vol. 104, pp. 37-52, 1979.

[9] S. A. Simon and T.J. McIntosh, "Depth of water penetration into bilayers," *Methods Enzymol.*, vol. 127, pp. 511-521, 1986.

[10] K. Olbrich, W. Rawicz, D. Needham and E. Evans, "Water permeability and mechanical strength of polyunsaturated lipid bilayers," *Biophys. J.*, vol. 79, pp. 321-327, 2000.

[11] G. Niggemann, M. Kummrow and W. Helfrich, "The bending rigidity of phosphatidylcholine bilayers: dependences on experimental method, sample cell sealing and temperature," *J. Phys. II France*, vol. 5, pp. 413–425, 1995.

[12] M. Mitov, P. Méléard, M. Winterhalter, M. I. Angelova and P. Bothorel, "Electric-field-dependent thermal fluctuations of giant vesicles," *Phys. Rev. E.*, vol. 48, pp. 628–631, 1993.

[13] P. Peterlin, S. Svetina and B. Žekš, "The prolate-to-oblate shape transition of phospholipid vesicles in response to frequency variation of an AC electric field can be explained by the dielectric anisotropy of a phospholipid bilayer", *J. Phys.: Condens. Matter.*, vol. 19, pp. 136220, 2007.

[14] H. Hyuga, K. Kinosita, Jr. and N. Wakabayashi, "Transient and steady-state deformations of a vesicle with an insulating membrane in response to step-function of alternating electric fields", *Jpn. J. Appl. Phys.*, vol. 30, pp. 2649–2656, 1991.

[15] H. Hyuga, K. Kinosita, Jr. and N. Wakabayashi, "Steady-state deformation of vesicle in alternating fields," *Bioelectrochem. Bioenerg.*, vol. 32, pp. 15-25, 1993.

[16] R. Dimova, K. A. Riske, S. Aranda, N. Bezlyepkina, R. L. Knorr and R. Lipowsky, "Giant vesicles in electric fields," *Soft Matter*, vol. 3, pp. 817-927, 2007.

[17] U. Zimmermann, "Electric field-mediated fusion and related electrical phenomena," *Biochim. Biophys. Acta.*, vol. 694, pp. 227–277, 1982.

[18] J. L. Griffin, "Orientation of human and avian erythrocytes in radio-frequency fields," *Exptl. Cell Res.*, vol. 61, pp. 113-120, 1970.

[19] F. J. Iglesias, M. C. Lopes, C. Santamaria and A. Dominguez, "Orientation of schizosaccharomyces pombe nonliving cells under alternating uniform and nonuniform electric fields," *Biophys. J.*, vol. 48, pp. 712–726, 1985.

[20] P. Sens and H. Isambert, "Undulation instability of lipid membranes under an electric field," *Phys. Rev. Lett*., vol. 88, pp. 128102, 2002.
[21] H. Hyuga, K. Kinosita, Jr. and N. Wakabayashi, "Deformation of vesicles under the influence of strong electric fields," *Jpn. J. Appl. Phys*., vol. 30, pp. 1141–1148, 1991.
[22] H. Hyuga, K. Kinosita, Jr. and N. Wakabayashi, "Deformation of vesicles under the influence of strong electric fields II," *Jpn. J. Appl. Phys*., vol. 30, pp. 1333–1335, 1991.
[23] E. Neumann, S. Kakorin and K. Toensing, "Membrane electroporation and eletromechanical deformation of vesicles and cells," *Faraday Discuss*. vol. 111, pp. 111-125, 1998.
[24] T. Griese, S. Kakorin and E. Neumann, "Conductometric and electrooptic relaxation spectrometry of lipid vesicle electroporation at high fields," *Phys. Chem. Chem. Phys*. vol. 4, pp. 1217-1227, 2002.
[25] S. Kakorin and E. Neumann, "Electrooptical relaxation spectroscopy of membrane electroporation in lipids vesicles," *Coll. Surf. A*., vol. 209, pp. 147-165, 2002.
[26] J. Teissie and T. Y. Tsong, "Electric field induced transient pores in phospholipid bilayer vesicles," *Biochemistry*, vol. 20, pp. 1548–1554, 1981.
[27] W. Harbich and W. Helfrich, "Alignement and opening of giant lecithin vesicles by electric fields," *Z. Naturforsch*., vol. 34a:, pp. 1063-1065, 1979.
[28] M. Mutz and H. Helfrich, "Bending rigidities of some biological model membranes as obtained from Fourier analysis of contour sections," *J. Phys. France*, vol. 51, pp. 991-1002, 1990.

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NOTES

Design of plasmids for gene therapy clinical trials

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Summary: The past several years have witnessed the evolution of gene medicine from an experimental technology into a viable strategy for developing therapeutics for a wide range of human disorders. Numerous prototype DNA-based biopharmaceuticals can now control disease progression by induction and/or inhibition of genes. These potent therapeutics include plasmids containing transgenes, oligonucleotides, aptamers, ribozymes, DNAzymes, and small interfering RNAs (siRNA) (Patil SD, Rhodes DG and Burgess DJ, The AAPS journal, 2005). Plasmid DNA offers multiple advantages over viral gene therapy vectors, including large packaging capacity, stability without integration and reduced toxicity. Furthermore, plasmid DNA can be delivered to many different tissues, using a variety of delivery techniques such as electroporation.
Plasmids are high molecular weight, double-stranded DNA constructs containing transgenes, which encode specific proteins. On a molecular level, plasmid DNA molecules can be considered pro-drugs that upon cellular internalization employ the DNA transcription and translation apparatus in the cell to biosynthetize the therapeutic entity, the protein. Plasmids contains not only the DNA coding the gene of interest but also sequences corresponding to the regulatory elements such as promoter and enhancer sequences that play an important role in regulating gene expression. Indeed, the therapeutic genes inserted inside the plasmid have to be expressed into the correct cells, at a correct intensity, at the correct time (transitory or long lasting) etc …The facility to direct tissue-specific expression of therapeutic gene constructs is so desirable for many gene therapy applications. In addition, spicing and polyadenylation sites are present in the transgene construct that help in the correct processing of the mRNA generated after transcription. Some vectors also have introns that may increase premRNA processing and nuclear transport.
For a specific application, such as a clinical trial several plasmids used to be tested and compared before selecting the best one.
The different system will be presented with their advantages and disadvantages and their current use in vitro, ex vivo and in vivo.
Different plasmids already used in clinical trials will be discussed.

INTRODUCTION

The success of gene therapy is mostly dependent on the development of the gene delivery method. Recently gene transfection into target cells using naked DNA which is a simple and safe approach has been improved by combinating several physical techniques, for example electroporation (the application of controlled electric fields to facilitate cell permeabilization). Electroporation can achieve short or long lasting expression of the transferred gene and can be used in various tissues. Electro-gene-therapy is currently used for the treatment of cancers, of hereditary pathologies, of cardiovascular diseases and for the prevention of infectious diseases (vaccination).

The therapeutic genes (naked DNA) which have to be transfected *in vivo* are inserted into DNA macromolecules called plasmids. The genes inserted inside the plasmid have to be expressed into the correct cells, at a correct intensity, at the correct time (transitory or long lasting) etc …

STRUCTURE AND FUNCTION OF A GENE: WARMING UP !

Structure:

DNA is a molecule which encodes genetic information. It is a long, coiled, double-stranded chain of interlocking base-pairs called a double-helix, negatively charged. The order of the bases in a DNA strand, called the sequence, creates a code for information: the DNA code. 'ATC' has a different meaning than the code 'TCA,' and so on. Each cell is equipped with special machinery used to read the sequence and use the information encoded.

Human genes show striking variation in size ranging from around 1 kb for the genes coding for β globin and insulin to 2.5 Mb for the dystrophine gene responsible for Duchenne muscular dystrophy (www.oup.co.uk).

Almost all the human genes contain coding regions known as exons, which are expressed, with intervening sequences, known as introns, which are not expressed. Introns are transcribed into primary RNA, also known as pre-mRNA in the cell nucleus but are spliced out of the mature mRNA in the

cytoplasm. For gene therapy protocols, genomic DNA (exons and introns) or cDNA (only exons) can be used.

In addition to exons and introns, each gene contains a closely adjacent upstream (5') regulatory promoter region and other regulatory sequences including enhancers, silencers, and sometimes a locus control region. These sequences are necessary for the expression of the gene of interest as they provide binding sites for transcription factors. The enhancer and silencer sequences fulfil a similar purpose, but are located at a greater distance from the coding sequences.

The first and last exons contain unstranslated regions known as the 5' UTR and 3' UTR respectively. The 5' UTR marks the start of transcription and contains an initiator codon which indicates the site of the start of translation. The 3'UTR contains a termination codon, which marks the end of translation, plus nucleotides which encode a sequence of adenosine residues known as the poly(A) tail (www.oup.co.uk).

Function :

The process linking DNA and its protein product is complex. For convenience this is usually considered as consisting of two stages, transcription and translation. Transcription involves the conversion of DNA to RNA. Translation refers to the synthesis of a polypeptide chain from RNA.

Transcription is a two steps process. First transcription begins at a transcriptional initiation site and involves the synthesis of a single strand of messenger RNA (mRNA) by RNA polymerase. This primary transcript is then processed to produce the mature mRNA for transportation into the cytoplasm. Processing involves the splicing out of the non-coding regions (introns) and the addition of a protective 5' guanine nucleotide to form a cap and of adenine residues at the 3' end to form a poly A tail. This processing is also called post transcriptional modifications.

In the case of a few genes, the RNA copy of the gene has a function on its own, usually by folding into a distinctive shape. We can mentionned as example the siRNA which will be presented at the end of the course. However, the majority of genes encode instructions for making specific proteins. The mRNA passes through the nuclear membrane to the ribosome in the cytoplasm, where it provides a template for the synthesis of a polypeptide chain in a process called translation. Translation always begins at a codon for methionine (AUG) which is referred to as the initiator codon and determines the reading frame of the mRNA. Three combinations (UGA, UAA and UAG) specify stop codons. These signal the end of the translation (www.oup.co.uk).

The polypeptide chain is then process through its passage to the endoplasmic reticulum, golgi asparatus etc … It could be glycosylated, phosphorylated, isoprenylated etc … All these process are needed to obtain a functional protein. There are called post translational modification.

DNA FOR ELECTROPORATION-BASED GENE THERAPY

Preparing DNA to be provided to the researchers involved in electroporation-based gene therapy is called genetic engineering. It involves the isolation, manipulation and production of DNA. Molecular cloning refers to the procedure of isolating a defined DNA sequence and obtaining multiple copies of it using prokaryots. DNA sequences are cloned into vectors. Those are most of the time plasmids which are high molecular weight, double-stranded DNA (up to 200 kb) constructs which usually contain at least an origin of replication, a sequence element capable of directing the propagation of itself and any linked sequence, and a gene coding for an antibiotic resistance. Cloning is frequently employed to amplify DNA fragments containing genes, but it is also used to amplify any DNA sequence such as promoters, non-coding sequences called enhancers, cis regulatory sequences, antibiotic response elements etc

The Plasmid design involves the cloning of DNA encoding :

- the gene of interest (the coding sequence) which can be genomic DNA or cDNA.
- Several regulatory signals (promoter and enhancers sequences)
- Splicing and polyadenylation sites
- Plasmids could be simple (one gene) or complex (several genes).

After genetic engineering, plasmids :

- should encode specific proteins and in this case plasmid DNA = prodrug which can correct genetic error or produce a drug (cytokine, growth factor etc …)
- or be transcripted in siRNA which can inhibit a cellular gene expression (knock down)

Regulatory signals:

Efforts have to be made to ensure that expression of therapeutic genes is restricted exclusively to the tissue of interest. This is particularly important for example for suicide gene strategies in which low level expression of toxic genes in normal tissues may lead to severe toxicity. A possibility to target gene expression to a specific tissue is to control gene

expression very tightly at the transcriptional level. It is mediated by the interaction between enhancer/promoters elements in the DNA and specific proteins which bind to them and which are present in the tissue (www.aaps.org/Siddhesh et al.). We can subclone in a plasmid and upstream of the open reading frame of the gene of interest :

- Constitutive promoters
- Tissue specific promoters
- Tumor specific promoters
- Inducible promoters
- Customized promoters.
- Each type of promoters have advantages and disadvantages depending on the purpose of the gene therapy protocol. The choice of the promoter is so crucial for success of gene therapy protocols.

Combination of coding sequences:

Several genes can be combined in the same plasmid.

They could be separated by :

- individual sequences (double or triple cassettes): these plasmids contain different independent cassette, each composed of a promoter, a coding sequence and a terminator.
- IRES sequences (internal ribosomal entry site): In this case the different coding sequences are transcribed from an unique promoter while IRES allow independent translation of each sequences.

Reporter gene:

In plasmids containing combination of gene, most of the time we clone the gene of interest (therapeutic gene) and a reporter gene. The reporter gene allows the researchers (or the clinicians) to follow transgenes expression and localization by the expression of an easy detectable protein such as a colour (green, red, blue, yellow) or an activity (chloramphenicol acetyl transferase). Indeed as mentioned already above, the genes inserted inside the plasmid and electroporated have to be expressed into the correct cells, at a correct intensity, at the correct time (transitory or long lasting) etc …Reporter genes allow researchers to verify these items.

KNOCK DOWN: DOWN REGULATION OF DISEASE-CAUSING GENES (RNA INTERFERENCE)

Principle:

The aim of a gene therapy trial could also be to suppress gene expression of a cellular targeted gene. A sequence-specific post-transcriptional gene silencing mechanism triggered by dsRNA (siRNA) causes degradation or inhibition of the translation of mRNAs homologous in sequence to the dsRNA.

The siRNA are short double strand RNA segments with typically 21-23 nt based complementary to the RNA sequence of the protein whose transcription is to be blocked. These short double strand RNA could be obtain from a plasmid which contains specific promoter recognized by RNA polymerase III, a DNA sequence encoding the short double strand RNA and a terminator. These plasmids are called shRNA plasmids. They are used as classical plasmids. Their expression induces the down regulation of the expression of the targeted gene.

CONCLUSION

The different system will be presented with their advantages and disadvantages and their current use *in vitro*, *ex vivo* and *in vivo*.

Different plasmids already used in clinical trials will be discussed.

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REFERENCES

[1] Siddhesh et al. The AAPS journal 2005 http://www.aapsj.org
[2] Bessis, N Gene Therapy 2004 11 S10-S17
[3] www.oup.co.uk
[4] www.wikipedia.com
[5] Scheule, RK : Adv Drug Deliv. Rev 44 (2000) 119-134
[6] Harrington, K : Adv Drug Deliv. Rev 44 (2000) 167-184
[7] Niidome and Huang : Gene Therapy (2002) 9 : 1647-1652

NOTES

Measurement of blood flow in tissues

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Abstract: A plethora of different methods based on different physical principles exist which can be used for measurement of blood flow in tissues on all levels from single large vessels to local tissue perfusion. No single measurement method is applicable to all levels of blood flow. This text provides a brief overview of some measurement techniques which are used in clinical and experimental setups.

INTRODUCTION

The circulatory cardiovascular system evolved as an adaptation to the increasing size and complexity of evolving organisms. Blood flow is an efficient convection system which provides a rapid transport and distribution of molecules and cells within the body. The circulatory system consists of three elementary parts: the heart, the vessels, and the blood. The demands imposed on the circulatory system fluctuate tremendously due to many factors (e.g. sleep versus strenuous activity). Hence the very complex regulation of blood flow to ensure adequate blood supply to tissues under variable conditions [1].

The roles of the circulatory cardiovascular system include: a) delivery and distribution of gases and nutrient molecules and removal of waste products (the primary role); b) transport of hormones and neurotransmitters (chemical signalling among cells and tissues); c) regulation of temperature (by carrying the blood from the core to the surface of the body); d) transport of molecules and cells responsible for inflammatory and defence response against infection.

The range of blood flows encountered in different blood vessels and tissues spans over more than nine orders of magnitude (Table 1). No single measurement method can be used over the whole range of flows. Thus different methods based on various physical principles exist which are applicable to different ranges of flow and different goals [3].

PROPERTIES OF BLOOD FLOW

In blood flow measurements we usually study either the flow in a single vessel expressed as volume (or mass) flow rate or the *perfusion* of tissue measured as flow rate per unit mass of tissue. Commonly used units in physiological measurements are ml/min and ml/min/100g respectively. Flow in a vessel can also be expressed in terms of velocity (e.g. in cm/s). For uniform flow the flow rate Q and flow velocity v are related as $Q = S \cdot v = \pi r^2 v$. S and r are the internal cross-section and radius of the vessel.

Blood flow velocity is never uniform in any vessel therefore there is always a distribution of velocities over the vessel cross-section. For steady laminar flow in a straight long tube the velocity profile is parabolic in shape. Blood flow in some vessels can be considered *laminar* under steady conditions. When not steady (e.g. in larger arteries, or near obstructions or bifurcations), the flow becomes *turbulent*. The dimensionless *Reynold's number Re* for the flow inside a vessel with diameter d, blood density ρ, viscosity μ and mean velocity $\bar{v}$ is defined as:

$$Re = \frac{\rho \bar{v} d}{\mu}. \qquad (1)$$

This number is a measure of general tendency for laminar ($Re < 200$) or turbulent flow ($Re > 2000$).

Tissue blood flow (perfusion) differs greatly for various tissues and physiological conditions. Some tissues are relatively uniformly perfused while others are not. Many malignant tumours, for example, exhibit great intra- and intertumoural variability in perfusion. Fig. 1 shows typical ranges for tissue blood flow in different organs.

Table 1: Typical mean values of circulatory parameters for a single vessel in humans [1,2].

vessel type	radius (cm)	flow (ml/s)	velocity (cm/s)	pressure (mmHg)
aorta	1.13	83	21	100
small artery	0.05	0.01	1.3	90
arteriole	15×10^{-3}	4×10^{-6}	0.6	60
capillary	3×10^{-3}	8×10^{-9}	0.03	17
vena cava	1.38	83	14	1

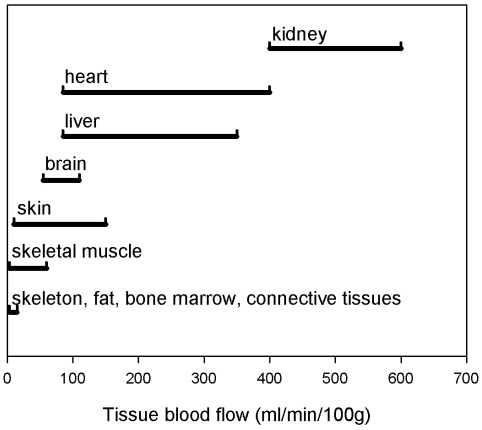

Figure 1: Physiological ranges of blood flow in tissues [3].

BLOOD FLOW IN SINGLE VESSELS

Electromagnetic flowmeters

The electromagnetic flowmeters are based on the electromagnetic induction principle. According to Faraday's induction law, moving a conductor (e.g. an electrolyte solution such as blood in a vessel) with velocity **v** in the direction perpendicular to the magnetic flux **B** induces a voltage difference between two electrodes placed perpendicularly to both the direction of movement and the direction of magnetic field as shown in Fig. 2. The induced voltage is:

$$v = |\mathbf{v}|, B = |\mathbf{B}| \rightarrow U = d \cdot v \cdot B, \qquad (2)$$

where d is the distance between the electrodes. Equation (2) is valid if the velocity profile is axisymmetrical about the longitudinal axis of the vessel. The flow rate can be expressed as:

$$Q = \frac{\pi d^2}{4} v = \frac{\pi d U}{4B}, \qquad (3)$$

where Q is expressed in m^3/s, d in meters (m), U in volts (V), and B in tesla (T).

Even though the principle of electromagnetic flowmeters is simple, there are important factors which significantly affect the sensitivity and accuracy of the method: the velocity profile, inhomogeneity of the magnetic field, and electric conductivities of the vessel wall and the media inside and outside the vessel. In modern electromagnetic flowmeters the magnetic field is produced with AC-driven electromagnets with excitation frequency typically in the 100 to 1000 Hz range.

Various types of probes and magnetic field excitation approaches exist. The perivascular probes (typically in the shape of a ring) exist for vessels down to 0.5 mm in diameter. The clinical use of these probes is mostly limited to surgical procedures. The catheter-type intravascular probes are designed for monitoring of blood velocity in larger vessels. For these two types of invasive probes the coils for magnetic field excitation and the electrodes are incorporated in the probe. However, the external magnetic field excitation from the outside of the body can also be applied. In this case the flow signal can be obtained by either intravascular electrodes or by surface electrodes (noninvasively) [3].

Ultrasonic flowmeters

The ultrasonic flowmeters are based on the principle that a sound wave propagating in a moving media (blood) is affected by the velocity of the medium and that the sound wave scattered on a moving particle is also affected by the velocity of this object [3]. The velocity of ultrasound in soft tissues is about 1500 m/s. The relationship between the sound velocity c (m/s), frequency f (Hz) and wavelength λ (m) is $c = f\lambda$. When ultrasound propagates in medium it is attenuated by absorption, scattering and reflection. Attenuation of ultrasound of frequency 1 MHz in soft tissues is typically about 1 dB/cm and in blood 0.18 dB/cm but it is much stronger in bone – 15 dB/cm.

Piezoelectric transducers are commonly used to generate and detect ultrasound.

Transit time and phase shift ultrasonic flowmeters

The apparent velocity of sound in a moving medium is different from the velocity in resting fluid because the velocity of sound wave is constant only relative to the medium (which is moving with its own velocity). For the basic arrangement in Fig. 3 and for uniform velocity profile the transit time T needed for the sound to travel between the transmitter and receiver is (± corresponds to downstream/upstream propagation of sound wave):

$$T = \frac{D}{c \pm v\cos\theta}. \qquad (4)$$

Therefore the *transit time difference* between upstream and downstream propagation of sound is:

$$\Delta T = \frac{2Dv\cos\theta}{c^2 - v^2\cos^2\theta} \xrightarrow{v \ll c} \simeq \frac{2Dv\cos\theta}{c^2}. \qquad (5)$$

The *phase difference* caused by this transit time difference is then:

$$\Delta\phi = \omega\Delta T. \qquad (6)$$

Various approaches are used to detect the transit time or phase differences. Corrections can be made to account for nonuniformity of the velocity profile.

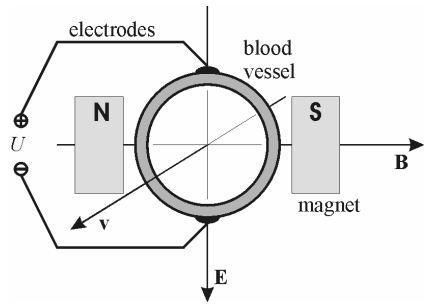

Figure 2: The principle of electromagnetic flowmeter. Vectors of magnetic field **B**, flow velocity **v** and induced electric field **E** are mutually perpendicular.

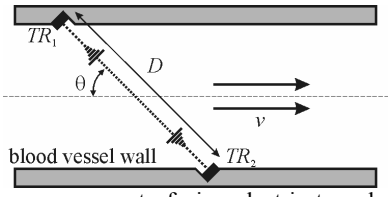

Figure 3: The arrangement of piezoelectric transducers TR_1 and TR_2 for transit time or phase shift measurement of blood velocity in a single vessel.

