November 14-20, 2005
Ljubljana, Slovenia

Proceedings of the

Electroporation based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Edited by:

Peter Kramar
Damijan Miklavčič

Organised by:

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

Sponsored by:

Bioelectrochemical Society
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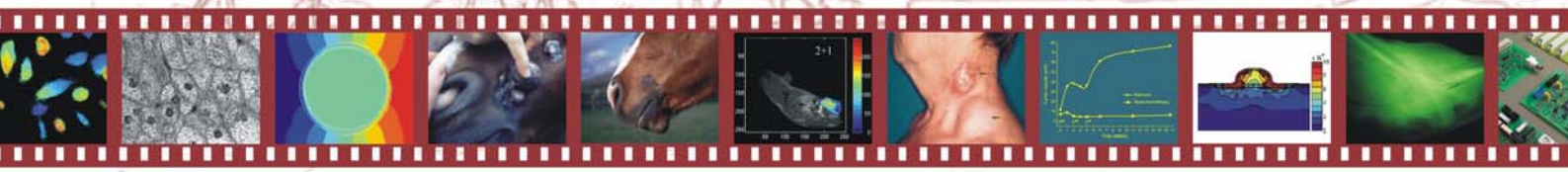

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CIP - Kataložni zapis o publikaciji
Narodna in univerzitetna knjižnica, Ljubljana

577.352.4(063)

ELECTROPORATION based technologies and treatments : proceedings of the international scientific workshop and postgraduate course / organized by University of Ljubljana, Faculty of Electrical Engineering ; edited by Peter Kramar, Damijan Miklavčič. - Ljubljana : Faculty of Electrical Engineering, 2005

ISBN 961-243-022-5
1. Kramar, Peter, 1977- 2. Fakulteta za elektrotehniko (Ljubljana)
222988032

Založila: Fakulteta za elektrotehniko, 2005
Urednik: mag. Peter Šega

Natisnil: BIROGRAFIKA BORI d.o.o., Ljubljana
Naklada: 100 izvodov
1. izdaja

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 has been developing for several years. After preliminary discussions, the Workshop and Course was organised for the first time two years ago. It is now for the second 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. Preeminent 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 result of exposing a cell to electric pulses is electroporation of its plasma membrane: transient permeabilization which facilitates 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 want 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.

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 and Bioelectrochemical Society, for their financial support.

Thank you for participating in our Workshop and Course.

Sincerely Yours,

Damijan Miklavčič and Lluis M. Mir

LECTURERS' ABSTRACTS

Biological Cells in Electric Fields

Tadej Kotnik

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

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 of the transport of specific ions across the membrane.

[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.

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, respecti-vely, 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.

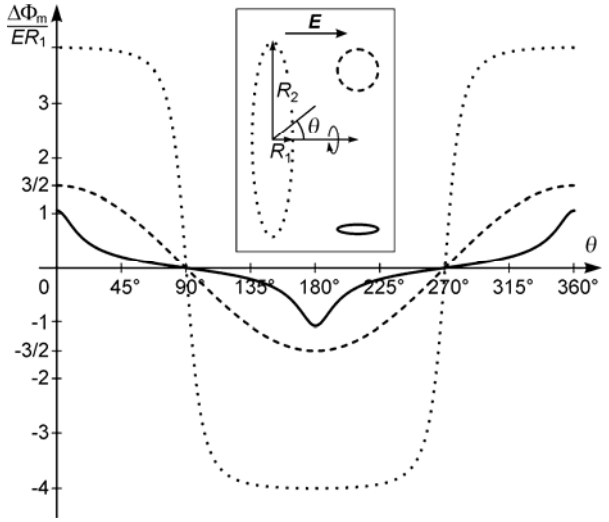

Figure 1: Normalized induced transmembrane voltage for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1$. Dotted: an oblate spheroidal cell with $R_2 = 5 \times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

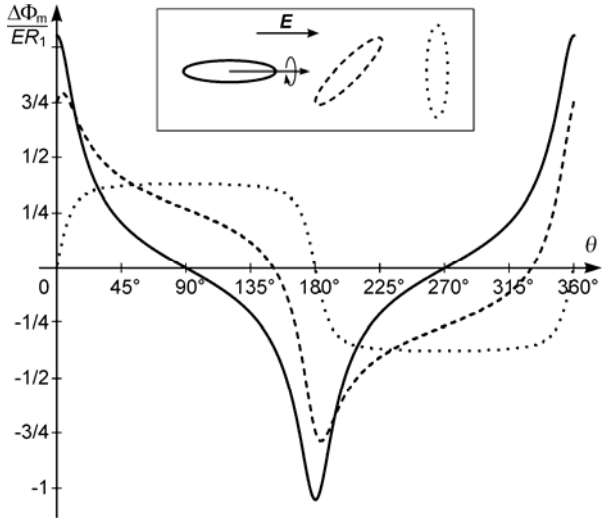

Figure 2: Normalized induced transmembrane voltage for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of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

[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.