With this type of ultrasonic flowmeters the sound beam is narrower than the diameter of the vessel and so the velocity rather than volume flow is measured. However, by using much larger transducer crystals we can obtain a uniform ultrasonic beam wider than the vessel diameter. In this case it is possible to measure the total volume flow in the vessel directly.

Ultrasonic Doppler flowmeters

When ultrasound in blood scatters on moving particles (red blood cells), the apparent frequency of scattered sound waves is changed. This is the so-called Doppler shift effect and can be used in flowmetry. For a particle moving with velocity v, and the arrangement of the transducers as shown in Fig. 4, the difference between the source and received frequencies is proportional to velocity of the particle:

$$\Delta f = f_r - f_s \simeq \frac{2v\cos\theta}{c} f_s \,. \qquad (7)$$

The Doppler shift Δf is very small. For example, $f_s = 5$ MHz, $c = 1.5\times10^5$ cm/s, and $\theta = 45°$ result in $\Delta f = 4.7$ kHz. Since there are many moving particles in the blood stream, in real situation we are always dealing with a *distribution* of Doppler shifts. Various methods for demodulation exist which can be used to obtain the velocity profile from the Doppler-shifted signal. The extraction of velocity profile also involves the spectrum analysis of the demodulated signal.

The major advantage of the ultrasound Doppler flowmeter is the ability to measure blood flow noninvasively. To measure the signal from a specified vessel and not from other surrounding vessels the so-called *range-discriminating* Doppler flowmeter must be used. Range discrimination is usually achieved by using either the so-called *pulse Doppler system* or the *random-signal Doppler system*. The most effective way of using Doppler flowmeters is in the *duplex scanner systems* in which the principles of flowmetry are combined with *ultrasound scanning imaging*.

Ultrasound Doppler flowmetry can also be performed invasively using either perivascular or intravascular (catheter-type) probes.

Indicator dilution method

This is a broad family of methods which involve an injection of an indicator into the bloodstream. The average blood flow is calculated from the time profile of the concentration of the indicator somewhere downstream from the injection site. Various indicators can be used such as dyes, radioactive isotopes, electrolytes, gases and heat. The injection is performed either very rapidly (e.g. bolus injection) or at a steady rate (constant injection) [3].

After bolus injection the concentration of the indicator at a downstream location will first rapidly increase and then decrease following the *indicator dilution curve* as shown in Fig. 5. The subsequent increases in the dilution curve are due to the recirculation of the indicator. In ideal situation the indicator does not leak from the system and there is no recirculation component present. If the blood flow rate Q is constant and the amount of injected indicator is I, the indicator dilution curve $c(t)$ is used to calculate blood flow rate:

$$Q = \frac{I}{\int_0^\infty c(t)dt} \,. \qquad (8)$$

In reality the recirculation of the indicator is inevitable therefore steps must be taken to minimize its effect on the calculation.

In the constant injection method the indicator is injected at a constant rate. After some time the concentration of indicator at a downstream site reaches an equilibrium value C. The flow Q can then be calculated from the injection rate J and the equilibrium concentration as:

$$Q = \frac{J}{C} \,. \qquad (9)$$

For this method to be valid the equilibrium must be reached before the first recirculation occurs.

In the *dye dilution technique* biological dyes such as *Indocyanine green*, *Evans blue*, and *Coomassie blue* can be used as the indicator and the dilution curve is determined optically by monitoring the absorption of light in blood at one or more wavelengths while taking into account the specific absorption coefficient of the dye used.

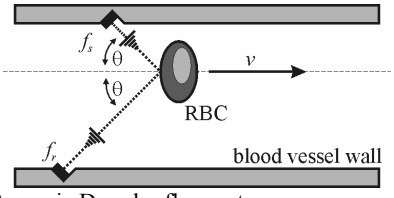

Figure 4: Ultrasonic Doppler flowmetry.

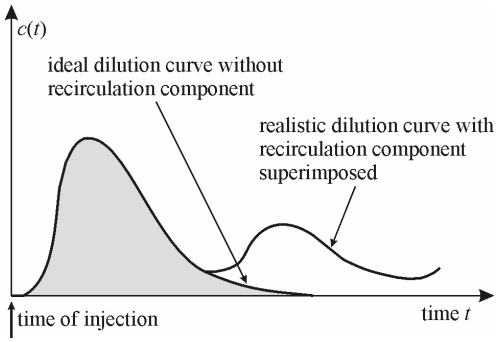

Figure 5: The dilution curve (concentration of the indicator) after bolus injection.

In the *thermodilution method* either hot or cold fluid can be used as the indicator and the corresponding temperature change is measured somewhere downstream. The method has several advantages over the use of dyes including complete nontoxicity and relative simplicity of measurement. A typical application is cardiac output measurement by using a special catheter-like probe which incorporates the injection and measurement parts.

The *Fick method* is yet another dilution method where gas is used as the indicator. Pulmonary blood flow is determined by measuring either gas uptake or extraction in the lung and the concentration change in the blood flowing though the lung. In the so-called direct Fick method oxygen is used as the indicator. The blood flow through the lung Q is calculated from oxygen uptake $\dot{V}_{O_2}$ and concentration of oxygen in pulmonary venous and arterial blood (C_{vO_2} and C_{aO_2} respectively) as:

$$Q = \frac{\dot{V}_{O_2}}{C_{aO_2} - C_{vO_2}}, \qquad (10)$$

This so-called *direct* Fick method is a very reliable method of cardiac output measurement. Its major disadvantage is that it requires cardiac catheterization for sampling of venous blood so it is not suitable for frequent measurements. The so-called *indirect* Fick method uses CO_2 as the indicator and is not as reliable but it does not involve invasive procedures.

BLOOD FLOW IN TISSUE

Methods described in this section are used to measure the amount of blood perfusing the bulk of a given organ or its part. Various methods exist and the selection of the most appropriate one depends on the requirements such as the tissue sampling volume (object size), spatial and temporal resolution of the measurement, and absolute or relative accuracy [3].

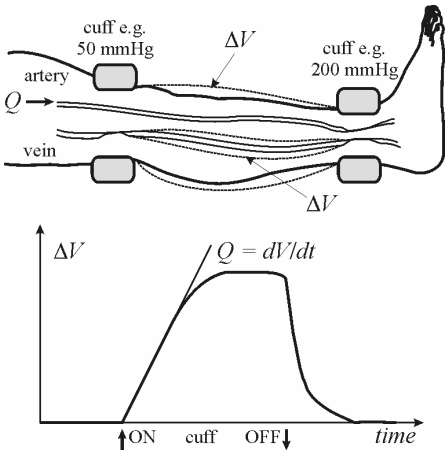

Figure 6: The principle of occlusion plethysmography.

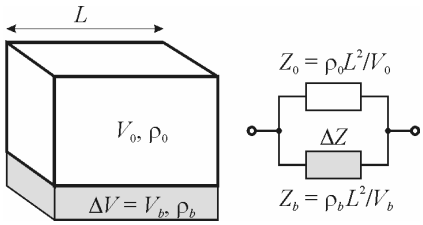

Figure 7: The parallel conductor model of a limb (or segment of a limb) assumed in impedance plethysmography.

Venous occlusion plethysmography

Plethysmography methods rely on the measurement of the tissue volume change caused by the blood flow in the tissue. This is a widely used method which yields quantitative information about blood flow in a limb or part of the limb. Its principle is illustrated in Figure 6. Two pressure cuffs are used. The distal cuff is inflated to a pressure above the maximal arterial pressure to completely block blood flow in the distal part of the limb. The proximal cuff is inflated to a pressure below the minimal arterial pressure so that only venous blood flow is occluded. The result is swelling of the tissue between the cuffs due to the arterial inflow and total blood flow Q can easily be determined by taking the derivative of the volume change curve ΔV in its initial linear phase.

The displacement of skin surface and hence ΔV caused by tissue swelling can be measured in variety of ways for example in water- or air-filled plethysmographs and capacitance plethysmo-graphs.

In *impedance plethysmography* the change in tissue volume is measured by the change in electrical impedance. A weak AC current (e.g. 1 mA) of constant amplitude and frequency is applied to the surface of the skin and the voltage drop is measured using another pair of electrodes thus enabling calculation of the impedance Z (Ω) or admittance Y (S). Impedance plethysmography measurement is based on on the parallel conductor model in Fig. 7 where V_0 is the initial volume of the limb and V_b is the blood volume increase due to venous occlusion.

For small changes in the impedance $\Delta Z \ll Z_0$, length of the limb L and known resistivity of blood ρ_b ($\Omega \cdot \mathrm{cm}$) the volume change equals

$$V_b = \frac{\rho_b L^2}{Z_0^2}\Delta Z \quad \text{or} \quad V_b = \rho_b L^2 \Delta Y . \qquad (11)$$

Clearance methods

When tissue containing some indicator is perfused by blood of lower indicator concentration, the indicator is washed out into the venous blood. Therefore the perfusion rate Q can be calculated from the time course of the indicator concentrations in venous and arterial blood c_v and c_a, because the change in the amount of indicator in tissue dI can be expressed at any time as:

$$dI = Q\left(c_a - c_v\right)dt . \qquad (12)$$

If the indicator is completely removed from the arterial blood before recirculation ($c_a = 0$), then the tissue concentration (equal to c_v for quickly diffusing indicator molecules), decreases exponentially and blood perfusion can be estimated from the rate of this decrease without absolute measurement of the indicator. Inert gases such as H_2 and N_2O can be used. Tissue perfusion can also be estimated by increasing the concentration of the indicator in the tissue. In this case the indicator must be delivered continuously into the arterial blood so long that the tissue concentration equilibrates with the arterial concentration.

Apart from inert gases some radioactive indicators such as isotopes of xenon ($^{133}\mathrm{Xe}$) and krypton ($^{85}\mathrm{Kr}$ and $^{79}\mathrm{Kr}$) are routinely used in various clearance applications for measurement of blood flow in brain, skin or muscle. The advantage of radioactive tracers is that their concentration can be measured externally using for example a gamma counter or emission computed tomography (ECT).

Tissue blood flow can also assessed by measuring the heat transfer. A localised region of tissue is either cooled or heated using a special probe. Blood in microcirculatory network acts as a very good heat exchanger and once the equilibrium between the blood and tissue temperature is established, blood flow perfusion rate can be calculated from

$$H = \rho c Q (T_t - T_a) , \qquad (13)$$

where H is the rate oh heat transfer (equal to power consumption of the heater), ρ and c are density and specific heat of the blood, Q is the flow rate, and T_t and T_a are tissue and arterial blood temperatures.

Laser Doppler flowmetry

Laser Doppler flowmetry and imaging (LDF and LDI) encompass methods for continuous and noninvasive monitoring of tissue microcirculation. LDF is based on the principle of Doppler shift (see Fig. 8) of monochromatic laser light which occurs in tissue due to scattering of photons on moving particles, predominantly the red blood cells (RBCs). LDF is used both in clinical and experimental setups. The areas of application include dermatology, transplantation and plastic surgery, gastrointestinal surgery, experimental oncology and other studies involving skin perfusion.

The interaction of light with tissue is a complex phenomenon and because of the heterogeneous structure of tissue it is also very difficult to study. Photons in tissue can undergo a variety of interactions with tissue components, the most important being absorption and scattering. Scattering occurs on all boundaries between media of different refractive indices. The nature of scattering depends very much on the relative size of the tissue particles in comparison to the wavelength of incident light.

The frequency (Doppler) shift Δf resulting from the relative motion of a scattering particle with respect to the source of electromagnetic radiation is:

$$\Delta f = \frac{v}{c} f_0 , \qquad (14)$$

where v is the relative velocity of the particle, c is velocity of light in the medium and f_0 is the unshifted frequency. Velocity of RBCs is in tissue is many orders of magnitudes below the velocity of light therefore only tiny Doppler shifts can occur which are not easy to measure. LDF is very sensitive method and offers excellent temporal and spatial resolutions (typically 1 mm^3).

The source of light in LDF instruments is usually the gas or the semiconductor laser. Optical fibres arranged in different configurations are used to bring the light to tissue and then back to the sensor, which usually consists of photomulipliers or semiconductor photodiodes. Different approaches to signal processing can be used to derive the blood flow signal from the recorded Doppler signals.

A disadvantage of LDF methods is lack of a "golden standard" tissue flow measurement which could be used for calibration of LDF instruments for all different tissues. This is why most LDF measurements do not provide blood perfusion

information in absolute units of flow but rather in dimensionless "perfusion units".

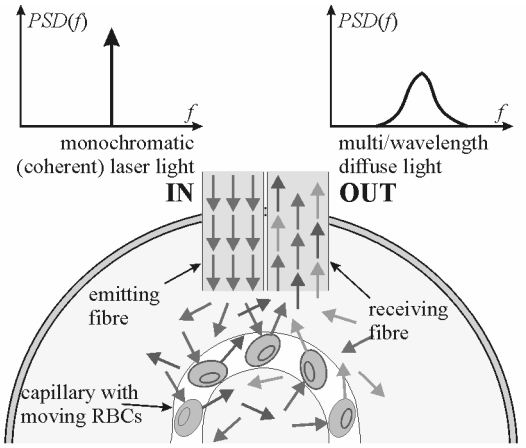

Figure 8: The principle of laser Doppler flowmetry.

A variant of the LDF technique is the LD imaging which can scan a larger area of tissue (normally skin) thus producing a 2D image of perfusion instead of a single measurement. But this method is inherently slower and cannot be used to monitor rapid changes in local perfusion.

Near-infrared spectroscopy

Near-infrared spectroscopy of tissue (NIRS) is a family of optical methods which were originally developed for noninvasive monitoring of brain oxygenation but are now used for measurement in different soft tissues. NIRS is based on two basic principles: the relative transparancy of tissues for near-infrared light; and the oxygenation/oxidation-dependent absorption of light by certain compounds in tissues (e.g. hemoglobin). The basic principle behind NIRS is described by the modified Beer-Lambert law:

$$A(\lambda) = \log_{10}\frac{I_0}{I} = \alpha(\lambda)\cdot c\cdot B\cdot d + G\,. \qquad (15)$$

Equation (15) describes a situation where a beam of light with intensity I_0 is delivered to a scattering and absorbing medium such as tissue. Some of the light with intensity I re-emerging from the tissue is measured. Then the total attenuation of light A at wavelength λ can be expressed as a sum of a term describing the light loss due to absorption of a known chromophore (e.g. oxygenated hemoglobin) with specific attenuation coefficient α and concentration c, and a term G accounting for all other losses of light at the distance d between the transmitter and receiver. Term B is the optical pathlength factor which accounts for average lengthening of the path travelled by the received photons due to multiple scattering events in the tissue. Equation (15) can be expanded into a system of n equations at n wavelengths, if n different chromophores are being observed (e.g. oxygenated and deoxygenated hemoglobin). Thus the changes in concentrations of these chromophores can be expressed as a linear combination of changes in attenuation of light at different wavelengths.

NIRS can be used for measurement of oxygenated and deoxygenated hemoglobin concentrations in blood and can thus also be used for indirect measurement of blood volume and flow in tissue [4]. Tissues such as skin, brain, and muscle can be accessed noninvasively.

Nuclear magnetic resonance

The phenomenon of nuclear magnetic resonance (NMR) is observed when certain nuclei having magnetic moment are first aligned with a strong static magnetic field and then perturbed by an alternating excitation magnetic field with a specific frequency perpendicular to the static field. Under appropriate conditions the resonant magnetic field signal emitted by the excited nuclei picked by a coil perpendicular to both the static and the excitation magnetic field coils will depend on the velocity of the nuclei and will thus enable measurement of the flow. NMR has numerous clinical and experimental applications. In blood flow measurements the hydrogen nuclei ^{1}H are observed because of their abundance in water and hence in blood.

Either continuous or pulsed wave excitation can be used in NMR measurements of blood flow. The principles of NMR can also be implemented in NMR imaging techniques which can be used for 2D or 3D spatial distribution of tissue blood flow.

FURTHER READING

[1] W.F. Boron, E.L. Boulpaep, *Medical physiology*. Philadelphia, Saunders, 2003.

[2] A.C. Guyton, J.E. Hall, *Textbook of medical physiology (9th ed.)*. Philadelphia, Saunders, 1996.

[3] T. Togawa, T. Tamura, P.Å. Öberg, *Biomedical transducers and instruments*. Boca Raton, CRC Press, 1997.

[4] B. Chance, C.E. Cooper, D.T. Delpy, E.O.R. Reynolds (editors), *Phil. Trans. R. Soc. Lond. (special issue on Near infrared spectroscopy and imaging of living systems)*, vol. 352 no. 1354, pp. 643-761, 1997.

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NOTES

Electrochemotherapy in Veterinary Oncology

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Abstract: Electropermeabilization is a method that uses electric field pulses to induce an electrically mediated reorganization of the plasma membrane of cells. Electrochemotherapy combines local or systemic administration of chemotherapeutic drugs bleomycin or cisplatin that have poor membrane permeability, with electropermeabilization, by direct application of electric pulses to the tumors. Preclinical studies have demonstrated excellent antitumor effectiveness of electrochemotherapy on different animal models and various tumor types, minimal toxicity and safety of the procedure. Based on results of preclinical studies the clinical studies that were conducted in human cancer patients, have demonstrated pronounced antitumor effectiveness of electrochemotherapy with 80 to 85% objective responses of the treated cutaneous and subcutaneous tumors, predominantly in treatment of progressive disease in patients with different malignancies. Clinical studies in veterinary oncology have demonstrated that electrochemotherapy is very effective in treatment of cutaneous and subcutaneous tumors of different histology in cats, dogs and horses. Results of these studies have also demonstrated ~ 80% long lasting objective responses of the electrochemotherapy treated tumors. Predominantly primary tumors of different histology were treated. Electrochemotherapy in veterinary oncology proved to be highly effective treatment, which can be used for treatment of cutaneous and subcutaneous tumors of different histologies, either for primary tumors or recurrent and metastatic disease.

INTRODUCTION

Electropulsation is the direct delivery of electric pulses on cells. Under controlled conditions, it brings a targeted permeabilization of the cell membrane (electropermeabilization, electroporation) [1, 2]. This is true not only for cells in culture but can be used *in vivo* by the direct electric field pulse delivery on the organ or across the skin of the animal or patient. It has, therefore, received much attention in the last 15 years as an emerging new way for increased delivery of chemotherapeutic drugs into the cells of different types of tumors *in vivo* [3-7]. Application of electric pulses to the tumors causes a transient permeabilization of the plasma membrane and thus allows exogenous chemotherapeutic drugs to enter the cells. This therapy was named electrochemotherapy [2]. Clinical studies performed in veterinary medicine started soon after the beginning of the first clinical trials in human clinical oncology. To date, more than 10 papers have been published dealing with electrochemotherapy in treatment of companion animals and horses.

CLINICAL STUDIES ON ELECTROCHEMOTHERAPY

In the first veterinarian clinical trial, conducted in 1997, 12 cats with spontaneous large soft-tissue sarcomas and relapse after treatment with conventional therapies were treated with electrochemotherapy associated with immunotherapy consisting of intratumoral injection of $30x10^6$ xenogeneic CHO (IL-2) living cells, which makes this study significantly different from the other studies. Electrochemotherapy combined bleomycin injected intravenously followed by application of electric pulses. This treatment approach resulted in one partial response and disease stabilization lasting from 2 weeks to 7 months for the others [8].

In 2001, groups from Ljubljana and Toulouse reported the successful use of electrochemotherapy with cisplatin for the treatment of tumors in dogs, cats and horses [9, 10]. In the study that used electrochemotherapy with cisplatin in dogs and cats, cutaneous and subcutaneous tumors of various histological types were included. Three cats with mammary adenocarcinoma and fibrosarcoma, and 7 dogs with mammary adenocarcinoma, cutaneous mast cell tumour, hemangioma, hemangiosarcoma, perianal adenocarcinoma and neurofibroma were treated with electrochemotherapy. Seven patients were treated with electrochemotherapy (19 nodules); five dogs responded completely to the treatment, one with partial response and one (cat with mammary adenocarcinoma) with progressive disease. On the other hand, only one patient that received cisplatin alone, responded partially, while the other two did not respond to treatment [9].

Electrochemotherapy also proved to be a very successful treatment of equine sarcoids. Only cisplatin was used in horses because of its broad spectrum [11]. These non-metastatic skin tumors are commonly treated with cisplatin intratumorally; however the

main disadvantage of this treatment is the poor drug diffusion into the tumor even when the drug is dissolved in oil. Combination of intratumorally injected cisplatin (multisided injection) and subsequent application of electric pulses using contact electrodes increase cisplatin concentration in sarcoids, thus enhance cytotoxic effect and consequently increase treatment effectiveness. In the first report, complete regression of 10 sarcoids was observed in all 3 horses treated with electrochemotherapy with cisplatin. Two years after the last electrochemotherapy no relapse of treated sarcoids was observed [10].

In most of the studies on electrochemotherapy in companion animals, cisplatin was used as the chemotherapeutic drug. In these cases, electrochemotherapy was used as single treatment and not as an adjuvant treatment. Only recently, studies using intratumorally injected bleomycin were performed either alone or as an adjuvant treatment to surgery.

Results of study on electrochemotherapy with bleomycin injected intratumorally in companion animals (9 dogs, 11 cats) with spontaneous tumors of different histological types were reported in 2003. Electrochemotherapy had a high response rate (overall response > 80%) and was superior to the group of patients treated only with intratumoral injection of bleomycin ($p < 0.01$) [12]. In another study ten dogs with locally aggressive spontaneous oral melanomas were treated by electrochemotherapy with bleomycin intratumorally. High effectiveness was observed with 80% overall response rate and 50% long-term control, however, survival rate was not improved compared to other treatment options and was less that 20% at 3-years observation periods. Other treatment options for this type of tumor include wide surgical excision and radiotherapy. Chemotherapy and immunotherapy were tested as adjuvant treatments or with palliative intent, however with no positive results [13]. Electrochemotherapy proved to be very effective also for treatment of mast cell tumors in dogs, where it was used as an adjuvant to surgery. Twenty-eight dogs with incompletely removed mast cell tumors were treated with intratumoral injection of bleomycin followed by application of electric pulses. The overall response rate in this study was 85% with a mean estimated time to recurrence of 52.76 ± 6.5 months, median survival time had not been reached by the time article was published [14]. This result is, compared to literature data available for recurrence rate after surgical treatment alone, very encouraging, since the recurrence rates after surgical excision are ranging from 22-45% [15, 16]. However, a controlled randomized study need to be performed to fully confirmed these findings. A case report described a successful electrochemotherapy treatment of a cat with haemangioperycitoma. The cat, which was previously unsuccessfully treated with two surgical resections, was treated with electrochemotherapy using intratumoral injection of bleomycin. Electrochemotherapy was repeated one week after the first treatment session to improve local control, and one year after, the cat was still in complete response [17]. Another case report was dealing with feline lingual squamous cell carcinoma that was treated with electrochemotherapy with cisplatin. The treatment of choice at early stages is radical surgery, but due to the location of the tumor and also the fact that the cats do not tolerate radical lingual surgery as well as dogs, the outcome is usually poor. The cat stayed stable until about 4 months after the treatment, which is long term response compared to cryotherapy, where partial response of the tumors is also observed [18].

Another use of electrochemotherapy as adjuvant treatment to surgery was described for feline soft tissue sarcomas. Electrochemotherapy was performed intra- or postoperatively. The median time to recurrence was 4 months for cats treated with surgery alone, 19 months for the electrochemotherapy postoperative group and 12 months for the intraoperative group. The results of this study suggest that electrochemotherapy is a well tolerated and potentially useful addition to surgery in controlling high-grade sarcomas, which is a very aggressive tumors with short recurrence time (60 to 270 days) after surgery alone [19].

In a recent study electrochemotherapy with either cisplatin or bleomycin injected intratumorally was evaluated in dogs with perianal tumors. These tumors are very common in older dogs. Perianal adenomas can be effectively treated with surgical removal and castration and can regress following estrogen therapy, while adenocarcinomas do not regress following castration, and are not responsive to the hormonal therapy with estrogens. Dogs with perianal carcinomas without lymph node involvement and distant metastases treated by wide surgical excision have survival of approximately 70% at 2 years. In the case of more progressive disease the survival of dogs is between 7 (dogs with distant metastases) and 24 months (dogs with lymph node involvement only). Twelve dogs with 26 tumor nodules of perianal tumors of different size, and clinically expected to be of different histological type, were treated with electrochemotherapy. Electrochemotherapy proved to be highly effective regardless of the chemotherapeutic drug used. Responses to treatment at the end of

observation time, ranging from 1 to 34 months, were 92% objective responses (complete response=65%, partial response=27%), 8% no change and no progressive disease (Figure 1) [20].

Figure 1: Antitumor effectiveness of electrochemotherapy with cisplatin in perianal tumors in dogs. The nodule was treated 2-times with ECT in 4 weeks interval at total cisplatin dose of 1.3 mg and 5 trains of 8 electric pulses (plate electrodes, 7 mm inner distance, 1300 V/cm, 100 μs, 1 Hz). After 8 weeks complete response was obtained, this lasted more than 10 months.

Besides in companion animals, several clinical trials on larger numbers of horses with sarcoids confirmed that electrochemotherapy with cisplatin is a highly effective treatment with long-lived antitumor effects and good treatment tolerance [21, 22]. Complete sarcoids eradication was systematically obtained for tumors with size up to 5 cm diameter. Sarcoids in horses that were treated more than 6 years ago never relapsed.

SUMMARY ON TREATMENT PROTOCOLS

Drug administration was in all above mentioned studies, except for the first study on cats, very uniform. In all of the studies intratumoral injection of very low doses of chemotherapeutic drugs was used in order to obtain high enough concentration of the drug in tumor cells following electropulsation and at the same time to avoid the occurrence of systemic side effects.

Furthermore, the choice of electropulsation protocols was also quite similar between the groups. All the groups used uni or bipolar square wave electric pulses, with amplitudes above 1000 V/cm, pulse duration up to 100 μs with the repetition frequency 1 Hz. The main problem associated with electropermeabilization of tissues is always how to get an effective field value inside the tissue where the tumor is growing. Square waved pulses are always applied to get rid of the problems linked to the changes in the tissue impedance during the pulse. These protocols vary mostly in the choice of electrodes; plate and needle electrodes were used for electrochemotherapy of dogs and cats and wire contact electrodes for the treatment of sarcoids in horses [9, 21]. Needle electrodes give a high field deep in the tissue but with a heterogeneous distribution. Of course the needles are invasive and their use is difficult when the skin is tough (as in the case of horses). Plate electrodes are suitable for surface tumors of different sizes, as the electrodes can be moved around the tumor in order to cover the whole tumor areas. The electric field distribution is better compared to the electric field distribution obtained with needle electrodes [23].

Especially for treatment of horses, contact wire electrodes are easy to bring in contact with the shaved skin (the electric contact being obtained with a conductive gel) (**Fig 2**) [22]. They can be moved easily on the tumor surface in different orientation to take advantage of the increase in drug delivery obtained with crossed orientation of the field. Their drawback is that only a limited layer of the tissue is affected by the field discharge and successive treatments are required for eradication. There is still a need for a proper design of the electrodes and an accurate evaluation of the field distribution in the tissue.

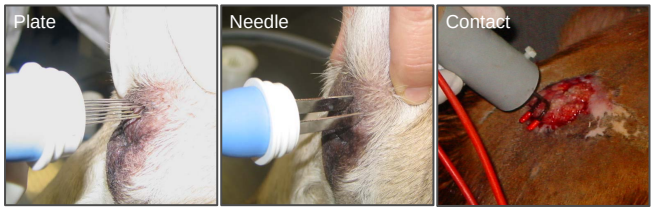

Figure 2. Application of electric pulses using different types of electrodes. plate, needle and contact electrodes.

Electrode configuration affects electric field distribution in tissue. But due to its anatomy and its electrical properties, tissue reacts to the applied external electric field making it difficult to choose the optimal electrode configuration and pulse parameters for the particular target tissue. Empirical methods of design are frequently developed [24, 25, 26]. A safe approach is to compute in advance the electric field distribution in tissue by means of modeling [27]. This is demanding due to heterogeneous material properties of tissue and its shape. In most cases numerical modeling techniques were used.

CONCLUSION

Electrochemotherapy proved to be highly effective treatment of different primary tumor types in companion animals and sarcoids in horses. This treatment approach can be used for treatment of cutaneous and subcutaneous tumors of different histologies, either for primary tumors or recurrent and metastatic disease. Due to the comparable clinical results to other standard treatment approaches and inexpensiveness and relative easiness of the procedure, it can be foreseen as a very suitable treatment option for veterinary oncology.

FURTHER READING AND REFERENCES

[1] Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J 1982;1:841-845.

[2] Mir LM. Bases and rationale of the electrochemotherapy. Eur J Cancer Suppl 2006;4:38–44.

[3] Okino M, Mohri H. Effects of a high-voltage electrical impulse and an anticancer drug on in vivo growing tumors. Jpn J Cancer Res 1987;78:1319-1321

[4] Mir LM, Orlowski S, Belehradek JrJ, Paoletti C. Electrochemotherapy potentiation of antitumor effect of bleomycin by local electric pulses. Eur J Cancer, 1991;27:68-72.

[5] Salford LG, Persson BRR, Brun A, Ceberg CP, Kongstad PCh, Mir LM. A new brain tumor therapy combining bleomycin with *in vivo* electropermeabilization. Biochem Bioph Res Co 1993;194:938-943.

[6] Sersa G, Cemazar M, Miklavcic D, Mir LM. Electrochemotherapy: variable anti-tumor effect on different tumor models. Bioelectrochem Bioenerg 1994;35:23-27.

[7] Heller R, Jaroszeski M, Leo-Messina J, Perrot R, Van Voorhis N, Reintgen D, Gilbert R. Treatment of B16 mouse melanoma with the combination of electropermeabilization and chemotherapy. Bioelectrochem Bioenerg 1995;36:83-87.

[8] Mir LM, Devauchelle P, Quintin-Colonna F, et al. First clinical trial of cat soft-tissue sarcomas treatment by electrochemotherapy. Br J Cancer 1997;76:1617-1622.

[9] Tozon N, Sersa G, Cemazar M. Electrochemotherapy: Potentation of local antitumour effectiveness of cisplatin in dogs and cats. Anticancer Res 2001;21:2483-2488.

[10] Tamzali Y, Teissie J, Rols MP. Cutaneous tumor treatment by electrochemotherapy: preliminary clinical results in horse sarcoids. Revue Med Vet 2001;152:605-609.

[11] Theon AP, Pascoe JR, Carlson GP, Krag DN. Intratumoral chemotherapy with cisplatin in oily emulsion in horses. J Am Vet Med Assoc 1993;202:261-267.

[12] Spugnini EP, Porello A. Potentation of chemotherapy in companion animals with spontaneous large neoplasms by application of biphasic electric pulses. J Exp Clin Cancer Res 2003;22:571-580.

[13] Spugnini EP, Dragonetti E, Vincenzi B, et al. Pulse-mediated chemotherapy enhances local control and survival in a spontaneous canine model of primary mucosal melanoma. Melanoma Res 2006;16:23-27.

[14] Spugnini EP, Vincenzi B, Baldi F, et al. Adjuvant electrochemotherapy for the treatment of incompletely resected canine mast cell tumors. Anticancer Res 2006;26:4585-4590.

[15] Michels, G.M., Knapp, D.W., DeNicola, D.B., Glickman N & Bonney, P. (2002). Prognosis following surgical excision of canine cutaneous mast cell tumors with histopathologically tumor-free versus nontumor-free margins: a retrospective study of 31 cases. *J Am Anim Hosp Assoc* 38, 458-466

[16] Weisse, C., Shofer, F.S. & Soremne, K. (2002). Recurrence rates and site for grade II canine cutaneous mast cell tumors following complete surgical excision. *J Am Anim Hosp Assoc* 38, 71-73

[17] Baldi A, Spugnini EP. Thoracic haemangiopericytoma in a cat. Vet Rec 2006;159:598-600.

[18] Pavlica Z, Petelin M, Nemec A, et al. Treatment of feline lingual squamous cell carcinoma using electrochemotherapy – a case report. Proceedings of the 15th European congress of veterinary densitry 2006;19-22.

[19] Spugnini EP, Baldi A, Vincenzi B. Intraoperative versus postoperative electrochemotherapy in high grade soft tissue sarcomas: a preliminary study in a spontaneous feline model. Cancer Chemother Pharmacol 2007;59:375-381.

[20] Tozon N, Kodre V, Sersa G, et al. Effective treatment of perianal tumors in dogs with electrochemotherapy. Anticancer Res 2005;25:839-845.

[21] Rols MP, Tamzali Y, Teissie J. Electrochemotherapy of horses. A preliminary clinical report. Biolectrochemistry 2002;1-2:101-105.

[22] Tamzali Y, Teissie J, Rols MP. First horse sarcoid treatement by electrochemotherapy: Preliminary experimental results. AEEP Proceedings 2003;49:381-384.

[23] Miklavcic D, Beravs K, Semrov D, et al. The importance of electric field distribution for effective in vivo electroporation of tissues. Biophys J 1998; 74: 2152-2158.

[24] Spugnini EP, Citro G, Porrello A. Rational design of new electrodes for electrochemotherapy. J Exp Clin Cancer Res 2005;24:245-254.

[25] Liu F, Huang LA. Syringe electrode device for simultaneous injection of DNA and electrotransfer.Mol Ther 2002;5:323-238.

[26] Tjelle TE, Salte R, Mathiesen I, Kjeken R. A novel electroporation device for gene delivery in large animals and humans.Vaccine 2006;24:4667-4670.

[27] Sel D, Mazeres S, Teissie J, Miklavcic D. Finite-element modeling of needle electrodes in tissue from the perspective of frequent model computation. IEEE Trans Biomed Eng 2003;50:1221-1232.

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Nataša Tozon was born in Koper, Slovenia in 1965. She graduated at Veterinary Faculty in Ljubljana in 1991. She finished her PhD study of veterinary medicine in 1998 at the same faculty, University of Ljubljana. She works as a clinician at Small Animal Clinic for Internal Medicine and Surgery but she is also assistant professor for Internal Veterinary Medicine at Veterinary Faculty in Ljubljana. She is mainly interested in veterinary clinical oncology and infection diseases in dogs and cats. In 2006 she received the Award of Veterinary Faculty for development of clinical oncology department at Small Animal Clinic for Internal Medicine and Surgery.

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NOTES

Electrically Mediated Gene Therapy: Translation to the Clinic

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INTRODUCTION

Gene medicine has held great promise for effective treatment of many diseases. Treatment of cancer, metabolic disorders, cardiovascular diseases and genetic disorders as well as vaccines for infectious diseases and cancer are being developed (1-2). The vast majority of gene therapy clinical trials are Phase I safety and tolerability studies. Many trials are not successful in Phase I due to either adverse reactions or low efficacy. About one third of these studies advance to Phase II studies and even fewer to Phase III. One critical aspect in these studies is efficient delivery of the DNA encoding the therapeutic gene or cDNA (1-2).

The transgene is contained within a vehicle which facilitates its delivery to an appropriate target. *In vivo* gene delivery methods can be broadly divided into two categories. Biological delivery depends on the ability of a genetically engineered virus to deliver the desired gene to the tissue of interest for expression. In non-viral gene therapy, the gene is delivered in a plasmid to specific tissues by simple injection or with chemical or physical assistance. The selection of the appropriate delivery vehicle depends on the therapeutic application. Several factors should be considered including efficiency and potential adverse effects (Table 1).

TABLE 1.IMPORTANT ATTRIBUTES FOR ENHANCED GENE DELIVERY

Reproducible
Delivery to non-dividing cells
Ability to perform repeat applications
Non immunogenic
Specific expression levels
Specific targeting
Minimal adverse effects (safe)
Ease of manufacture

PROTOCOL DEVELOPMENT

A number of criteria should be evaluated when developing a therapeutic application. Initially, the objectives of the application and the transgene expression profile required to fulfill those objectives should be considered. This includes the levels, duration, and location of transgene expression. Based on these criteria, an appropriate target tissue must be selected for delivery. Subsequently, the appropriate delivery approach, considering safety as well as efficacy, should be chosen.

The majority of gene therapy clinical trials utilize viral vectors for delivery. Viral delivery is highly efficient and appropriate in a range of situations. However, due to considerations such as potential immunogenicity, integration into the genome and environmental spread, plasmid based gene transfer (non-viral) protocols have been developed. A major shortcoming of plasmid-based delivery is the lack of efficient delivery methods. Both chemical and physical techniques have been tested to increase the efficiency of DNA uptake. While calcium phosphate precipitation has been used for *in vitro* DNA transfer (3), cationic liposomes are more effective at *in vivo* gene delivery as well as *in vitro* delivery (4-6). In particle bombardment or "gene gun" transfer, the target tissue is bombarded with DNA coated gold particles, which penetrate the cells (7-8). Hydrodynamic delivery or hydroporation has been shown to enhance *in vivo* delivery and expression (9-11).

One physical method that has emerged as a means to effectively facilitate delivery of plasmid DNA is *in vivo* electroporation or electropermeabilization (12). The biotechnological advances in the application of electric fields suggest that it is possible to control the delivery of plasmid DNA to impart a desired expression pattern (12-14). Most accessible tissues may be targeted for electrically mediated delivery. Selection of the delivery site and the electrical parameters allow control over the level and duration of expression of the transgene.

PRECLINICAL STUDIES FOR TRANSLATION OF ELECTRICALLY MEDIATED GENE DELIVERY

Once the transgene and the delivery parameters have been selected, the efficacy and safety of delivery in an appropriate animal model should be evaluated. This assessment should be performed utilizing the same protocol, transgene (animal counterpart), delivery vehicle and instrumentation that would be used in the clinic. The study should be conducted on

both male and female animals and contain sufficient numbers to obtain statistically significant results.

Prior to initiating the efficacy studies, it is important to determine a minimum level of response. For example, if the study is for an anti-tumor therapy the acceptable level may be a 50% response rate. This would be broken down further based on acceptable complete or partial response rates. These numbers would be based on the currently used standard of care for a particular form of cancer and what has previously been accomplished in preclinical and clinical studies.

With respect to assessment of safety, multiple time points should be evaluated after delivery of relevant doses or treatments. Evaluation should include hematological and histological data (15). In addition, it is important to determine the distribution and persistence of the plasmid within the animal following therapy. If the preclinical studies demonstrate effectiveness and safety, the protocol can be submitted to the appropriate regulatory agencies for approval to initiate the clinical trial.

SUMMARY

The use of electric fields to deliver plasmid DNA has seen tremendous growth over the last 5 years (12-14). It is quickly being recognized as an important tool for performing effective gene transfer studies. The versatility inherent in this delivery system is an important reason for this growth. The key to successfully translating electroporation protocols into the clinic is understanding the type of expression that is needed for the specific therapeutic application being investigated. Careful selection of tissue target and delivery parameters including electrical conditions allows an investigator to obtain the type of transgene expression necessary for a particular therapeutic application. The true evaluation of this delivery approach as a means for moving from gene transfer to gene therapy will be based on the results from clinical studies. During the past few years several clinical trials utilizing electroporation to deliver plasmid DNA have been initiated (12). Once results from these studies are released electrically mediated plasmid delivery will either expand with additional trials or it will regress to use as a preclinical or laboratory tool to evaluate potential targets.

REFERENCES

[1] Gao, X., Kim, K.S. and Liu, D. Nonviral gene delivery: what we know and what is next. AAPS J. 23;9(1):E92-104, 2007.

[2] Preuss, M.A., Curiel, D.T. Gene therapy: science fiction or reality? South Med J 100(1):101-104, 2007.

[3] Benvenisty, N. and Reshef, L. (1986). Direct introduction of genes into rats and expression of the genes. Proc. Natl. Acad. Sci., USA 83:9551-9555.

[4] Nicolau, C. and Sene, C. (1982) Liposome-mediated DNA transfer in eukaryotic cells: Dependence of the transfer efficiency upon the type of liposomes used and the host cell cycle stage. Biochim. Biophys. Acta 721:185-190.

[5] Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M.and Danielsen, M. (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci., USA 84:7413-7417.

[6] Li, S.D. and Huang, L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther. 13(18):1313-9, 2007.

[7] Yang, N-.S., Burkholder, J., Roberts, B., Martinell, B. and McCabe, D. (1990) *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. Proc. Natl. Acad. Sci., USA 87:9568-9572.

[8] Sun, W.H., Burkholder, J.K., Sun, J., Culp, J., Turner, J., Lu, X.G., Pugh, T.D., Ershler, W.B. and Yang, N-S. *In vivo* cytokine gene transfer by gene gun reduces tumor growth in mice. Proc. Natl. Acad. Sci., USA 92:2889-2893.

[9] Liu F, Song Y, Liu D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999 6(7):1258-66.

[10] Liu D, Knapp JE. (2001). Hydrodynamics-based gene delivery. Curr Opin Mol Ther. 2001 3(2):192-7.

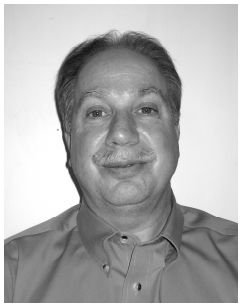
[11] Herweijer, H. and Wolff, J.A. Gene therapy progress and prospects: hydrodynamic gene delivery. Gene Ther. 14(2):99-107, 2007.

[12] Heller, L.C. and Heller, R. (2006). In vivo electroporation for gene therapy. Human Gene Therapy, 17(9):890-897.

[13] Favard, C., Dean, D.S. and Rols, M.P. Elecrtrotransfer as a non viral method of gene delivery. Curr Gene Ther 7(1):67-77.

[14] Heller, L.C., Ugen, K.E. and Heller, R. Electroporation for targeted gene transfer. Expert Opinion in Drug Delivery, 2(2):255-268, 2005.

[15] Heller L, Merkler K, Westover J, Cruz Y, Coppola D, Benson K, Daud A, Heller R. (2006). Evaluation of toxicity following electrically mediated interleukin-12 gene delivery in a B16 mouse melanoma model. Clin. Cancer Res. 12(10):3177-83.



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delivery procedures for non-viral gene therapy. The approach utilizes pulsed electric fields to facilitate targeting and uptake of the molecules to be delivered. The current gene therapy approach is geared toward the development of appropriate vaccine and immunotherapy protocols for the delivery of DNA vaccines for cancer or infectious disease.

NOTES

STUDENTS' ABSTRACTS

Electroendocytosis using Bipolar Electric Pulses

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INTRODUCTION

Using electric pulses (EP) in the range of 2.5-20V/cm or even pulsed electromagnetic fields (EMF) (envelope frequency of 217 Hz, carrier frequency of 900 MHz , pulsed duration of 580 μs and exposure time exceeding 10 min.) at least two groups have shown that there is a stimulation of the endocytosis [1-3]. Endocytosis is a fundamental physiological process that mediates essential cell processes such as: extracellular molecules uptake, membrane renewal and surface area control, antigen presentation, membrane receptor expression, etc. The stimulation described in references [1-3] was achieved using monopolar electric pulses. In the first step of the studies on electroendocytosis, we intend to reproduce this stimulation of the endocytosis using bipolar electric pulses.

MATERIALS AND METHODS

DC-3F cells suspended in PBS or in low conductive medium (250 mM sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7) were exposed to a train of bipolar EP with field intensity of 4.5 V/cm to 12 V/cm, frequency of 100-500Hz, pulse duration of 100 μs to 500 μs and total time of exposure of 2-10 min. 10^6 cells in 100 μl containing 2 mM Lucifer Yellow (LY) were deposited between two stainless steel parallel plates. To determine the LY uptake, cells were washed twice in 0.9% NaCl and lysed. Fluorescence was measured using a spectrofluorimeter set at 423 and 525 nm for the excitation and emission wavelengths. Actual cell concentration in the samples was determined by protein quantity determination. All the data obtained were normalized by the cell number.

RESULTS AN DISCUSSION

Preliminary results actually showed an increase in the uptake of LY by endocytosis in the exposed cells. This increase depends on pulse repetition frequency, individual pulse duration, field intensity and total exposure time. Increases by a factor of two have been regularly achieved with short exposure times (1 or 2 minutes of exposure). The interaction between these parameters is being analyzed. Previous findings on electroendocytosis are thus being reproduced using bipolar asymmetric pulses which allow to rule out mechanisms such as electrophoresis of membrane components or global polarization of the suspension when monopolar pulses are delivered. Mechanisms will be analyzed later.

REFERENCES

[1] N. Mahrour, R. Pologea-Moraru, M. Moisescu , S. Orlowski, P. Leveque and L. M. Mir, "In vitro increase of the fluid-phase endocytosis induced by pulsed radiofrequency electromagnetic fields: importance of the electric field component," *Biochimica et Biophysica Acta*, vol. 1668, pp. 126-137, 2005.

[2] Y. Antov, A. Barbul and R. Korenstein, "Electroendocytosis: stimulation of adsorptive and fluid-phase uptake by pulsed low electric fields," *Experimental Cell Research*, vol. 297, pp. 348-362, 2004.

[3] Y. Antov, A. Barbul, H. Mantsur and R. Korenstein, "Electroendocytosis: Exposure of Cells to Pulsed Low Electric Fields Enhances Adsorption and Uptake of Macromolecules," *The Biophysical Journal*, vol. 88, pp. 2206-2223, 2005.

Apoptosis of cultured cells exposed to 2.45 GHZ-EMFs

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INTRODUCTION

Effects of electromagnetic field (EMFs) on different cell lines have been studied for many years leading often to contradictory results regarding the mutagenic potential of EMFs. In this study we examine the genotoxic effect of 2.45GHz electromagnetic fields (EMFs) on consecutive generations of cells from a murine embryonic fibroblast cell line (NIH3T3 cells) using Single Cell Gel Electrophoresis (Comet Assay) and DNA Agarose Gel Electrophoresis.

MATERIALS AND METHODS

Murine embryonic fibroblast cell were cultured at 370C and 5% CO_2 in DMEM supplemented with fetal bovine serum, L-glutamine and antibiotics. The cellular culture was trypsinised and resuspended in DMEM supplemented with fetal bovine serum. Cellular suspension was exposed to 2.45 GHz-EMF increasing temperature with 15 ^{0}C. Four types of samples were used: A-control (non-heated); B-warmed by thermostat, C-warmed by exposure to microwaves and D-samples obtained by irradiation in the same conditions of two consecutive cell generations.

Cellular apoptosis was studied by two techniques: Single Cell Gel Electrophoresis (SCGE) and DNA Agarose Gel Electrophoresis. For DNA Agarose Gel Electrophoresis we used a regular agarose which separated DNA fragments between 200 and 50.000 kb and a high resolution agarose which separated fragments under 1000 kb of DNA.

RESULTS AND FIGURES

In controls (samples A) the band corresponding to DNA molecules with high molecular weight was observed. The same situation can be seen in samples B (warmed by thermostat). The samples exposed to EMF show a scalariform band pattern suggesting cell apoptosis. The cells exposed to EMF twice at 72 h interval (D samples), show a different DNA migration pattern comparatively to controls and samples exposed just once to EMF. On comet assay both controls and B samples showed a similar migration pattern.

CONCLUSION

2.45 GHz – EMF irradiation may have a non-termal genotoxic effect on transformed fibroblasts, inducing an apoptosis-like fragmentation of nuclear DNA. Further studies are required to confirm this effects both on NIH3T3 cells and on other cell lines. The re-exposure of the cells to MW induces a completely different pattern of DNA migration.

REFERENCES

[1] Sabine IVANCSITS, Alexander PILGER ı, Elisabeth DIEM, Oswald JAHN, Hugo W. RUDINGER, Cell type-specific genotoxic effects of intermittent extremely low-frequency electromagnetic fields. ELSEVIER, Mutation Research 583 (2005) 184–18.

[2] Narendra P. SINGH, A Simple Method for Accurate Estimation of Apoptotic Cells. Experimental Cell Research 256, 328–337 (2000)

[3] Tomas GICHNER, Anita MUKHERJEE, Elizabeth D. WAGNER, Michael J. PLEW, Evaluation of the nuclear DNA Diffusion Assay to detect apoptosis and necrosis. ELSEVIER, Mutation Research 586 (2005) 38–46

Effects of a Nuclear Permeabilizing High Voltage Micropulse on Nucleus of Living Cells *in vitro*

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INTRODUCTION

Conventional electropermeabilization, employing long lasting pulses and low electric fields, is widely used to permeabilize the plasma membrane and allow the entry of molecules into the cytoplasm of living cells while leaving intact organelles [1]. Previous studies suggested that the application of high electric fields with ultra short duration allowed permeabilizing the membranes of intracellular organelles like the nuclear membrane [2,3]. Intense pulsed electric fields could enhance gene delivery to the nucleus [3].

In the present study, we focused on the modifications induced by electric fields on nuclear morphology and permeabilization of CHO (Chinese Hamster Ovarian) cells.

METHODS

Cells were cultured over night at 37°C on labtek slides for adhesion and, the following day, were electropermeabilized in a pulsing buffer (10 mM Phosphate Buffer, 250 mM sucrose, 1 mM MgCl2) with a GHT electropulsator. The permeabilization efficiency was followed with use of propidium iodide (PI, 0.1 mM). In the same time, cells were imaged by fluorescence on an inverted digitized videomicroscope with a 63X objective (Leica, Roper camera, Metavue software).

For the double pulsation procedure we used the following protocol: 0.7kV.cm^{-1}, 5 ms, 1Hz, X10 and 2 min later a single 5kV.cm^{-1}, 5 µs pulse. We acquired an image before the procedure, every 20 s after the first pulse train and every 1 min during 10 min after the second pulse condition. By image processing (Optimas 6.5), we calculated the changes of nucleus area and mean fluorescence during the procedure.

RESULTS

The electropulsation induced membrane permeabilization brought an enhancement of the fluorescence in the nucleus. The first train of pulses induced a slight swelling of cells nucleus (Fig 1). The mean fluorescence of the nucleus was increased and reached a saturation level in less than 2 minutes (Fig 2). This level was maintained along the 10 following minutes when the nucleus slowly recovered its initial size (Fig 1 & 2).

The application of a second high voltage pulse did not significantly affect the enhanced mean fluorescence of the nucleus but delayed its return to its initial size (Fig 1 & 2).

CONCLUSIONS

The electropulsation induced nuclear swelling reflects a perturbation of the nuclear envelope. This can be a direct or a by effect of the pulse [4]. The second pulse apparently "stabilizes" this perturbation. The PI fluorescence results suggest that the chromatin structure is not significantly affected.

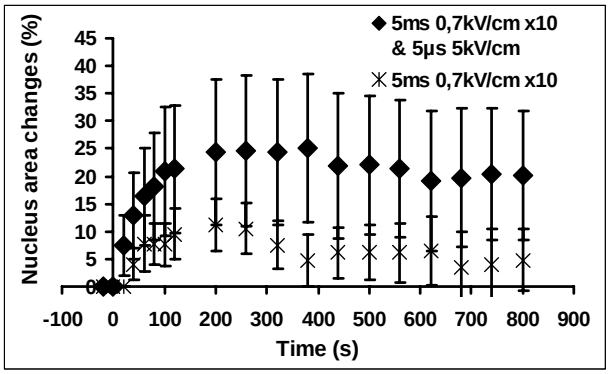

Figure 1: Nucleus area changes (%) during permeabilized state

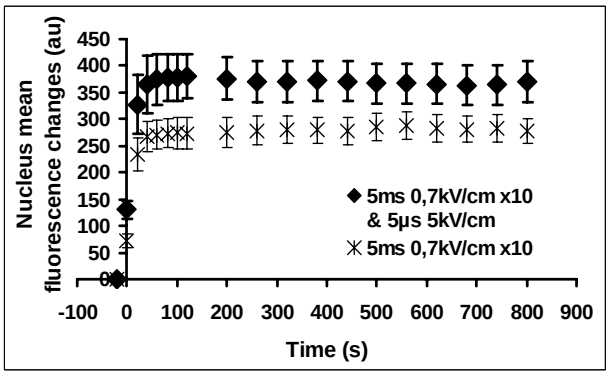

Figure 2 : Mean fluorescence (au) of the nucleus during permeabilized state

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REFERENCES

[1] M.Golzio, M.P. Rols and J. Teissie, "In vitro and in vivo electric field-mediated permeabilization, gene transfert, and expression," *Methods*. vol. 33, pp. 126-135, 2004.

[2] K.H. Schoenbach, S.J. Beebe and E.S. Buescher, "Intracellular effect of ultrashort electrical pulses," *J. Bioelectromagnet.* vol. 22, pp. 440, 2001.

[3] S.J. Beebe, et al, "Diverse effects of nanosecond pulsed electric fields on cells and tissues," *DNA and Cell Biology*. vol. 22 (12), pp. 785-796, 2003.

[4] ML Escande-Geraud et al, "Reversible plasma membrane ultrastructural changes correlated with electropermeabilization in Chinese hamster ovary cells", *Biochim Biophys Acta*. vol 939(2) pp. 247-59, 1988.

Range Analysis on a Circuital Model of a Eucariotic Cell Subjected to High Amplitudes Nanosecond-Duration Pulsed Electric Fields

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INTRODUCTION

The behaviour of an eucariotic cell can be modified by the application of an external electrical field pulse. The fast structural modifications of the plasma membrane, known as electro-permeabilization or electroporation has been proposed as a tool for non-thermal pasteurization of fluid food, in medicine for cancer chemotherapy, drug delivery, etc. The most remarkable phenomena associated to electroporation are linked to the formation and growth of pores in the lipid bilayer membrane which allow the transport of ions and water-soluble species through the membrane. The flux of the different ionic species is driven by Trans-Membrane Voltage (TMV). Depending on the characteristics of the applied pulses (amplitude, duration, rise time) it is also possible to affect the membranes of some internal cell structures. In particular, when high-intensity, nanosecond-duration pulsed electric fields (nsPEF) are applied, the voltage on such organelles membranes may temporarily exceed that on the plasma membrane giving the very interesting possibility to interact with the cell internal structures.

Several papers have been recently published concerning either experimental studies [1-2] or theoretical analysis [3-4] on the response of biological cells subjected to nanosecond-duration pulsed electric fields. As it concerns the theoretical studies, both field or circuit-based models approaches have been used. The field-based models, usually solved by means of suitable numerical schemes (FEM, BEM, FDTD, etc.) provide detailed information on the relevant physical quantities, but require considerable computational resources. The circuit-based approach, although less accurate, is more flexible and allows a more straight association of the electrical quantities to the biological phenomena. A relevant subject of these studies is the determination of the role of the different geometrical and physical parameters in the time evolution of the voltages on outer and inner membranes. The evaluation of these parameters can be of paramount importance for example in the design of the pulse generators or in the choice of the experimental conditions in real applications. However, the variability of the relevant parameters, due to different cell characteristics or uncertainties on the measured values, is either not considered (nominal values analysis), or taken into account in oversimplified conditions.

In [5] the authors have proposed a lumped parameters circuit based on the so called Hodgkin-Huxley (HH) model for the evaluation of the time evolution of the voltage across the plasma membrane. In this model the membrane is represented by a capacitance, the ionic channels as linear or nonlinear conductances and the voltage generators are linked to the so called Nernst equilibrium potential, determined by the ratio of the specific ionic concentrations inside and outside the cell.

In this paper we intend to present the results of simulation studies performed on a more accurate circuit model representing the different regions of a cell. The model consists of five sections (external bio solution, plasma membrane, cell cytosol, organelle membrane, organelle cytosol), each one characterized by a suitable electric circuit. In particular, the three fluids are represented by parallel connections of a resistor and a capacitor, whereas the two membranes are modelled through a non linear time variant circuit shown in Figure 1.

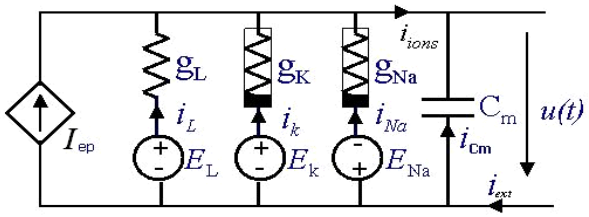

Figure 1. Non linear time variant circuit adopted for modelling the plasma and organelle membranes.

The voltage controlled current generator accounts for the pores density dynamics *N(t)* described by the so called Smoluchowsky equation:

$$\frac{dN(t)}{dt} = \alpha e^{\left(u(t)/V_{ep}\right)^2}\left(1-\frac{N(t)}{N_0}e^{-q\left(u(t)/V_{ep}\right)^2}\right)$$

A systematic approach is used in order to study the influence of the different physical and geometric parameters on the dynamics of the voltage across the plasma and the organelle membrane voltages. The response of the considered circuit to trapezoidal voltage pulses is considered.

REFERENCES

[1] E.H. Hall, K.H. Schoenbach, S.J. Beebe, Nanosecond Pulsed Electric Fields Have Differential Effects on Cells in The S-Phase, DNA and Cell Biology. 2007, 26(3): 160-171.

[2] S.J. Beebe et al., Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis induction and tumor growth inhibition, IEEE Trans. Plasma Sci., 2002, 286–292.

[3] R.P.Joshi, Q. Hu, K.H. Schoenbach, S.J. Beebe. 2004. Energy landscape- model analysis for irreversibility and its pulse-width dependence in cells subjected to a high-intensity ultrashort electric pulse, Phys. Rev. E. 69:051901.

[4] T. Kotnik, D. Miklavcic, Theoretical Evaluation of Voltage Inducement on Internal Membranes of Biological Cells Exposed to Electric Fields, Biophysical Journal, Volume 90, Jan. 2006, pp. 480–491.

[5] N. Citro, V. Tucci: "A detailed analysis of the electrical dynamics of the Hogkin-Huxley cellular model including electroporation", Proc. of the 4th Int. Workshop on Biological Effects of EM Fields, Oct. 2006, pp. 853-860.

Duration and number of electric pulses effects on DNA electrotransfection *in vitro*

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INTRODUCTION

Gene therapy consists in the safe and efficient DNA transfer in organism in order to treat diseases. However, cell membrane is a barrier to the free diffusion of DNA between external medium and cytoplasm. Nevertheless, the permeability of membrane can be transiently increased when external electric field pulses are applied, a process called **electropermeabilization**.

This physical method allows gene delivery in several organs (muscle, tumour, brain…). But the safe and efficient use of this method for clinical applications requires the knowledge of the mechanism underlying that electropermeabilization phenomenon[1].

It is known that when the electric field is higher than the permeabilization threshold, it induces DNA interaction with the permeabilized membrane[2]. In this study, we research to identify the effects of others electric field parameters: the duration and the number of electric pulses on the membrane permeabilization, cell viability, DNA/membrane interaction and gene transfection.

METHODS

In our group, we develop a single cell visualization set-up. This set-up is composed of an inverted fluorescence microscopy and a Jouan electropulsateur. This set-up gives us a time and spatial resolution[3].

We use the CHO WTT line cell. The propidium iodide uptake enables us to follow the electropermeabilization efficiency. Moreover, we use peGFP-C1 plasmid labelled with fluorescent probe (TOTO-1) to evaluate the DNA/membrane interaction and transfection efficiency to quantify by measuring of the eGFP fluorescence by flow cytometer. The cell viability is determined by violet crystal assay over 24h.

RESULTS

We study the effects of electric pulses duration on membrane permeabilization and gene transfection. When we increase the pulses duration (0 ms, 0.1 ms, 1 ms, 5 ms and 10 ms), we observe a 4-times increase for the percentage of electropermeabilized cells and a 10-times increase for associated fluorescence intensity. The cell viability decreases slightly by 20%. One can propose that the increase of pulses duration induces more membrane defaults (number and/or size), so the propidium iodide penetration rises in cells. The percentage of cells expressing the eGFP protein increases until 20% at 5 ms and decreases slightly by 5% at 10 ms. But the associated fluorescence intensity rises with the pulses duration. These results suggest that the amount of DNA entered in the cells increases with the pulses duration. We also show the amount of DNA which interacts with the membrane increases with pulses duration. So this hypothesis is plausible. However, at 10 ms, the amount of DNA would induce a decrease of cell viability.

As for as the effect of pulse nimber is concerned, we observe that the percentage of electropermeabilized cells reaches 60-70% after the first pulse. It remains constant when the number of electric pulses rose from 1 to 10. However, the associated fluorescence intensity linearly increases with the pulses number. The cell viability decreases by 30%. Like for pulse duration, we suggest that increase of pulses number induces more membrane defaults. Between the first and the last pulse, the percentage of eGFP positives cells and the associated fluorescence intensity increase by a 4 times and 7 times, respectively. We demonstrate this raising of transfection efficiency is correlated with the increase of 3 fold of DNA/membrane interaction.

CONCLUSIONS

In this work, we study the effects of number and duration of electric pulses on membrane permeabilization and gene transfection. We show that the electropermeabilization efficiency increase with the number and the duration of pulses. We can suppose that the raising of number and duration of pulses increase the number and/or the size of membrane defaults.

We demonstrate that the raising of transfection efficiency induced by the increase of duration and number of pulses is correlated with the increase of DNA amount which interacts with the permeabilized membrane.

REFERENCES

[1] J.M. Escoffre, D.S. Dean, M. Hubet, M.P. Rols, C. Favard, "Membrane perturbation by an external electric field: a mechanism to permit molecular uptake" (in press)

[2] M. Golzio, J. Teissie, M.P., Rols, "Direct visualization at the single-cell level of electrically mediated gene delivery". *Proc. Natl. Acad. Sci. USA,* Vol. 99, pp1292-1297, 2002

[3] C. Faurie, E. Phez, M. Golzio, C. Vossen, J.C. Lesbordes, C. Delteil, J. Teissie, M.P. Rols, "Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells". *Biochim. Biophys. Acta,* Vol. 1665, pp92-100, 2004

The influence of ions on the efficiency of gene electrotransfer

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INTRODUCTION

Gene electrotransfer is a method that enables delivery of plasmid DNA into the cell. To date, only few studies have experimentally investigated the effect of ionic composition of the electroporation buffer in vitro. Because of negatively charged DNA, divalent cations (Mg^{2+}, Ca^{2+}) act as a bridge between polyanionic DNA and negatively charged exterior of the cell [1]. But higher concentration of divalent cations affects the ionic strength of the plasmid DNA solution, and alters stability and physical properties of DNA molecules [2]. Main objective of this study was to compare gene electrotransfer efficiency in two commonly used electroporation buffers (potassium and sodium buffer) and to analyze effect of higher concentration of Mg^{2+} ions.

MATERIALS AND METHODS

Electropermeabilization was performed on 24 hours old Chinese hamster ovary cells grown as a monolayer culture. Before application of pulses we removed culture medium from cells and incubated them with the plasmid DNA that codes for GFP (green fluorescent protein) in a specific electroporative buffer (potassium, sodium, 10 mM Mg and 50 mM Mg). Trains of four rectangular pulses with amplitudes from 60 to 280 V were applied. After the pulsing fetal calf serum (FCS) was added (25% of sample volume). Treated cells were incubated for 5 minutes at 37° C to allow cell membrane resealing and then grown for 24h in cell culture medium at 37° C in a humidified 5% CO_2 atmosphere in the incubator. Cell concentration in all media was 5×10^4 cells/ml and plasmid DNA concentration was 10 μg/ml. Efficiency of gene electrotransfection was determined by fluorescent microscopy (Zeiss 200, Axiovert, Germany).

RESULTS AND DISCUSSION

In order to investigate the role of ionic composition of different media on gene electrotransfection we examined the effect of different electroporation buffers (sodium and potassium medium with 1 mM $MgCl_2$) and the effect of different Mg^{2+} concentration (1 mM, 10 mM and 50 mM) in the potassium buffer. The effect of buffer composition was followed by determining the expression rate of the transferred GFP gene. The cell expressing GFP protein is presented in Figure 1.

In general, electroporation efficiency increased with increasing field strength in all media. The transfection efficiency was similar in sodium and potassium buffer. For higher concentration of $MgCl_2$ we obtained lower transfection, which could be due to the fact, that divalent cations either interact with plasmid DNA and alter the topology and consequently the stability of a DNA molecule [2], or as it was shown in some experiments divalent ions (mostly Mg^{2+}) increase activity of DNAase, which consequently decreases the transfection rate [3].

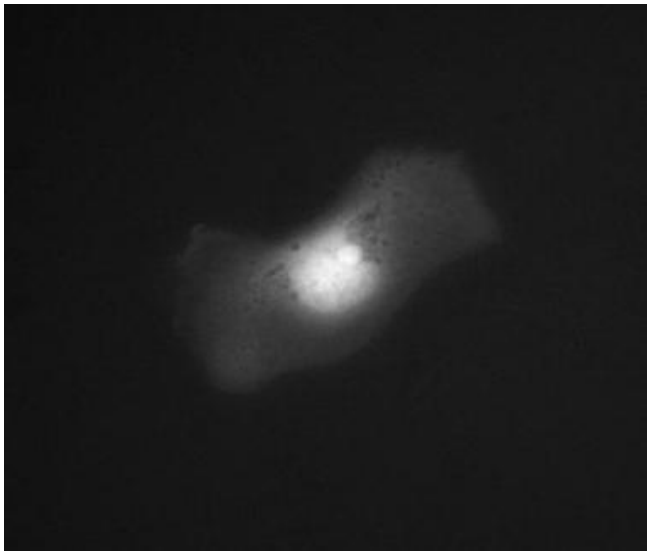

Figure 1: The cell expressing GFP protein

CONCLUSIONS

We have shown that the transfection efficiency depends on medium divalent ionic composition. The higher concentration of divalent cations affects the ionic strength of the plasmid DNA solution and decreases transfection efficiency. Divalent cations at some point interact with plasmid DNA and affect direct the stability and of DNA molecule or increase activity of DNAase and therefore influence efficiency of transfection [2]. No significant difference in gene electrotransfer was observed in potassium and sodium medium.

REFERENCES

[1] E. Neumann, S. Kakorin, I. Tsoneva, B. Nikolova, T. Tomov. "Calcium-mediated DNA adsorption to yeast cells and kinetics of cell transformation by electroporation," *Biophys. J.*, vol. 71, pp. 868-877, 1996.

[2] Y.G. Zhao, H. Lu, J. Peng, Y. Xu. "Inhibitory effect of Ca^{2+} on in vivo gene transfer by electroporation "*Acta Pharmacol. Sin.,* vol. 27(3), pp. 307-310, 2006.

[3] Z. Brnakova, A. Godany, J. Timko. "An exodeoxyribonuclease from Streptomyces coelicor: Expression, purification and biochemical characterization " *Biochim. Biophys. Acta,* vol. 1770, pp. 630-637, 2007

Enhancement of gene expression by nanosecond electric pulses

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INTRODUCTION

Pulsed electric fields (in the range of 100 microseconds to 100 milliseconds and 25 to 1500 V/cm about) are known to permeabilize the cell membrane and they are used to allow the entry of genes or molecules in cells. Recently, technology allow the application of ultra short pulses (10 to 300 ns) of higher electric field strength (20 to 150 kV/cm) without increase of temperature. They are termed nanosecond pulsed electric field (nsPEF).

Several studies have been published on effects of nsPEF on cells, reporting an induction of apoptotic markers [1,2] and a release of calcium [1,3,4]. In one paper [1], one figure showed that the application of 1 nsPEF (10 ns, 150 kV/cm) 30 min after the GFP gene electrotransfer allows an increase of 3-fold of GFP expression compared to electrotransfer only.

The aim of our study was to explore whether nsPEF affect the expression of electrotransferred genes *in vitro*.

METHODS

DNA coding for the luciferase was electrotransferred into cells using classical procedures (Cliniporator, IGEA, Carpi, Italy). Then cells were exposed or not to nsPEF (20 ns duration) (FID Technology, Russia), and incubated for 24 to 48 hours. Measurements were performed using a luminometer and results were expressed as pg luciferase / µg protein.

RESULTS

Results show an increase in the expression of the electrotransferred luciferase gene after exposure of the cells to nsPEF compared to electrotransfer only. This increase seems to be dependent on several parameters that are still under investigation: the number of nsPEF, the delay between the gene electrotransfer and the nsPEF, the amplitude of the nsPEF and the concentration of the DNA.

Studies are still under investigation to improve the exposure system (cuvette, incubation medium,…).

CONCLUSION

An increase of 3-fold of the luciferase gene expression can be achieved with the application of just 1 nsPEF of 20 ns and 60 kV/cm, 60 minutes after gene electrotransfer, in an électroporation cuvette with 1 mm of distance between the electrodes. No loss of viability is associated to the exposure t these nsPEF.

Thus cell manipulation by means of electric nsPEF delivery may efficiently increase the overall efficacy of gene electrotransfer.

REFERENCES

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

[2] S.J. Beebe, J.A. White, P.F. Blackmore, Y. Deng, K. Somers and K.H. Schoenbach, "Diverse effects of nanosecond pulsed electric fields on cells and tissues", *DNA Cell Biol*, vol. 22, pp. 785-796, 2003.

[3] P.T. Vernier, Y. Sun, L. Marcu, S. Salemi, Craft C.M. and M.A. Gundersen, "Calcium bursts induced by nanosecond electric pulses", *Biochem Biophys Res Commun*, vol. 310, pp. 286-295, 2003.

[4] J.A. White, P.F. Blackmore, K.H Schoenbach and S.J. Beebe, "Stimulation of capacitive calcium entry in HL-60 cells by nanosecond pulsed electric fields", *J Biol Chem*, vol. 279, pp. 22964-22972, 2004.

Electrodeformation and electroporation of giant vesicles in the gel phase

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INTRODUCTION

Gel phase membranes have very different mechanical properties as compared to fluid phase membranes. Thus, different dynamics of vesicle deformation in electric fields and poration are expected. Investigations on vesicles in the gel phase date from already 25 years ago [1]. Small vesicles (100 nm in size) made of dipalmitoylphosphatidylcholine (DPPC) were studied. The results showed that no global damage of the vesicles occurs and that the induced pores are fully reversible. Thus, the conclusion was that the lifetime of the pores must be very short and "… may just exist as long as the membrane potential is greater than its threshold value".

Because, these vesicles were of much smaller size as compared to cell dimensions, here we address our attention to giant unilamellar vesicles, or GUVs (several tens of micrometers), in order to test the above statement for membranes on the cell-size scale. DPPC is a suitable lipid for such studies because its phase transition temperatures are both well above room temperature. Our results show the opening of large and stable pores in the vesicle membrane.

METHODS

The vesicles were prepared using the method of electroformation as given in details in [2]. Briefly, GUV were grown from a DPPC film on conductive glasses in 0.1 M sucrose solution at ~ 60 °C. AC-field of 1,1 V and frequency 10 Hz was first applied for ~ 1 h, after which the field was changed to 1.5 V; 5 Hz for 2 hours. At the end, the chamber was slowly cooled down (0.5 °C/min). Then the vesicles were diluted 1:20 with 0.1 M glucose and placed in an electroporation chamber (electrode spacing: 0.5 mm). The vesicle response to the applied pulses was observed on an inverted microscope (Zeiss, Axiovert 135) equipped with an ultra fast camera (Red Lake Sci, US) or a confocal microscope (Leica SP5). For confocal imaging the vesicles were fluorescently labeled with 0,1 mol% DPPE-Rhodamine.

RESULTS

We applied square-wave pulses with duration of 300 µs and increasing field strength (2-3 kV/cm). The degree of vesicle deformation was quantified by the ratio a/b, where a and b are the vesicle semiaxes along and perpendicular to the field direction, respectively. The relaxation processes could be fitted to single exponential decays. A typical deformation sequence is shown in Fig. 1. The maximal deformation increases with increasing field strength but the decay times decreased from 0.54 ms (2 kV/cm) to 0.25 ms (3 kV/cm). Within 2 ms the vesicle was observed to completely relaxed back to the initial deformation. These relaxation times are comparable to those measured for vesicles in the fluid phase [1].

Poration of the GUVs required the application of stronger DC pulses. Typically, for vesicles with a diameter of approximately 60 µm, pulses of strength above 4 kV/cm were required to induce poration. The pores observed here are several micrometres in size and are stable with time (they were observed to remain open for more than 10 min after applying the pulse). They resemble cracks on a rough solid shell, see Fig. 1 inset. In some occasions, the porated vesicles adhere to the cover slide of the observation chamber after poration and tend to collapse.

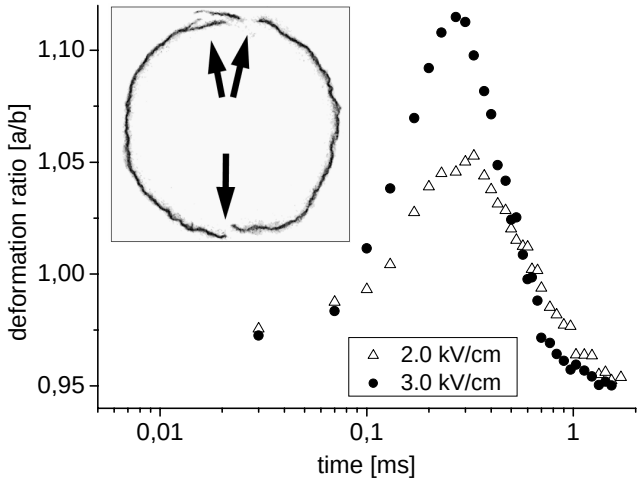

Figure 1: Deformation of DPPC-GUV subjected to pulses of different field strength (see legend) and pulse duration of 300 µs. The vesicles were recorded at 30 000 fps for 2 ms, (since the initial shape of DPPC GUV is not sphere [3] the ratio a/b differ from 1);
inset: Poration of a DPPC-GUV, equatorial section, pores are visible at both sides of the GUV and marked by arrows

CONCLUSIONS

Compared to fluid phase GUV [2], in gel phase vesicles, one requires much higher field strength to obtain similar degree of deformation. However, the decay times of the vesicle relaxation dynamics are comparable. In contrast to a previous work [1], we show that pores on DPPC-vesicles in the gel state usually do not reseal within the pulse duration.

REFERENCES

[1] Teissie & Tsong, "Electric field induced transient pores in phospholipid bilayer vesicles," *Biochemistry*, vol. 20, pp. 1548-54, 1981.

[2] Riske & Dimova, "Electro-deformation and poration of giant vesicles viewed with high temporal resolution," *Biophys. J.*, vol. 88, pp. 1143-55, 2005.

[3] Ly & Longo, "Probing the interdigitated phase of a DPPC lipid bilayer by micropipette aspiration," *Macromol. Symp.*, vol. 219, pp. 97-122, 2005.

DC-DC converter with digital setting of the output for charging output amplifiers of electroporator

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INTRODUCTION

The electroporator is an electronic device – electric pulse generator for performing electroporation. The DC-DC converter represents an important constituent of the electroporator. The main task of the DC-DC converter is to ensure the desirable voltage output which can be set via microcontroller. The output voltage on the electrodes ensures establishing of the electric field in the sample/tissue between the electrodes. Living cells are thus exposed to the electric field which causes their membranes to become permeable to substances which otherwise hardly or never permeate the plasma membrane.

The main feature of our DC-DC converter is that we can set output voltage digitally via microcontroller. The other important feature is galvanic separation of the output from input.

Available commercial DC-DC converters which one can buy of the shelves namely do not have both features.

DC-DC CONVERTER STRUCTURE

We based the design of our DC-DC converter on two transistor forward converter. For pulse width modulation we used PIC18F45J10 microcontroller. Measuring element is constructed of a 10-bit A/D converter and two resistors. The resistors present voltage divider. The power part of the DC-DC converter provides currents peak and overvoltage protection.

We have to ensure, that the control circuit was isolated from other parts of the DC-DC converter. We galvanically separated control signals by using optocouplers HCPL-2200 and digital isolators ISO721d. These elements have high voltage breakdown (several kV) and their task is to protect sensitive elements of the control circuit from high voltage. Operating output voltage of our DC-DC converter is 50-250 V.

Operating frequency of our DC-DC converter is 156 kHz. Therefore we used EMI filter FN 402-4 to prevent high frequency from getting to the distribution network.

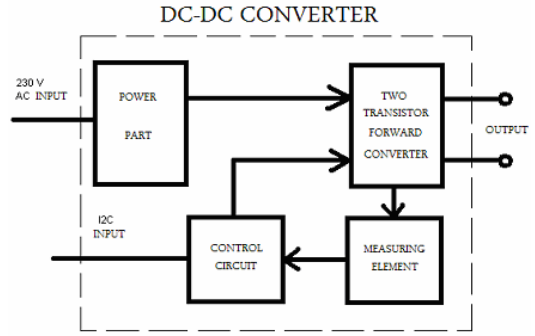

Figure 1: Block diagram of the DC-DC converter

CONTROL

Our DC-DC converter is operated by a microcontroller. All parameters of DC-DC converter (output voltage, frequency of the measuring element, relay switch) are set digitally and can thus be controlled by computer. A PIC18F45J10 microcontroller that we used satisfies all the design requirements. It receives output voltage from the measuring element (A/D converter) and on that basis controls the duty cycle of the two transistors (T_1,T_2).

For safety of the operator and of the patient microcontroller sends a signal to the relay and turns off the switch so that network voltage is held by the relay and can not go to the output.

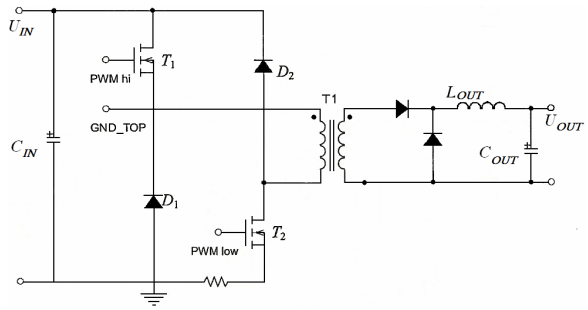

Figure 2: Electrical scheme of two transistor forward converter

RESULTS

This DC-DC converter was successfully build and tested. Microcontroller works as we predicted. It drives transistors flawlessly as demonstrated by output voltage being stable during tests (load: resistor decade). Output voltage oscillates only ± 5 V on the whole design range (50-250 V).

Courses of the input and output magnitudes were satisfying although DC-DC converter was not performing at maximum power.

With carefully chosen elements our device did not overheat at any time during tests. Next step is to increase output voltage to a higher range (200-1000 V). Only then can be build in electroporator.

REFERENCES

[1] Schmidt-Walter H., Cories R., *Taschenbuch der elektrotehnik*. Published by Harri Deutsch ISBN 3-8171-1563-6, 1998.

[2] Keith Billings, *Switch mode power supply handbook*. Mc Graw Hill, ISBN 0-07-005330-8, 1989.

Electroporation in a Current-Controlled Mode

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INTRODUCTION

Under application of a high electric field nanopores appear in the cell membrane, increasing its conductivity. Depending on the method of the field application, one or more electropores are induced. Nevertheless, the control over electropores is still impeded by instability of their size and lifetime. Under application of the high electric field of the fixed intensity, the electropores extend their surface breaking the plasma membrane and damaging the cell. Therefore, in medical applications of electroporation, the electric field is delivered by impulses preventing uncontrollable extension of the electropores. This method, however, does not allow for a precise control of a number, diameter, and lifetime of the induced electropores. The study on planar lipid membranes shows possibility of applying different electroporation methods that permit creating a single long-lived nanopore of a predictable size: current-controlled mode [1]-[3] and CACC electroporation [4].

METHODS AND RESULTS

In the current-controlled experiment, the stability of electroporated membrane is maintained by a negative feedback automatically decreasing the electric field when the electropore diameter extends. Chronopotentiometric measurements (Fig.1) [1], [3] on planar membranes under current-clamp conditions show that the electropore can be kept open for an hour or above maintaining approximately the same diameter. The only problem is stochastic fluctuations of the pore diameter that depend on the experimental conditions showing certain statistical regularity [3] that is called self-similarity.

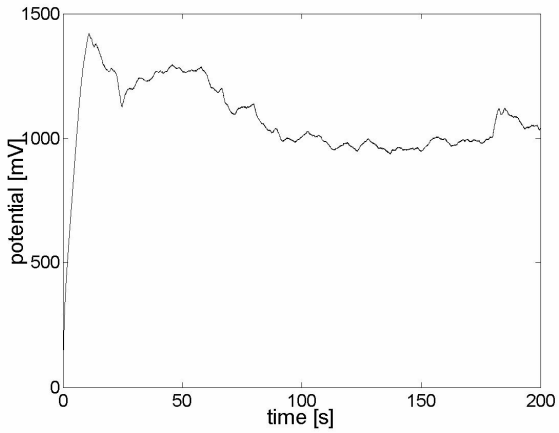

Figure 1: Electroporation under current-clamp conditions.

The fluctuations can be reduced applying another method based on the current-clamp electroporation – Chronopotentiometry After Current Clamp (CACC) (Fig.2) [4], in which the electropore is created under constant current and then maintained under constant voltage. Such an electropore remains stable because the current clamp electroporation provides an efficient method for stabilizing the electropore edge even if the pore is maintained under constant voltage afterwards.

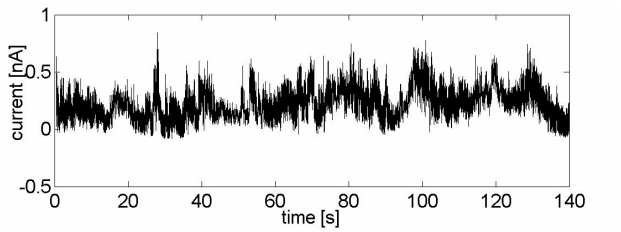

Figure 2: Stochastic fluctuations of the electro-nanopore in CACC electroporation.

CONCLUSIONS

Application of the current based methods to cells *in-vivo* could improve our control over the electroporation parameters and, possibly, enhance medical applications of the electroporation. Until now, both stabilizing methods have been studied mostly on planar lipid membranes and their usefulness for cell membranes needs further research.

REFERENCES

[1] S. Kalinowski, G. Ibron, K. Bryl, Z. Figaszewski, „Chronopotentiometric studies of electroporation of bilayer lipid membranes." *Biochim. Biophys. Acta*, vol. 1369, pp. 204-212, 1998.

[2] M. Robello, A. Gliozzi, "Conductance transition induced by an electric field in lipid bilayers." *Biochim. Biophys.* Acta, vol. 982, pp.173-176, 1989.

[3] M. Kotulska, S. Koronkiewicz, S. Kalinowski, "Self-similar processes and flicker noise from a fluctuating nanopore in a lipid membrane." *Phys. Rev. E.*, vol. 69, pp. 031920-30, 2004.

[4] M. Kotulska, "Natural fluctuations of an electropore show fractional Lévy stable motion." *Biophys. J.*, vol. 92, pp. 2412-21, 2007

Electrical module for commutating between high- and low- voltage pulses in gene electrotransfer of skin cells

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INTRODUCTION

Gene electrotransfer is a method using electric pulses to temporarily and reversibly permeabilize the cell membrane and to drive the DNA into the cell electrophoretically [1]. Recent experiments of gene electrotransfer suggested the use of a pulsing protocol, consisting of a high voltage, permeabilizing pulse, followed by a low voltage, electrophoretic pulse [2].

Electrical module for commutating between high and low voltage pulses or also known as voltage commutator presents a significant electrical module in realization of successful gene electrotransfer. Pulses are delivered through the microelectrodes to the skin cells. Final value of the amplitude of the voltage pulse is determined by a voltage commutator as a last component between microelectrodes and the rest of electrical components (Figure 1).

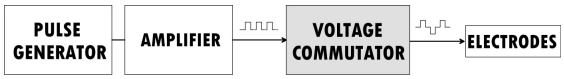

Figure 1: Block scheme of electrical components required for successful gene electrotransfer.

ELECTRICAL SPECIFICATIONS OF ELECTRICAL MODULE

For a successful gene electrotransfer it is important that a voltage commutator (Figure 2) meets the following requirements:

- Driving rectangular high voltage pulses with an amplitude up to 200 V and duration ranging between 20 µs and 20 ms.
- Driving rectangular low voltage pulses with an amplitude up to 20 V and duration ranging between 10 ms and 500 ms.
- Commutating between pulses at frequency ranging from 1 Hz to 5 kHz.
- Capability of switching between polarities of pulses.
- At least 10 individual outputs for microelectrodes.

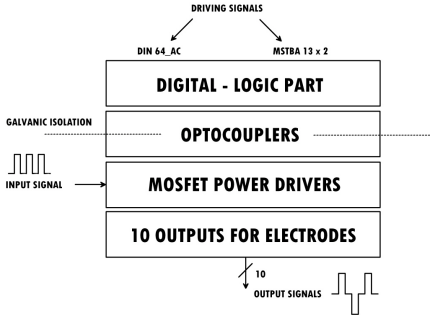

Figure 2: Block diagram of voltage commutator.

PERFORMANCE AND EXPERIMENTAL RESULTS

The demonstration of voltage commutator ability to commutate voltage pulses with required duration and amplitude was verified with the following experiment. We measured the voltage commutator performance of driving voltage pulses with maximum duration at certain amplitude of the pulse. At the beginning, we used one rectangular voltage pulse for an input signal with amplitude of 600 V. Due to the limited value of the capacitance of the voltage commutator capacitors, the amplitude of the output signal started to decrease after a certain time. Therefore, when we decreased the amplitude of the input signal, the duration of the output signal prolonged. We measured the maximum duration of the output signal at amplitudes decreasing from 600 V towards 0 V. The results are shown in figure 3. As we compare voltage commutator performance area with required high and low voltage pulse area, we can observe that voltage commutator is capable to meet the pulse duration and amplitude requirements of gene electrotransfer.

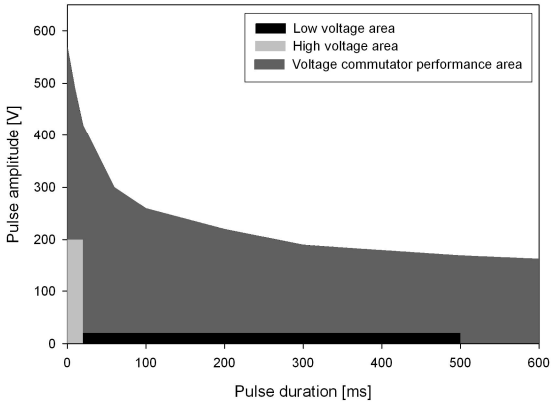

Figure 3: Voltage commutator performance area in comparison with required high and low voltage area of gene electrotransfer pulses.

REFERENCES

[1] S. Somiari, J. Glasspool-Malone, J. J. Drabick, R. A. Gilbert, R. Heller, M. Jaroszeki, R. W. Malone, Theory and in vivo application of electroporative gene delivery, *Molecular Therapy,* 2 (3) 178-187, 2000.

[2] S. Satkauskas, F. Andre, M. F. Bureau, D. Scherman, D. Miklavčič, L. M. Mir, Electrophoretic component of electrical pulses determines the efficacy of in vivo DNA electrotransfer, *Human Gene Therapy*, 16:1194-1202,200.

A First Approch to Microdosimetry Studies Related to Wide Band Signals

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INTRODUCTION

A fundamental task to approach the bioelectromagnetic interaction studies is to quantify the electromagnetic (EM) field distribution inside the biological target down to the microscopic cell level. This is important both for evaluating possible effects induced by the EM exposure and for looking at specific medical applications, as those based on nano-pulses. In particular a number of biological relevant phenomena has been associated to pulsed EM signals at plasmatic and sub-cellular membrane level. In this context a microdosimetric study based on a single spherical three-layered cell, able to deal with the main parameters involved in the solution of the EM problem (i.e., cell size or dielectric model) seems to be of interest.

METHODS

Here, the solution of the Laplace equation on a single cell model has been proposed to address the issue. Since it is necessary to describe the relaxation phenomena implied by the wide frequency content of the incident signals (up to tens of GHz in case of ultra short pulses). The cell model takes into account the dispersive properties of the dielectrics describing each of the three layers (cell membrane, cytoplasm, extra-cellular medium) through Debye equation and conductivity term. In order to highlight the importance of such proper modelling, a comparison with the EM solution based on a non-dispersive dielectric cell model is proposed. Variations due to the cell radius are also reported. The applied signal is transformed by DFT (Discrete Fourier Transform) in the frequency domain and at each frequency of the spectrum the EM solution is calculated, obtaining E field within the membrane. Afterwards an inverse DFT is used to re-construct the time behaviour of the membrane field.

RESULTS

The pulsed signal adopted was a bi-exponential one: amplitude 2×10^4 V/m, width 10 ns, rise time 1.5 ns. Fig. 1a shows the temporal behaviour of the EM field in the cell membrane obtained dealing with three different dielectric models [1, 2, 3], confirming that the choice of the dielectric properties plays a fundamental role in the final results. One of the models [1] accounts for a membrane with frequency independent behaviour. Conversely, the other two exhibit a frequency dependency: one with two relaxation terms [2] and the other with one [3]. For all the dielectric models the cytoplasm and extra cellular medium are described by a single relaxation term with slightly different values for the relaxation frequency value. The figure evidences good accordance between the E field results for the two frequency dependent dielectric models of the membrane. A strong difference is obtained in comparison with the model in [1]. The E field dependence on cell radius has also been studied. Fig 1b reports the comparison among the E field for three different radii (5, 10, 25 μm) considered within the range of human cells. The main differences are observed in the decay times related with the low frequency components of the incident signal; this result seems to be consistent since cell size is relevant to the EM field solution only in the low frequency range.

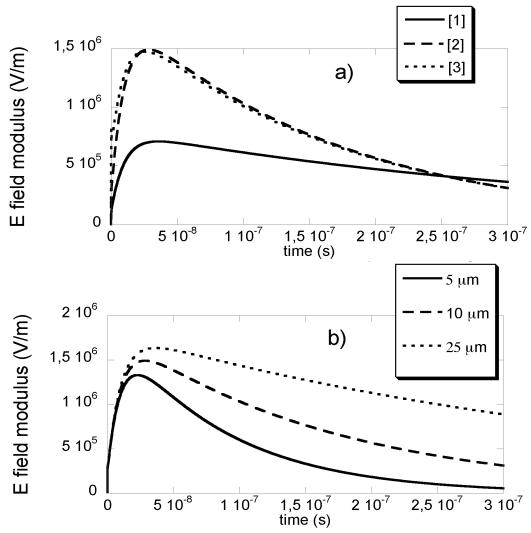

Figure 1: Time behaviour of the EM field in cell membrane: (a) dependence on different dielectric cell model adopted, (b) dependence on different cell radius considered.

CONCLUSIONS

A picture of the most important parameters acting on the EM field induced in cell membranes by a pulsed signal has been reported. The choice of the dielectric model resulted of great importance in case of accurate and quantitative analysis of pulsed EM field on biological structures.

REFERENCES

[1] L. M. Liu, S. F. Cleary, "Absorbed energy distribution from radiofrequency electromagnetic radiation in mammalian cell model: effect of membrane-bound water", *Bioelectromagnetics,* vol. 16, pp. 160–171, 1995.

[2] T. Kotnik and D. Miklavcic, "Theoretical evaluation of distributed power dissipation in biological cells exposed to electric fields", *Bioelectromagnetics*, vol. 21, pp. 385–394, July 2000.

[3] C. Merla, M. Liberti, A. Ramundo Orlando, F. Apollonio, G. D'Inzeo, "A theoretical and experimental methodology for identifying dielectric models of biological cells", *Proc. 16th International Zurich Symposium on Electromagnetic Compatibility, EMC,* Zurich, 2005.

Electro-Transfer *in vitro* and *in vivo* of siRNA into Tumor Cells

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INTRODUCTION

Gene silencing by RNA interference in mammalian cells through the use of siRNAs represents a powerful tool for reverse genetics in mammalians and possibly for gene therapy in humans. This is supported by accumulated published data describing the use of RNAi in the treatments against various diseases. Some of these new therapies were shown to involve off target responses such as undesirable immune reactions. It is thus of primary importance to study the biological mechanisms of transfer of molecules in cells to obtain a safe use in a whole organism.

The aim of this work is to study the electrotransfer of a synthetic siRNA directed specifically against mRNA of gene coding the GFP, expressed constitutively in a B16 model of murine melanoma.

RESULTS

The inactivation of the post transcription of gene coding the EGFP by electrotransfer of siRNA, results in a significant reduction in the percentage of cells expressing GFP and in the associated fluorescence intensity as well *in vitro* as *in vivo*. The results show a transient inactivation with a maximum 72 hours after the electrotransfer of the siRNA (under EGT conditions [1]).

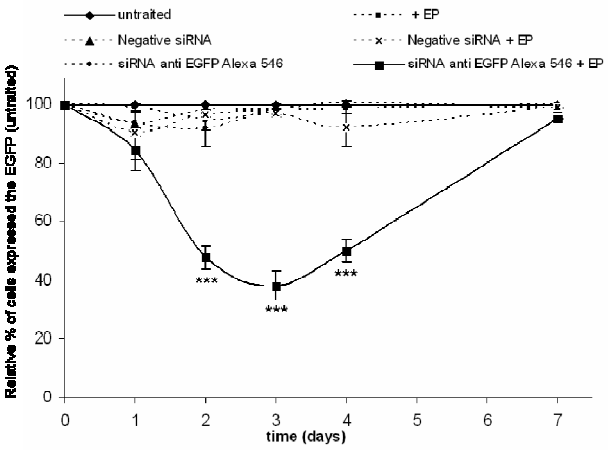

Figure 1: Effectiveness of siRNA electrotransfer *in vitro*.

The siRNA being labelled by the fluorophore Alexa 546, its cellular localization and its biodistribution in subcutaneous tumor cells iss studied by fluorescence microscopy. *In vitro* and in absence of electric field, the siRNA does not penetrate into the cells. When appropriated electric pulses are applied, the siRNA is found in diffuse form in the cytoplasm, where it is detected during 48 hours. The siRNA penetrates directly into the cell cytoplasm during the application of electric field, without interacting with the plasma membrane by a mechanism different from what was described with plasmid DNA [2].

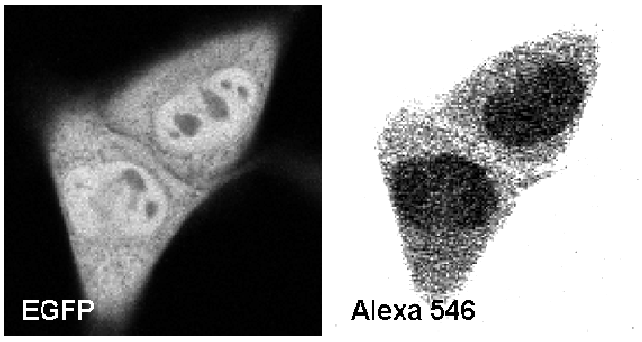

Figure 2: Localisation of siRNA-Alexa546 in electrotransfected cells.

The results obtained *in vivo* show that application of electric pulses, after intratumoral injection siRNA, is essential for penetrating into tumoral cells and for effective action on its intracellular target. SiRNA is visualized in a relatively homogeneous way in the electropermeabilized tumors. Moreover, a diffuse signal in cytoplasm of cells is observed on histological slices of tumors visualized in confocal microscopy 72h after electrotransfer.

The electropermeabilization of subcutaneous tumors is a promising development in cancer gene therapy by providing a targeted treatment in a relatively homogeneous way restricted on tumors. For example, it has successfully been used by others to silence the Mitf gene expression in mice tumors leading to reduction in the outgrowth of subcutaneous melanoma [23].

ACKNOWLEDGEMENTS

This work was supported by grants from the Region Midi Pyrenees, the AFM, the ANR, the ITAV and by a Slovenian French PICS Program.

REFERENCES

[1] Golzio M et al, In vivo gene silencing in solid tumors by targeted electrically mediated siRNA delivery, *Gene Therapy*, vol. 14, pp. 752-759, 2007.

[2] Golzio M, Teissié J and Rols MP, "Direct visualisation at the single cell level of electrically mediated gene delivery", *PNAS,* vol. 99, pp. 1292-1297, 2002.

[3] Nakai N, Kishida T, Shin-Ya M, Imanishi J, Ueda Y, Kishimoto S and Mazda O, "Therapeutic RNA interference of malignant melanoma by electrotransfer of small interfering RNA targeting Mitf", *Gene Therapy*, vol. 14, pp. 357-365.

Temperature based radiofrequency ablation

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INTRODUCTION

In this work we briefly present cardiac arrhythmias and their treatment, but the main goal of the study is to built realistic three-dimensional (3D) model of the human heart and to evaluate distribution of the temperature around the ablation catheter.

RADIOFREQUENCY ABLATION

Radiofrequency (RF) refers to alternating current in the range of 0.1 to 100 MHz. When used for ablation of cardiac arrhythmias, continuous, unmodulated sine wave outputs in the range of 100 to 750 kHz are used [1]. Electrical current flows from RF generator to the catheter, through the heart muscle to the external surface electrode on the patient's back. RF ablation is usually described with Penn's equation, which describes heat flow through the tissue [2]:

$$\rho c \frac{\partial T}{\partial t} = \nabla \cdot k \nabla T + J \cdot E - Q_h \qquad (1)$$

where T is the temperature of heart muscle [ºC], ρ specific density [kg/m^3], c specific heat [J/kgºC], k heat conductivity [W/mºC], J current density [A/m^2], E intensity of electrical field [V/m] and Q_h heat loss because of the blood flow [W/m^3].

METHOD

3D model was designed using images from magnetic resonance scan of a human chest. 15 consecutive two-dimensional images, 7 mm apart, were used to constructed geometry of the heart muscle and the right atrium with the veins. Both objects were built by 3D Doctor software (Able Software Corp.) and imported into Femlab (COMSOL environment) (see Figure 1).

Object that presents the right atrium, was imported into the object that presents the heart muscle. With this procedure we were able to get the heart muscle and the cavity of the right atrium. We presumed that the cavity was filled with blood and consecutively we got two different physical properties for both objects [3]. The reason for designing only two objects was because the changes of temperature are small and localized. Also, time dependence was not considered.

Tip of the ablation catheter we presented with one selected surface finite element, located between the inner heart muscle wall and the right atrium. The electrical field and convection were neglected. We solved equation [4]:

$$\nabla \cdot k \nabla = 0 \qquad (2)$$

where k is heat conductivity [W/mºC].

After we defined physical properties and edge conditions for the model, model was automatically meshed with combined 85064 elements.

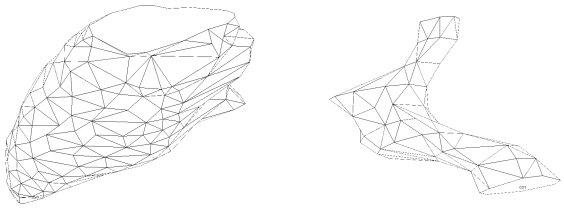

Figure 1: 3D objects designed in 3D Doctor and transferred into Femlab. Left – the heart muscle, right – the right atrium with veins.

RESULTS

We evaluated temperature distribution for three different catheter tip temperatures. The temperature, which made irreversible damage of the tissue, is T > 50 ºC [1]. Two parameters were evaluated: length and depth of the ablation lesion.

We measured the length of the surface lesion (d) from the edge of the catheter to the temperature tissue border T > 50 ºC. At the temperature (tip of the catheter) T = 55 ºC (70 ºC) the length was d ~ 0.8 mm (1,5 mm). In case of T = 55 ºC and k increased for 10 % in radius tendency (tissue fibre direction) the length was d ~ 0,8 mm.

Lesions in depth (h), from surface to temperature border T > 50 ºC, were measured with the same catheter tip temperatures. The results were h ~ 1,2 mm (at T = 55 ºC in both cases) and h ~ 1,2 mm (at T = 70 ºC).

CONCLUSIONS

The goal of this study was to build a 3D model of the heart and to show temperature distribution near the ablation catheter. Our results are in line with the results obtained by other researchers [1]-[3]. Complexity of the model was the main limitation for even better numerical computation of temperature distribution in the tissue.

REFERENCES

[1] S. J. Kalbfleisch, J. J. Landgberg, "Catheter Ablation with Radiofrequency Energy: Biophysical Aspects and Clinical Applications," in Journal *of Cardiovascular Electrophysiology*, Vol. 3, No. 2, 1992.

[2] J. Berjano, F. Hornero, "Thermal-electrical modeling for epicardial atrial radiofrequency ablation," in *IEEE, Transactions on biomedical engineering*, Vol. 48, No. 4, 2001.

[3] S. Tungjitkusolmun, D. Haemmerch, H. Cao, J. Z. Tsai, Y. B. Choy, V. R. Vorperian, J. G. Webster, "Modeling bipolar phase-shifted multielectrode catheter ablation," in *IEEE Transactions on biomedical engineering*, Vol. 49, No. 1, 2002.

[4] Femlab V3.1, Comsol Multiphysics.

Optimal Parameters in Electroporation of Malignant Cells

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INTRODUCTION

We intend to perform experiments on membrane parameters and apoptosis of electroporated cells. First we optimized the electrical parameters (pulse amplitude, pulse duration and number of pulses) for our electroporation set-up and the B16F10 cell line in order to obtain the highest percentage of porated and viable cells. The poration yield and the viability of electroporated cells were evaluated by the Propidium Iodide (PI) uptake [2].

MATERIAL AND METHODS

B16F10 Murine metastatic melanoma cells were used for electroporation experiments in suspension of 1×10^6 cell/ml. The attached cells were first washed with EBSS (Earl's buffer salt solution) twice, then detached with trypsin (1 min, 0.5g/L) [1]. After centrifugation (200g, 5 min) the pellet was suspended in Manitol solution, with a pH of ~ 7.3 and a conductivity of ~ 4.8 mS/cm. The electroporation was done in cuvettes (BioRad, 2 mm gap) using an Eppendorf electroporator that delivers only exponential decay electric pulses (Hofmann G.A. et al, 2000).

Protocols of 1 or 2 pulses of 100 μs or 200 μs length and electric field intensity ranged from 1 to 5kV/cm were studied. The 0.5mM PI was added before and 10min after the pulse delivery. The labelled cells were counted under a luorescence microscope (Zeiss Axiovert 200) equipped with an appropriate filter set. The percentage of viable porated cells was calculated by subtracting the cells labelled 10min after the pulse delivery from the bulk labelled cells obtained by pulse delivery in the presence of PI.

RESULTS

We obtained the highest percentage of viable porated cells for one pulse of 100 μs of the amplitude of 2.5 to 3 kV/cm.

CONCLUSIONS AND PERSPECTIVES

We exploited the handy PI protocol to find the optimal electrical parameters for electroporation of B16F10 cells in our experimental set-up. Fluorescence depolarization measurements (using TMA-DPH) will be subsequently performed to determine the membrane fluidity of the cells before and after electropermeabilization [3]. Similarly cell membrane water content will be checked by generalized polarization measurements (with Laurdan) in order to evaluate the membrane biophysical parameters of electroporated cells. In parallel the cell apoptosis will be quantified by classical methods (Annexin V, "DNA diffusion" assay).

REFERENCES

[1] Pavlin M, Leben V, Miklavcic D. Electroporation in dense cell suspension – Theoretical and experimental analysis of ion diffusion and cell permeabilization. *Biochim. Biophys. Acta* 1770: 12-23, 2007.

[2] Pucihar G, Kotnik T, Teissié J, Miklavcic D. Electroporation of dense cell suspensions. *Eur. Biophys. J.* 36: 173-185, 2007.

[3] Roxana Pologea-Moraru, Eugenia Kovacs, Karina Roxana Iliescu, Violeta Calota, Gheorghe Sajin The effects of low level microwaves on the fluidity of photoreceptor cell membrane. *Bioelectrochemistry* 56 (2002) 223-225.

Electrical characterization of electroporation cuvettes used for the application of nanosecond electric pulses on cells

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INTRODUCTION

Nanosecond pulsed electric fields (nsPEF) are a new kind of electric pulses used in biology. Several studies showed effects of the nsPEF on cells (from 10kV/cm to 150kV/cm in the range of 10 ns to 300ns) [1-2]. Among these results, a figure reported that the GFP electrotransferred gene expression could be increased 3 times under some conditions (10ns, 150 kV/cm and 30 min between the electrotransfer and the application of the nsPEF) [1]. Our team has started to work to replicate these results on cells *in vitro* but with DNA coding for the luciferase.

During the first experiments, we observed a variability in the biological results depending on the pulses delivered (as a function of the electrical parameters : pulses number, repetition frequency...) as we expected, but not only. It appeared that the results we obtained depended also on the material configuration of the electrical system for the nsPEF application (FID Technology, Russia) and on the kind of electroporation cuvettes we used (Molecular BioProducts (MBP), CytoPulse Science (CPS) and Bridge). To obtain the same normalized expression of luciferase in MBP 1mm-gap cuvettes and in CPS 2mm-gap cuvettes, we had to apply respectively 1nsPEF and 20nsPEF under the same experimental (electrical parameters) conditions.

The aim of our study was to characterize the electroporation cuvettes, which are used here outside of their initial application field.

METHODS

Impedance of electroporation cuvettes was measured with an impedancemeter HP4194 and its probe HP41941A (bandwidth from 10kHz to 100MHz). We compared cuvettes of MBP and CPS, respectively 1mm-gap and 2mm-gap, containing respectively 50µL and 200µL of solution of sucrose and cells (10^6 cells per cuvette). Results were analysed with Matlab™.

Energy was calculated with the Fast Fourier Transform (FFT) algorithm of Matlab™ applied on the acquisition of the delivered signal on cuvettes. This signal was acquired with an oscilloscope LeCroy WavePro 7000 and two high voltage probes Tektronik P6015A.

Electric fields were modelled and simulated by the finite element method (FEM) with Comsol Multiphysics 3.3™.

RESULTS

Results show an increase of 20% in the impedance between the 1mm-gap MBP cuvettes and the 2mm-gap CPS cuvettes. This increase seems to be caused by the difference of distance gap and the difference of volume of solution.

The increase in the energy received by the cuvettes seems to be of the same order than the variation of impedance between the two cuvettes reported here above.

But these two results cannot explain the dispersion of the pulses number that we have to apply to obtain the same biological result (a factor 20 between the MBP 1mm-gap cuvettes and the CPS 2mm-gap cuvettes). Calculated energy was total energy and it corresponds to the sum of at least??? two terms : losses by Joule effect and the energy directly absorbed by the cells.

FEM analysis did not show any interesting results yet, due to the lack of mathematical models of new appeared phenomenon linked to the application of nsPEF and high voltage amplitude pulses on an aqueous solution with metal electrodes (the aluminium electrodes of the electroporation cuvettes).

CONCLUSION

These three electrical analysis can't explain the dispersion in biological results between experiences using 1mm-gap MBP cuvettes and 2mm-gap CPS cuvettes. This lack of explanation is mainly due to precarious models of the electroporation cuvettes in the new use we made of them. Some linked phenomena, like electrolysis along aluminium electrodes [3] which can promote the salting out of ions in the solution, have not yet been modelled with classical tools.

REFERENCES

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

[2] S.J. Beebe, J.A. White, P.F. Blackmore, Y. Deng, K. Somers and K.H. Schoenbach, "Diverse effects of nanosecond pulsed electric fields on cells and tissues", *DNA Cell Biol*, vol. 22, pp. 785-796, 2003.

[4] [3] F. Loste, Etude biophysique de l'intéraction des ondes électromagnétiques GSM avec les gliomes C6, Thesis, University Paul Sabatier de Toulouse, 2000.

Gene Electrotransfer as Therapy for Psoriasis Vulgaris

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INTRODUCTION

Psoriasis represents a chronic inflammatory skin disease with an approximate prevalence of 3% in Scandinavian countries [1]. The symptoms vary from mild to severe. Despite that psoriasis primarily is a lymphocyte driven disease the prominent dermal microvascularity suggest that psoriasis is angiogenesis-dependent [2,3]. Therapy of severe psoriasis often involves a systemic treatment regime, which if used over a period may cause serious side effects. Treatment regimes with minimal side effects are needed. Gene electrotransfer therapy is a possible novel treatment candidate.

By applying an appropriate electrical field to the cells, they become reversibly permeable (electroporated) and by applying a constant electric field (electrophoresis) charged molecules like siRNA, miRNA, RNA and DNA, can enter the cells (gene electrotransfer). The standard operating procedure applied to *in vivo* tissues considering one high and one low voltage pulse is suggested to be efficient and not to cause any gene expression alterations and therefore to be considered safe in a clinical perspective[4-6]. An anti-angiogenic gene administered by gene electrotransfer therapy applied to the skin is suggested as a novel treatment candidate of psoriasis, and will be carried out in two different human SCID mouse xenograft models for psoriasis vulgaris.

MATERIALS AND METHODS

SCID Chase Fox mice were xenografted with psoriatic vulgaris skin biopsies (1.5 cm x 1.5 cm). Fourteen days after transplantation the grafts were treated with plasmid pCAGGS-lacZ (50 µg) and electroporated with a 4x2 needle electrode (length 18 mm and 4 mm distance) applying eight pulses of 1000 V/cm 100µs. Two days after the gene electrotransfer the mice were euthanized and the grafts were harvested in formaldehyde. ß-Galactosidase expression was determined with X-gal for 24 hour and incubated in sodium-azide in three weeks. The grafts were paraffin embedded and counterstained with haematoxylin and eosin.

PRELIMINARY RESULTS AND DISCUSSION

Figure 1 indicates β-Galactosidase was expressed in the endothelial cells and fribroblasts of the dermis on day 2 after the pCAGGS-lacZ electrotransfer. These preliminary results indicate that gene electrotransfer is a possible novel treatment candidate for psoriasis.

Skin-targeted gene electrotransfer may be suitable not only for chronic diseases but also for acute diseases requiring the short-term systemic delivery of a therapeutic protein.

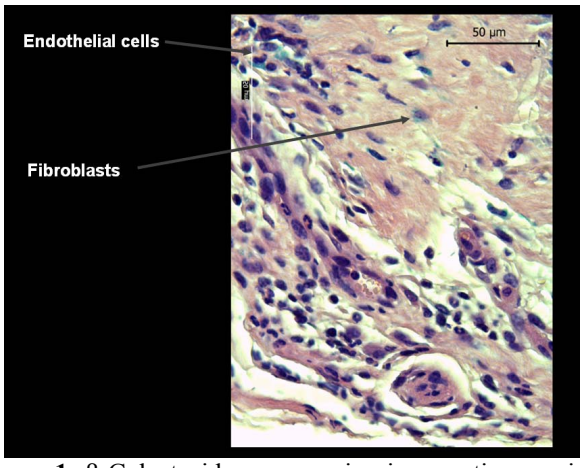

Figure 1: β-Galactosidase expression in an active psoriatic graft.

ACKNOWLEDGEMENTS

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REFERENCES

[1] G. LOMHOLT, "Prevalence of skin diseases in a population; a census study from the Faroe Islands," *Dan. Med Bull.*, vol. 11, pp. 1-7, Feb.1964.

[2] J. N. Barker, "The pathophysiology of psoriasis," *Lancet*, vol. 338, no. 8761, pp. 227-230, July1991.

[3] J. Folkman, "Angiogenesis in psoriasis: therapeutic implications," *J Invest Dermatol.*, vol. 59, no. 1, pp. 40-43, July1972.

[4] P. Hojman, J. R. Zibert, H. Gissel, J. Eriksen, and J. Gehl, "Gene expression profiles in skeletal muscle after gene electrotransfer," *BMC Mol. Biol.*, vol. 8, p. 56, 2007.

[5] N. Pavselj and V. Preat, "DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse," *J. Control Release*, vol. 106, no. 3, pp. 407-415, Sept.2005.

[6] D. Cukjati, D. Batiuskaite, F. Andre, D. Miklavcic, 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, May2007.

Modeling the electrophoresis and molecule cell membrane interaction in electroporation experiments

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INTRODUCTION

Electropermeabilization is a physical process based on the application of electrical pulses, which can induce a transient permeabilization of the cell membrane. That permeability increase allows different types of molecules to enter the cell, thus giving this technique a great potential in cancer treatment or in gene therapy [1,2].

Here, we model the transport of charged molecules in electroporation experiments and the interaction of molecules with spherical cells or vesicles in the system.

MODEL

With σ denoting the local electrical conductivity, Laplace's equation

$$\nabla \cdot (\sigma \nabla \varphi) = 0 \qquad (1)$$

for the electric potential φ between two electrodes with a spherical cell membrane in between is solved numerically in a number of cases, with and without pores.

It is assumed that the molecules have an effective electrophoretic charge (which can be estimated both experimentally and theoretically) and that their motion is dominated by Brownian motion and a drift caused by the electric field.

The cell membrane is modeled as a large potential barrier which prevents the molecules from entering the cell. The total energy of a molecule is denoted by E which is a sum of the electrostatic energy and the membrane barrier potential.

SIMULATIONS

This model is analysed both by solving the resulting diffusion equation for the concentration *c* of molecules

$$\frac{\partial c}{\partial t} = D\nabla \cdot (\nabla c + \beta c \nabla E) \qquad (2)$$

with the finite element software *COMSOL,* and via Monte Carlo simulations of the corresponding stochastic differential equation for a molecule position $\mathbf{x}(t)$

$$\mathbf{dx}(t) = -\beta D \nabla E dt + \sqrt{2D}\mathbf{dW}(t) \qquad (3)$$

The picture in (Figure 1) is an example of what is obtained while using *COMSOL* and (Figure 2) shows the positions of molecules for the second type of simulations.

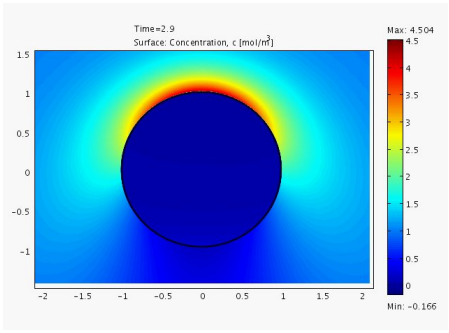


Figure 1: Concentration *c* of molecules

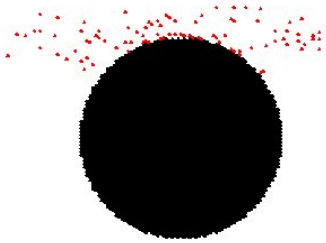


Figure 2: Positions of the molecules

RESULTS

When the membrane is not modified by the electric field we find that the molecules are pushed up against the membrane and then flow around the cell.

We also propose a mechanism to model the phenomenon of electroporation based on a lowering of the membrane barrier potential due to the local electric field. For sufficiently high applied fields this leads to an interaction of the molecules with the membrane.

CONCLUSION

It is hoped that this dynamical model can be exploited to optimise the efficiency of electrotransfer protocols. Indeed, a better understanding of mechanisms involved in electroporation would help to guarantee the safety of new therapies.

REFERENCES

[1] C. Favard, D.S. Dean and M.-P. Rols, "Electrotransfer as a non viral method in gene delivery" *Current Gene Therapy*, vol. 7, pp. 67-77, 2007.

[2] J.-M. Escoffre, D.S. Dean, M. Hubert, M.-P. Rols and C. Favard, "Membrane perturbation by an external electric field : a mechanism to permit molecular uptake" to appear in *European Biophysics Journal, 2007*

WORKBOOK

Laboratory safety – Read Carefully!

***Duša Dolenc**; University of Ljubljana, Faculty of Electrical Engineering*

BEFORE ADMITTED TO THE LABORATORY WORK YOU NEED TO PASS THE LABORATORY SAFETY TEST ON THE INTERNET: www.cliniporator.com/ect/safetytest.asp

CHEMICAL HAZARD SYMBOLS

Be aware what you are working with; know the symbols of chemicals and how to handle them to prevent any accidents!


(T) Toxic


(C) Corrosive

(T^+) Very toxic


(E) Explosive


(O) Oxidizing

(F^+) Extremely flammable

(Xi) Irritant


(F) Highly flammable


(Xn) Harmful


(N) Dangerous for the environment

When working with laboratory equipment be careful not to get electrocuted.

OTHER SAFETY PRECAUTIONS

1. Wear laboratory coat and gloves. Wear shoes (rather than sandals) and tie back loose hair.
2. **Never taste laboratory materials.** Food or drinks should not be brought into the laboratory. If you are instructed to smell something, do so by fanning some of the vapor toward your nose. Do not place your nose near the opening of the container.
3. Be careful not to spill cell medium or microbe culture on the bench, floor or clothes. Do not touch cell suspensions or colonies with bare hands. **Contaminated surface should be covered with paper towels and sprayed over with disinfection solution; we leave it to work for at least 20 minutes.** Contaminated spots on clothes or body can be sprayed and washed with water.
4. Contaminated material should be put into special containers or designated area before autoclaved.
5. Every solution you make and want to store should be properly labeled (name, date, contents).
6. After you finish with your work put everything back to its place and disinfect the surfaces you worked on.
7. Wash your hands before and after your work in the laboratory. Disinfect your hands if necessary.
8. The work in laboratories is not permitted without the presence of an instructor.

NOTES

L1 - Gene electrotransfer with different electric field orientations

Mojca Pavlin, Saša Haberl, Matej Reberšek; *University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiments: appr. 2×45 min
Max. number of participants: 4
Location: Cell Culture Laboratory 1

THEORETICAL BACKGROUND

Gene electrotransfer is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses - electroporation. An exposure of a cell to an adequate amplitude and duration of electric pulses leads to a temporary increase of the cell membrane permeability which allows various otherwise nonpermeant molecules, including DNA, to cross the membrane and enter the cell.

Efficiency of the gene electrotransfer depends on variety of parameters: cell type, temperature, parameters of electric pulses, post-pulse manipulation, DNA concentration and electroporation buffer. The mechanism of the process are not fully explained, however it was shown that three steps are crucial for gene electrotransfer: interaction of DNA molecules with the cell membrane, translocation and expression. The surface of the cell where DNA interacts with the membrane can be increased by changing electric field orientation between the consecutive pulses. Since DNA molecule is a negatively charged molecule it is dragged due to the electrophoretic force in the opposite direction of the electric field thus making contact with the cell membrane on one side of the cell surface. If the direction of the electric field is reversed DNA molecules interact with the cell surface also on the other side which increases total transfection efficiency.

The aim of this practical exercise is to demonstrate and present a concept and system (electroporator and electrodes) for pulse delivery that improves gene electrotransfer by automatic change of electric field direction between the electrical pulses.

EXPERIMENT

Chinese hamster ovary cells (CHO) are grown in multiwells as a monolayer culture in culture medium (HAM-F12) at 37° C. Cells are plated 24 hours before the experiment in concentration 4×10^4 cells per well. On the day of the experiment remove culture medium and replace it with 150 µl of electroporative buffer (10 mM phosphate buffer Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose; pH = 7.4) containing plasmid DNA (pEGFP-N_1) that codes for GFP (green fluorescent protein) with concentration 10 µg/ml. Incubate cells with plasmid for 2-3 minutes at the room temperature. Then apply a train of eight electric pulses with amplitude of 200 V (+100 V, -100 V), duration 1 ms and repetition frequency 1 Hz to each sample. Cells in the control are not exposed to electric pulses. Use two different electric field protocols: single polarity (SP) – the direction of the electric field is the same for all consecutive pulses (Fig. 1a) and orthogonal both polarities (OBP) – the direction of the electric field is changed between the pulses as shown in Fig. 1b.

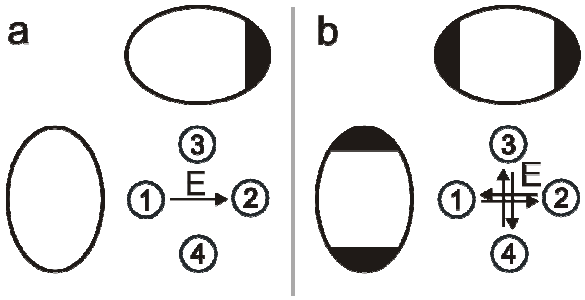


Figure 1: Two different pulsing protocols which will be used: a) single polarity (SP) and b) orthogonal both polarities (OBP). Dark regions schematically represent regions where DNA molecules interact with the cell membrane.

After exposure of cells to electric pulses add 37 µl fetal calf serum (FCS). Incubate treated cells for 5 minutes at 37° C and then add 1 ml of culture medium. After 24h of incubation at 37° C determine the efficiency of gene electrotransfer by fluorescent microscopy (Zeiss 200, Axiovert, Germany).

View samples using a fluorescence microscope at 20x magnification using GFP filter with excitation at 488 nm. Count number of cells under phase contrast and number of fluorescent cells. Determine the percentage of fluorescent cells for both orientations of the applied electric field.

FURTHER READING:

1. Reberšek M, Faurie C, Kandušer M, Čorović S, Teissié J, Rols MP, Miklavčič D. Electroporator with automatic change of electric field direction improves gene electrotransfer in vitro. *Biomed. Eng. Online* 6: 25, 2007.
2. Golzio M, Teissie J, Rols MP. Direct visualization at the single-cell level of electrically mediated gene delivery. *PNAS* 99: 1292–1297, 2002.

NOTES & RESULTS

Electric field protocol	Single polarity	Orthogonal both polarities
Number of cells		
Number of fluorescent cells		
[%]		

L2 - Monitoring cell membrane electropermeabilization with ratiometric fluorescence dye Fura-2AM

***Gorazd Pucihar**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiments: appr. 45 min
Max. number of participants: 4
Location: Cell Culture Laboratory 1

THEORETICAL BACKGROUND

Exposure of a biological cell to a sufficiently strong external electric field leads to a detectable increase of the membrane permeability, a phenomenon termed electropermeabilization. Because it is assumed that an increased permeability is related to the occurrence of hydrophilic pores in the membrane, the phenomenon is often termed electroporation. Provided that the parameters of the electric field (amplitude, duration, number of pulses, frequency) are moderate, the increased permeability is reversible, and cells recover within a few minutes after the exposure. During the state of high permeability the molecules for which the membrane is otherwise impermeable (e.g. drugs, DNA) can be transported across the membrane. Electropermeabilization is nowadays used in biochemistry, molecular biology, and different fields of medicine. It has already become an established method in oncology for electrochemotherapy of tumors, and holds great promises in gene therapy.

The efficiency of electropermeabilization is influenced by the parameters of the electric field, cell size and geometry, and physiological characteristics of the medium surrounding the cell. Different fluorescence dyes (e.g. Propidium Iodide, Lucifer Yellow, Fura-2, Fura-3,…) can be employed to investigate the influence of these parameters on electropermeabilization and the same dyes can be used to monitor electropermeabilization.

EXPERIMENT

You will monitor cell membrane electropermeabilization using a fluorescent calcium sensitive dye Fura-2AM. From the images acquired during the experiment, you will try to determine the effect of cell size and cell shape on the efficiency of electropermeabilization.

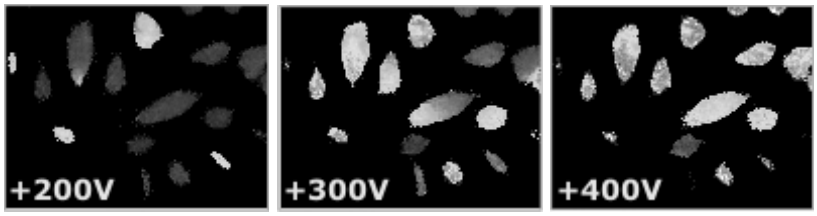


Figure 1: Cells stained with Fura-2AM and exposed to electric pulse with increasing amplitude.

Protocol: The experiments are performed on Chinese hamster ovary cells (CHO) grown on a cover glass in the culture medium (HAM-F12) containing 8% fetal calf serum, 0.15 mg/ml L-glutamine, 200 units/ml crysticillin and 16 µg/mg gentamicin in 5% CO_2 at 37°C. Plate 2×10^4 cells/ml on cover glass of Lab-Tek chamber and keep them for 24 hours in the incubator. Before experiments, replace the culture medium with SMEM medium containing 1.5 µM Fura-2 AM and 0.02 % pluronic acid. After 30 minutes of incubation at 21°C wash the cells with pure SMEM and add HAM-F12 (containing 441 mg/l of $CaCl_2 2H_2O$). Calcium ions, present in the extracellular medium, do not readily cross an intact (nonpermeabilized) cell membrane. Electropermeabilization results in the entry of Ca^{2+} ions into the cells, where the binding of Ca^{2+} to Fura-2AM changes the excitation and emission spectrum of the dye.

Place two parallel Pt/Ir wire electrodes with a 4 mm distance between them to the bottom of the chamber. Using a Cliniporator device, deliver one electric pulse of 100 µs with voltages varying from 150 to 300 V. Immediately after the pulse, acquire two fluorescence images of the cells at 540 nm, one after excitation with 340 nm and the other after excitation with 380 nm. Divide these two images in MetaFluor to obtain the ratio image ($R = I_{340}/I_{380}$). Wait for at least 3 minutes and apply a pulse with higher amplitude. Observe which cells become electropermeabilized first.

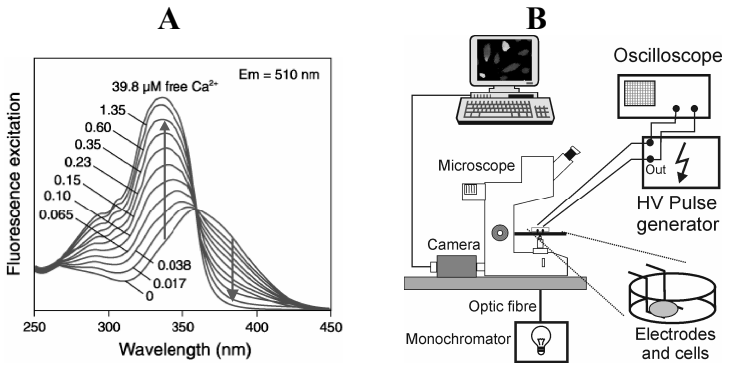


Figure 2: (A) Fluorescence excitation spectra of Fura-2 for different concentrations of Ca2+ (image from *http://probes.invitrogen.com/handbook/figures/0554.html*). (B) Schematic of the experiment.

FURTHER READING:

1. Grynkiewicz, G., M. Poenie, and R.Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450, 1985.
2. Teruel M.N., and T. Meyer. Electroporation-induced formation of individual calcium entry sites in the cell body and processes of adherent cells. *Biophys. J.* 73:1785-1796, 1997.
3. 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.

NOTES & RESULTS

voltage [V] E [V/cm]	150 (375)	200 (500)	250 (625)	300 (750)
Number of permeabilized cells				
permeabilization [%]				

L3 – Electropermeabilization detection with propidium iodide

***Maša Kandušer, Marko Ušaj**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiments: appr. 45 min
Max. number of participants: 4
Location: Cell Culture Laboratory 2

THEORETICAL BACKGROUND

When the cell is exposed to electric field above the threshold value, its membrane is permeabilized. Increasing amplitude of electric pulses increases propidium iodide uptake into the cell and the number of cells that are permeabilized (Figure 1). Intact cell is not permeable for propidium iodide and is therefore non-fluorescent; enhanced fluorescence is a direct consequence of cell membrane permeabilization and binding of propidium iodide to nucleic acids in the cell nucleus.

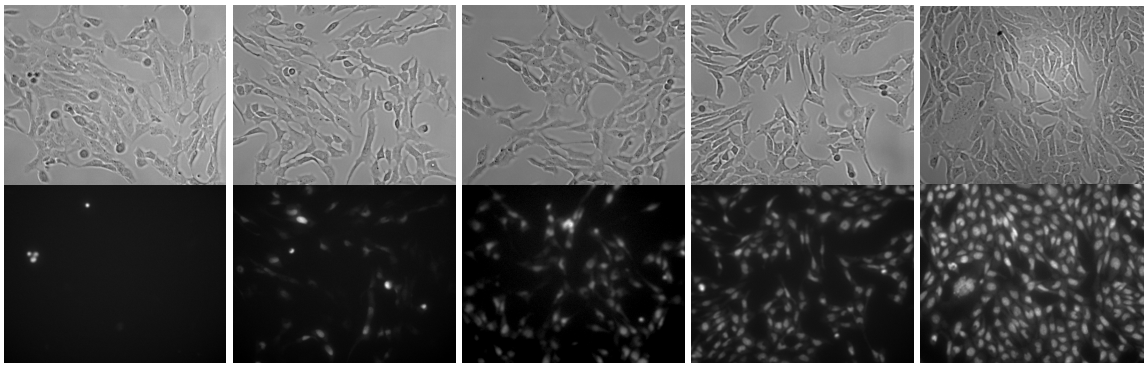


Figure 1: Sequence of phase contrast (top) and fluorescence images of cells (bottom) obtained during electropermeabilization: from negative control (on the left) to permeabilization at maximum pulse amplitude (on the right).

EXPERIMENT

You will detect cell membrane electropermeabilization using fluorescent dye Propidium iodide. From the data obtained during experiment, you will determine the effect of pulse amplitude on the efficiency of electropermeabilization.

Protocol: Chinese hamster ovary cells (CHO), plated previous day in 24 multiwell dish in concentration $2.5*10^5$ cells per well, are used for the experiment. Cells are attached to the culture dish surface. As medium for electropermeabilization isosmolar buffer with pH 7.4, composed of 10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$ and 250 mM sucrose is used. Immediately before electric pulse application 0.15 mM propidium iodide is added to the medium. For electropermeabilization electrodes with 5 mm distance between the electrodes are used and electric pulses are applied with electric pulse generator Cliniporator.

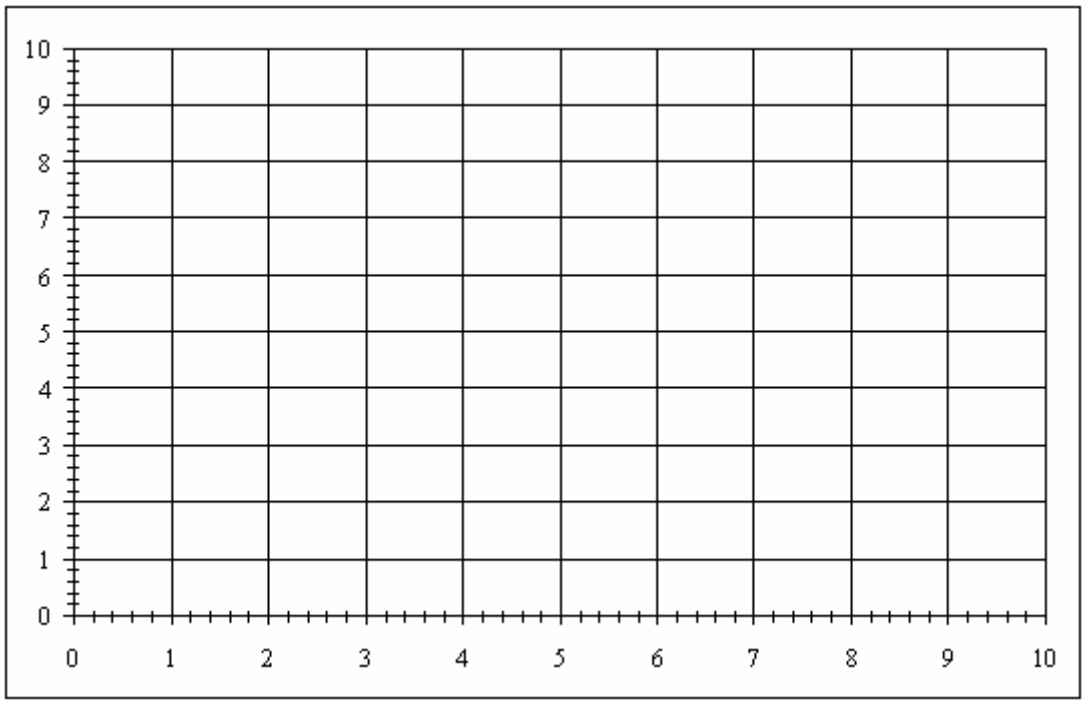
Remove the growth medium from the cell culture and replace it with 300 µl of electropermeabilization medium that contains 0.15 mM propidium iodide. Apply electric pulses for treated cells and no electric pulses for control cells. Electric pulse parameters are: 8 pulses, duration 100 µs and repetition frequency 1Hz. The pulse amplitude is increased from 0 V for control treatment, to 200 V, 300 V, 400 V and 500 V. The 0 V represents negative control as its florescence is not related to increased cell membrane permeability. For positive control permeabilize cells with 1 % Triton X-100, at this concentration all the cells are permeabilized and represent maximum uptake of propidium iodide. Leave the cells for 5 to 10 minutes at room temperature than replace the medium with 1 ml of fresh electropermeabilization medium. Determine fluorescence intensity for different parameters of electric field in spectrofluorometer. Excitation λ for propidium iodide is 490 and emission λ is 620 nm. From the data obtained calculate the percentage of permeabilized cells. Negative control represents 0 % permeabilization cells while positive control represents 100 % permeabilization.

FURTHER READING:

1. Rols M.P. Electropermeabilization, a physical method for the delivery of therapeutic molecules into cells. *Biochim. Biophys Acta* 1758: 423-428, 2006.
2. Sixou S, Teissie J. Exogenous uptake and release of molecules by electroloaded cells: a digitized videomicroscopy study. *Bioelectrochemistry and Bioenergetics* 31: 237-257, 1993

NOTES & RESULTS

voltage [V] E [V/cm]	0 (0)	200 (400)	300 (600)	400 (800)	500 (1000)	1% Triton X-100
raw data [RFU]						
permeabilization [%]						



L4 – Electric field orientation and uptake of propidium iodide

***Maša Kandušer, Marko Ušaj, Matej Reberšek**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiments: appr. 45 min
Max. number of participants: 4
Location: Cell Culture Laboratory 2

THEORETICAL BACKGROUND

The induced transmembrane potential is position dependant and reaches maximal values at the poles of the cell facing the electrodes. Besides, it is dependent on the shape and orientation of the cell exposed to the external electric field. Changing the orientation of electric field improves the efficiency of electropermeabilization (Figure 1).
Fluorescence probe propidium iodide is membrane unpermeant and generally excluded from viable cells. When cell membrane is permeabilized we detect the uptake of propidium iodide and enhanced fluorescence due to its binding to nucleic acids in the cell nucleus.

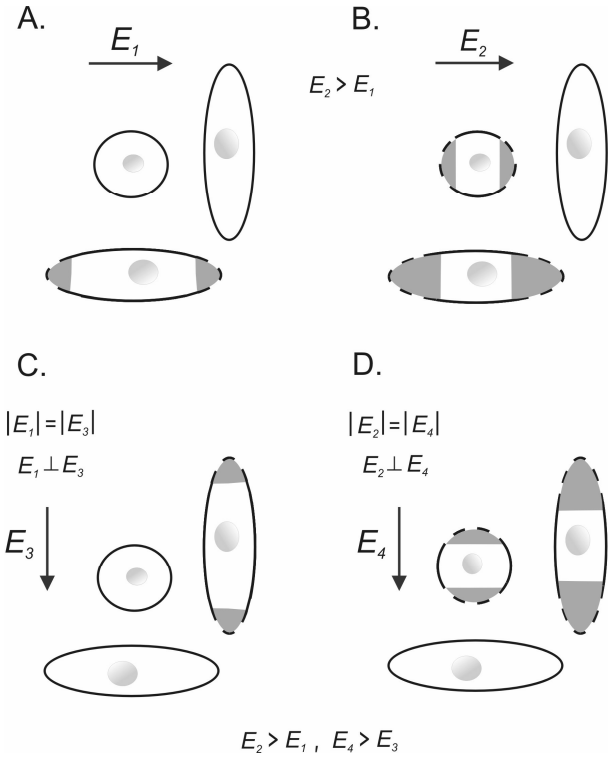


Figure 1: The positions dependence of the induced transmembrane potential and the effect of the cell orientation in the external electric field.

EXPERIMENT

You will detect the effect of electric field orientation on the uptake of fluorescent dye Propidium iodide at two different electric pulse amplitudes. From the data obtained during experiment, you will determine the effect of changing the orientation of electric field on the efficiency of electropermeabilization.

Protocol: Chinese hamster ovary cells (CHO) plated previous day in 24 multiwell dish in concentration 2.5×10^5 cells per well are used for the experiment. Cells are attached to the culture dish surface. As medium for electropermeabilization isosmolar buffer with pH 7.4, composed of 10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$ and 250 mM sucrose is used. Immediately before electric pulse application 0.15 mM propidium iodide is added to the medium. For electropermeabilization electrodes with 5 mm distance between the electrodes are used and

electric pulses are applied with prototype electric pulse generator that enables electric field orientation during pulse application.

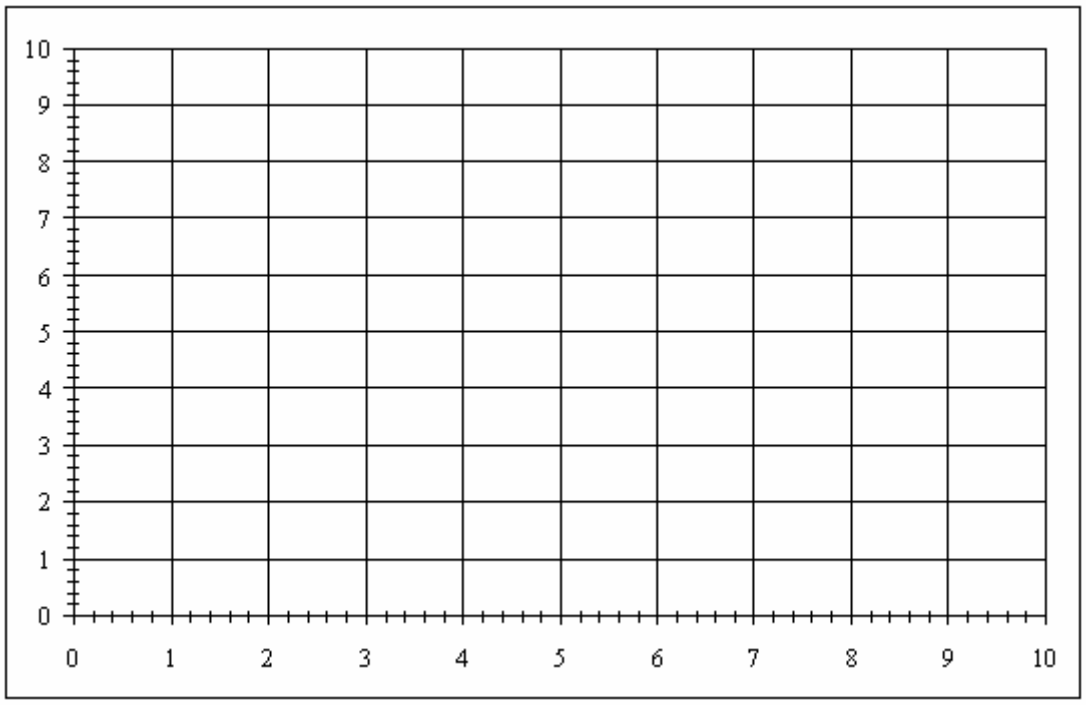
Remove the growth medium and replace it with 300 µl of the medium for electropermeabilization that contains 0.15 mM propidium iodide. Apply electric pulses for treated cells and no electric pulses for control cells. Electric pulses: 8 pulses, duration 100 µs, repetition frequency 1Hz, pulse amplitudes: 0 V (control), 200 V, 300 V in one direction or apply two pulses, in each of four different directions of electric field. For positive control permeabilize cells with 1 % Triton X-100, at this concentration all the cells are permeabilized and represent maximum uptake of propidium iodide. After electropermeabilization wait for 5 to 10 minutes than remove the medium and replace it with 1 ml of phosphate buffer. Spectrofluorometricaly determine fluorescence intensity for different parameters of electric field. Propidium iodide has excitation λ at 490 and emission λ at 620 nm. From the data obtained, calculate the percentage of propidium iodide uptake.

FURTHER READING:

4. 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.
5. Faurie C, Phez E, Golzio M, Vossen C, Lesbordes JC, Delteil C, Teissie J, Rols MP. Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells. *BBA* 1665: 92-100, 2004.
6. Reberšek M, Faurie C, Kandušer M, Čorović S, Teissie J, Rols MP, Miklavčič D. Electroporator with automatic change of electric field direction improves gene electrotransfer in vitro. *Biomed. Eng. OnLine* 6: 25, 2007.

NOTES & RESULTS

	negative control	one direction	four directions	one direction	four directions	positive control
voltage [V] E [V/cm]	0 (0)	200 (400)	200 (400)	300 (600)	300 (600)	1 % Triton X-100
raw data [RFU]						
uptake of PI [%]						



D1 – Electrochemotherapy with cisplatin of solid tumors in mice

Maja Čemazar[1], Gregor Serša[1], Justin Teissié[2]; *[1]Institute of Oncology Ljubljana, Slovenia, [2]Institut de Pharmacologie et de Biologie Structurale, Toulouse, France.*

Duration of the experiment: app. 45 min
Max. number of participants: 8
Location: Animal laboratory at the Institute of Oncology Ljubljana

THEORETICAL BACKGROUND

In cancer treatment, electrochemotherapy utilises electric pulses to potentiate delivery of chemotherapeutic drugs into cells. Exposure of cells or tissues to short intense electric pulses increases permeability of plasma membrane without impairing cell viability. This nonselective plasma membrane permeabilization enables drugs to diffuse into the cells and reach their intracellular targets. The cytotoxicity of cisplatin is due to the formation of different DNA adducts. Thererefore, the cytotoxicity of the drug dependens on its intracellular concentration and also upon membrane permeability. The aim of this practical exercise it to demonstrate electrochemotherapy with intratumoural injection of cisplatin.

EXPERIMENT

Solid subcutaneous tumors growing subcutaneously in A/J mice will be used for experiment. The tumors will be transplanted 1 week before the experiment by subcutaneous injection of 5x105 cells/0.1 ml PBS. Prior to the electrochemotherapy mice will be anesthetized intraperitoneal injection (10 ml/kg) of physiologic saline containing Xylasine (1mg/ml) and Ketamine (5 mg/ml).
Cis-Diamminedichloroplatinum (II) (Cisplatin; Cisplatyl, Aventis, Paris, France) is obtained as a crystalline powder and dissolved in sterile H_2O at a concentration of 1 mg/ml. Further dilutions are made in 0.9 % NaCl. Cisplatin at a dose of 5 mg/kg will be injected intratumorally. Injection volume will be 100 µl. A measure of good intratumoral injection is tumor whitening.
One minute after administration, 8 square-wave electric pulses (unipolar) will be delivered in two sets of 4 pulses in perpendicular directions, of 780 V (1300 Vcm^{-1} voltage to distance ratio), with pulse width of 100 µs and repetition frequency 1 Hz. They will applied either by two parallel stainless-steel plate electrodes 6 mm apart (two stainless-steel strips: length 15 mm, width 7 mm with rounded corners (IGEA, Italy), which will placed percutaneously at the opposite margins of the tumor or by contact electrodes. Good contact between the electrodes and the skin will be assured by means of conductive gel (Eco gel, Italy). Electric pulses will be generated by an electropulsator Beta tech (France) or Cliniporator (Italy). All pulse settings will be obtained from a PC, which will record and store the pulses delivery.
Antitumor effect of electrochemotherapy will be followed by daily measurements of 3 mutually orthogonal diameters by means of a caliper. Calculation of tumor volumes ($V= a x b x c x \pi/6$) and construction of tumor growth curves in time will be available after the course.

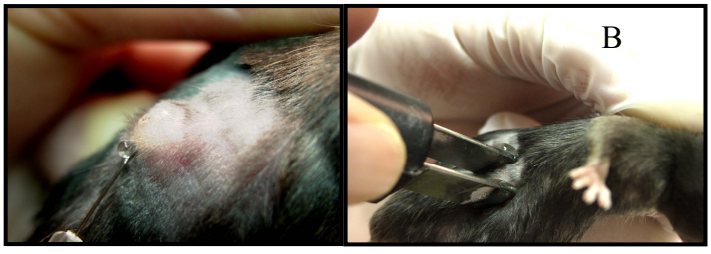
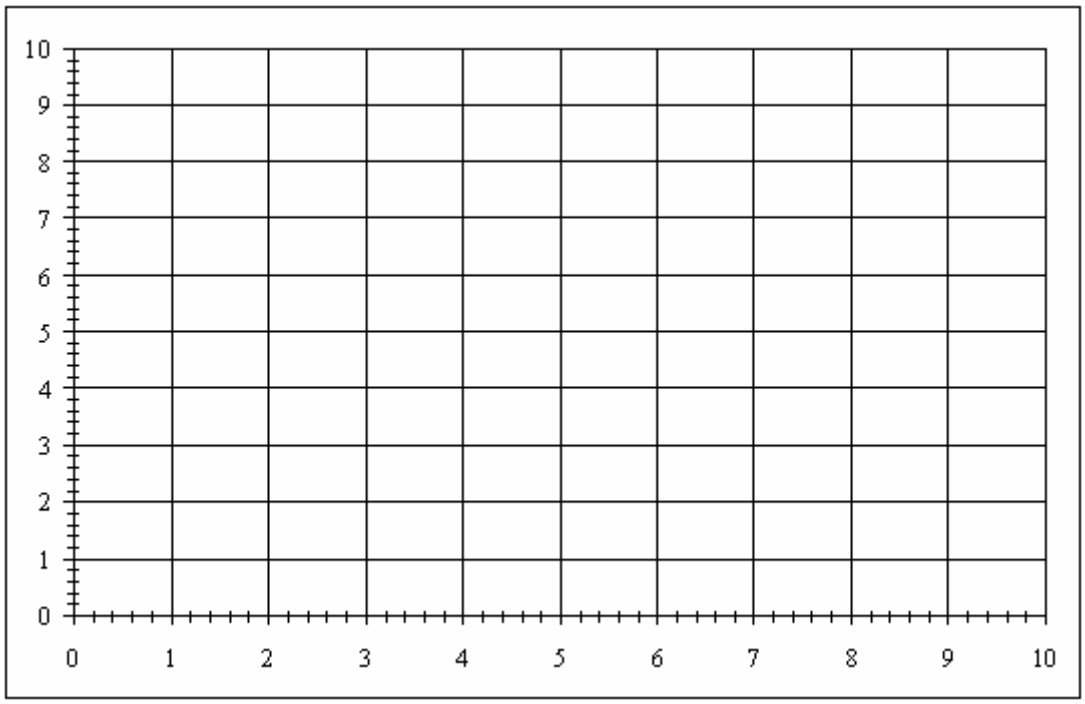


Figure 1: Intratumoral injection of cisplatin (A) and placement of the parallel electrodes on the each side of subcutaneous tumor (B).

FURTHER READING:

1. Cemazar M, Milacic R, Miklavcic D, Dolzan V, Sersa G: Intratumoral cisplatin administration in electrochemotherapy: antitumor effectiveness, sequence dependence and platinum content. *Anticancer Drugs* 1998; 9: 525-30.
2. Sersa G, Cemazar M, Miklavcic D, Rudolf Z. Electrochemotherapy of tumours. *Radiol Oncol* 2006; 40(3): 163-74.
3. Cemazar M, Golzio M, Escoffre JM, Couderc B, Sersa G, Teissie J. In vivo imaging of tumor growth after electrochemotherapy with cisplatin, *Biochem Biophys Res Commun.* 2006; 348(3):997-1002

NOTES & RESULTS



C1 - Electrochemotherapy treatment planning: optimization of voltage and electrode position

***Anže Županič, Selma Čorović**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of experiment: appr. 45 min
Max. number of participants: 4
Location: Laboratory of Biocybernetics

THEORETICAL BACKGROUND

Electrochemotherapy is an efficient local treatment of cutaneous and subcutaneous tumors, which combines the delivery of nonpermeant, cytotoxic chemotherapeutics (e.g. bleomycin, cisplatin) and short high-voltage electric pulses. The pulses induce high electric fields inside the tumor, thereby increasing the tumor cell permeability (electropermeabilization) to otherwise nonpermeant chemotherapeutics. Efficient electrochemotherapy requires the electric field inside the tumor to be higher than the threshold value needed for reversible electropermabilization (E_{rev}) and lower than the tissue damaging threshold for irreversible electropermeabilization (E_{irrev}) in healthy tissue. Electrochemotherapy treatment planning methods should enable the physician to guarantee that the whole tumor is electropermeabilized, while the damage to healthy tissue is kept to the minimum. It is not necessary that the whole tumor is electropermeabilized in one pulse or pulse sequence - sometimes a combination of more than two pulse sequences applied on a combination of different electrodes is required.

EXPERIMENT

A finite element based numerical modeling program package COMSOL Multiphysics will be used to optimize voltage between the electrodes and position of the electrodes on a prepared simple 2D model of a spheroid subcutaneous tumor and surrounding tissue. (Figure 1a). The starting position and the voltage on the electrodes cause healthy tissue damage, while the whole tumor is actually not permeabilized (Figure 1b). Your goal is to optimize the electrode position and the electric potential on the electrodes, so that the tumor is permeabilized ($E_{tumor} > E_{rev} = 400\ V/cm$) and the damage to healthy tissue ($E_{healthy} > E_{irrev} = 900\ V/cm$) is kept to the minimum. To help you with your task, two objective functions are defined in the program - percentage of reversibly electropermabilized tumor volume (%$E_{tumor} > E_{rev}$) and percentage of irreversibly electropermeabilized healthy tissue (% $E_{healthy} > E_{irrev}$) You will calculate the electric filed distribution in the model after each change of the model geometry or electrode voltage and then calculate both objective functions. Your goal is to achieve 100 % $E_{tumor} > E_{rev}$ and less than 0.05 % $E_{healthy} > E_{irrev}$. After you have finished with the optimization, we will compare your results to the results of an optimization algorithm.

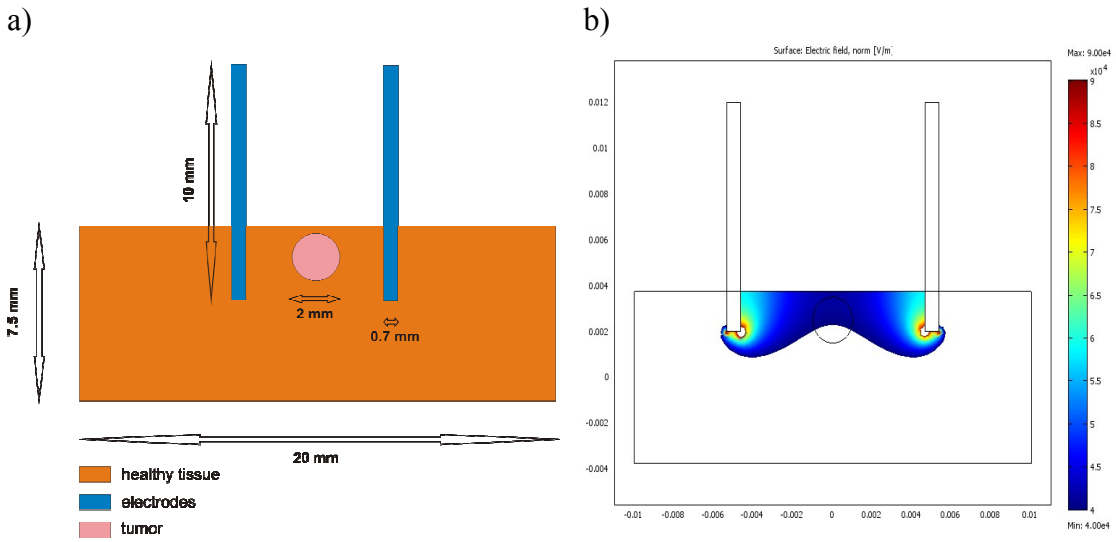


Figure 1: a) Simple 2D model of tumor and needle electrodes in healthy tissue; b) electric field over reversible threshold inside the healthy tissue and the tumor.

FURTHER READING

1. Miklavčič D, Čorović S, Pucihar G, Pavšelj N. Importance of tumor coverage by sufficiently high local electric field for effective electrochemotherapy. *EJC Supplements*, 4: 45-51, 2006b.
2. Miklavčič D, Šemrov D, Mekid H, Mir L M. A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. *Biochimica et Biophysica Acta,* 1523: 73-83, 2000.
3. Čorović S, Pavlin M, Miklavčič D. Analytical and numerical quantification and comparison of the local electric field in the tissue for different electrode configurations. *Bio. Med. Eng. OnLine*. 6: 37, 2007;.

NOTES & RESULTS

Distance between electrodes [mm]	Electrode depth [mm]	Voltage [V]	$E_{tumor}>E_{reversible}$ [%]	$E_{healthy}>E_{irreversible}$ [%]
10	1.75	450	68.7	0.16

C2 – Numerical modeling of electric field in a cell suspension

***Nataša Pavšelj**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of experiment: appr. 45 min
Max. number of participants: 4
Location: Laboratory of Biocybernetics

THEORETICAL BACKGROUND

A cell membrane is, in general, impermeable for larger molecules; however, the application of electric pulses to cells, either in suspension or tissue, causes the electroporation of cell membrane, increasing its permeability and making it possible for larger molecules that otherwise can not cross the membrane, such as drug molecules or DNA, to enter the cell. After pulsing, the cell membrane reseals provided the applied voltage was not too high to cause permanent cell membrane damage. Even a short electric pulse of a high enough voltage causes an increased transmembrane potential in the cell membrane. If the pulse is of adequate amplitude, the electric field strength and consequently the transmembrane potential are high enough for cell membrane permeabilization.

EXPERIMENT

A finite element numerical model of a cell suspension exposed to electric field will be made, using numerical modeling program package COMSOL Multiphysics. The geometry of the model will use a real in vitro experimental setup where cells in a suspension are subjected to electric pulses delivered through four cylindrical rods, allowing delivery of electric field in different directions (see Fig. 1)

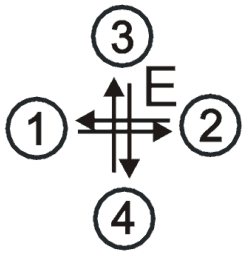


Figure 1: Electric field directions between opposite pairs of electrodes

In the first part of the experiment, the cell suspension will be modeled as a homogeneous material. Two different situations will be compared:

a) only one pair of opposite electrodes is used to deliver electric pulse(s)
b) both pairs of opposite electrodes are used.

Electric field intensities throughout the suspension between the electrodes for both situations will be compared. The aim of the experiment is to become familiar with the electric field distribution in such an experimental setup and to demonstrate the usefulness and necessity of knowing the theoretical background before planning such an experiment.

A cell suspension is not a homogeneous material, as it consists of a culture medium and cells of different shapes, sizes and distances between them. Also, the conductivities of a culture medium and of cells are significantly different. In the second part of our experiment a single cell will be modeled in a culture medium. We will show how each cell in electric field behaves as a dipole, which modifies the external electric field. The aim of this experiment is to get an insight into cell interactions in cell suspensions, aggregates and tissues and how this influences the induced transmembrane potential.

By taking advantage of the fact that the cell suspension is electrically insulated at the bottom and the fact that the vertical dimension of the electrodes is much larger than the cells, the model will be 2-dimensional. Boundary conditions will be set appropriately.

FURTHER READING:

1. Reberšek M, Faurie C, Kandušer M, Čorović S, Teissié J, Rols MP, Miklavčič D. Electroporator with automatic change of electric field direction improves gene electrotransfer in vitro. Biomed. Eng. Online 6: 25, 2007.
2. Pavlin M, Pavselj N, Miklavcic D. Dependence of induced transmembrane potential on cell density, arrangement, and cell position inside a cell system. *IEEE Trans. Biomed. Eng.* 49: 605-612, 2002.
3. Susil R, Semrov D, Miklavcic D. Electric field induced transmembrane potential depends on cell density and organization. *Electro. Magnetobiol.* 17: 391-399, 1998

NOTES & RESULTS

E1 - Electrochemotherapy - interactive learning

***Selma Čorović**; University of Ljubljana, Faculty of Electrical Engineering*

Duration of the experiments: appr. 90 min
Maximal number of participants: 18
Location: Computer room (CIT)

THEORETICAL BACKGROUND

Electrochemotherapy is an effective approach in tumor treatment employing locally applied high-voltage electric pulses in combination with chemotherapeutic drugs (bleomycin or cisplatin). Electrochemotherapy is performed using either intravenous or intratumoral drug injection, followed by application of electric pulses, generated by an electric pulses generator and delivered to the target tissue via appropriate electrodes. As a response to the high electric pulses delivery, a local electric field is established within the treated tissue. A sufficient magnitude of electric field initializes electropermeabilization of cell membranes, which allows for increased entrance of the drug into the cell and thus allows for improved effectiveness of electrochemotherapy (Figure 1).

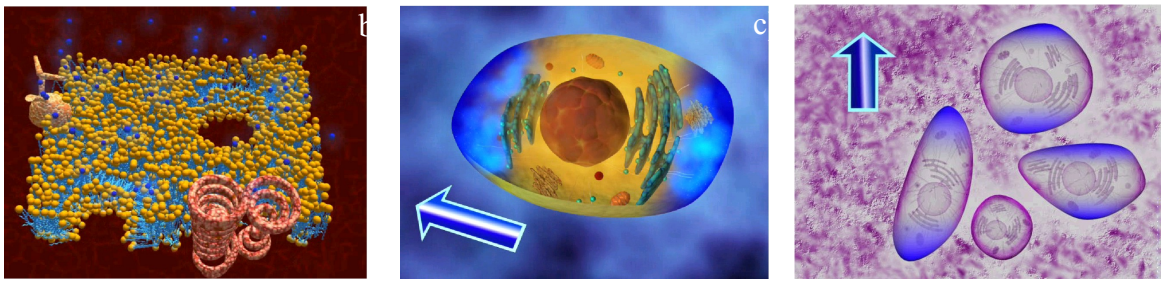


Figure 1: The molecules of the chemotherapeutic drugs a) cross the electropermeabilized cell membrane b) enter an electropermeabilized cell and c) enter the electropermeabilized cells within the tumor tissue

Such a modality of cell membrane permeabilization by means of electric field is named electropermeabilization. For successful electrochemotherapy the entire volume of the target/tumor tissue has to be subjected to the local electric field above reversible threshold value (E_{rev}). By appropriate selection of electrode configuration (plate or needles) and amplitude of electric pulses the needed electric field can be obtained only inside the target/tumor tissue, while the damages to the surrounding healthy tissue are prevented or minimized.
The key parameter for successful antitumor treatment outcome by means of electrochemotherapy is the sufficiently high local electric field (E) inside the target/tumor tissue. At a given number and duration of the pulses (e.g. 8×100 µs) the permeabilized area of the tissue is determined by the pulse amplitude, the geometry and orientation of the electrodes and electric properties of the tumor and surrounding tissue. The appropriate pulse parameters and electrode geometry are difficult to obtain experimentally, mostly because electric field distribution between the electrodes is inhomogeneous due to the different tissue properties (e.g. conductivity and geometry of the tissue) and the geometry of the electrodes. By means of numerical modeling, it is possible to calculate and visualize the electric field distribution in the treated tissue. Based on this the pulse amplitude and electrode configuration necessary for successful electropermeabilization of the whole tumor can be determined.
In the development of the electrochemotherapy treatment a multidisciplinary expertise is required. Therefore, the collaboration and the knowledge and experience exchange among the experts in the fields of medicine, biology and engineering is needed. To collect, organize and transfer the acquired knowledge web-based technologies are being an indispensable tool in modern teaching.

EXPERIMENT

Explore the problems of electric field distribution during electrochemotherapy using a web-based e-learning application. You will become familiar with different definitions used in the field of electrochemotherapy and with the impact of pulse intensity and electrode configuration on electric field distribution in the tissue. The main objectives of this exercise are to give inside into basic mechanisms underlying electroporation process on the level of cell membrane (Figure 1a), cells (Figure 1b), and tissues (Figure 1c) and to provide an interaction

with the educational content in order to simulate the "hands-on" learning approach about the parameters of the local electric field, being crucial for successful tissue electropermeabilisation. For example, by changing different parameters (e.g. applied voltage, electrode configuration, distance between electrodes) by a mouse click on the buttons in the navigation bar users have the possibility to design the needed electric field intensity according to the properties of the target tissue. In Figure 2 the electric field distribution inside the cutaneous protruding tumor obtained with two different amplitudes of applied voltage using two parallel plate electrodes is shown as example.

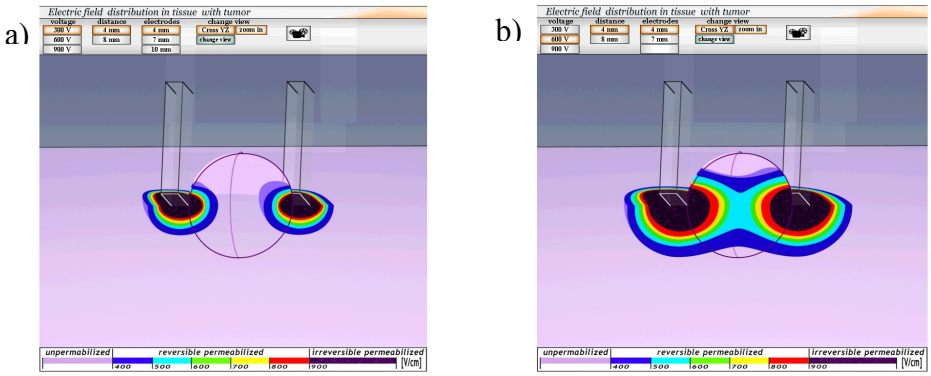


Figure 2: Electric field distribution inside the protruding tumor for two different the applied voltages (U) on the electrodes: a) U=300 V and b) U=600 V

FURTHER READING:

1. Čorović S, Pavlin M, Miklavčič D: Analytical and numerical quantification and comparison of the local electric field in the tissue for different electrode configurations. *Bio. Med. Eng. OnLine*, 6: 37, 2007.
2. Šemrov D, Miklavčič D: Numerical modeling for in vivo electroporation. *Meth. Mol. Med.*, 37:63-81, 2000.
3. Pavšelj N, Bregar Z, Cukjati D, Batiuskaite D, Mir LM, Miklavčič D: The course of tissue permeabilization studied on a mathematical model of a subcutaneous tumor in small animals. *IEEE Trans. Biomed. Eng.*, 52:1373-1381, 2005.
4. Miklavčič D, Šemrov D, Mekid H, Mir LM: A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. *Biochim. Biophys. Acta,* 1519:73-83, 2000.
5. Gilbert R, Jaroszeski M, Heller R: Novel electrode designs for electrochemotherapy. *Biochim. Biophis. Acta,* 1334: 9-14, 1997.
6. Miklavčič D, Pavšelj N, Hart FX: Electric Properties of Tissues. *Wiley Encyclopedia of Biomedical Engineering*, *John Wiley & Sons, New York*, 2006.

NOTES & RESULTS

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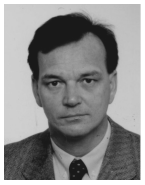

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