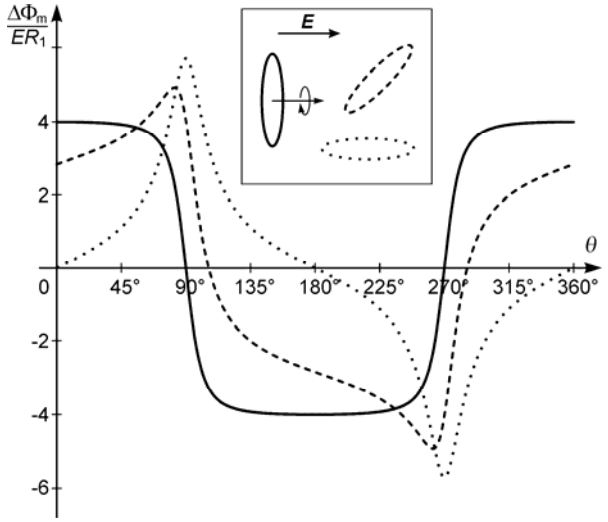

Figure 3: Normalized induced transmembrane voltage for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of the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

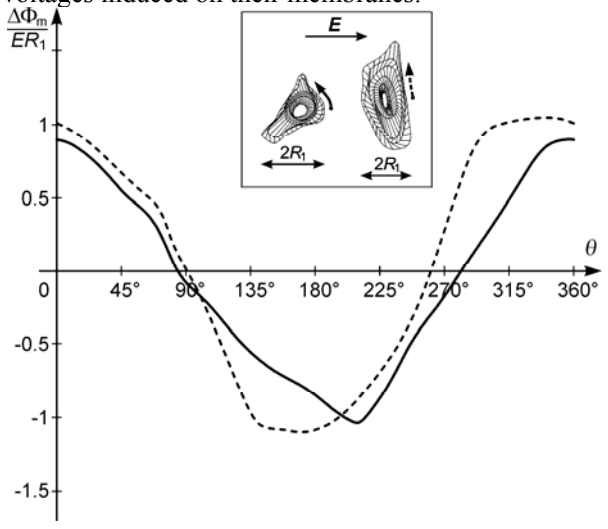

Figure 4: Normalized induced transmembrane voltage for two irregularly shaped cells growing on the flat surface of a Petri dish. Adapted from [12] with the permission of the authors.

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.

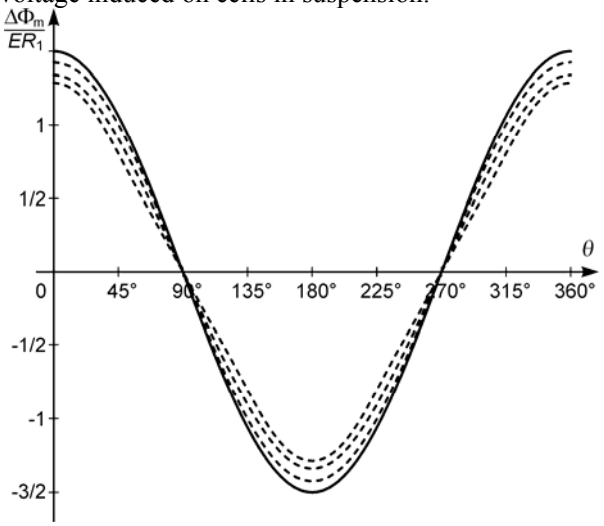

Figure 5: Induced transmembrane voltage normalized to electric field and cell radius. Solid: prediction of Schwan's equation, i.e., of formula (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (from top to bottom) 10%, 30%, and 50% of the total suspension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

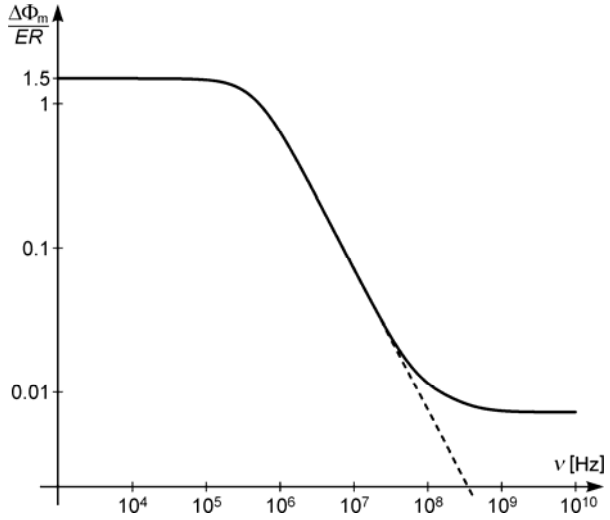

Figure 6: The amplitude of the induced transmembrane voltage, normalized to the amplitude of the field and cell radius, as a function of the frequency of the AC field. The solid curve shows the second-order, and the dashed one the first-order Schwan's equation. Note that both axes are logarithmic. Adapted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

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ACKNOWLEDGEMENT

This work was supported by the Ministry of Higher Education, Science and Technology of the Republic of Slovenia.

Tadej Kotnik was born in Ljubljana, Slovenia, in 1972. He received a Ph.D. in Electrical Engineering from University of Ljubljana, and a Ph.D. in Biophysics from University Paris XI, both in 2000. He is currently a Researcher at the Faculty of Electrical Engineering of the University of Ljubljana. His main research interest is in membrane electrodynamics, as well as in theoretical and experimental study of related biophysical phenomena, especially membrane electroporation (electropermeabilization).

Tadej Kotnik is the first author of 14 articles in SCI-ranked journals. In 2001 he received the Galvani Prize of the Bioelectrochemical Society.

NOTES

Biological Tissue in Electric Fields: From Weak Field Responses to Responses at Electroporative Field Strengths

Igor Lacković

University of Zagreb, Faculty of Electrical Engineering and Computing, Zagreb, Croatia

Abstract: Today, biological tissues are exposed to electric fields in order to induce certain biological effect - for instance, the electropermeabilization of cell membranes. The exposure is, however, often unintentional coming from numberless electrical devices and appliances. In this contribution we present basic methodology for modeling and understanding the electrical response of biological tissue to harmonic and pulsed electric fields of various intensities and frequencies. Focus is on short-term electrical and thermal response of tissue. Methodology is based on basic concepts of electromagnetic theory. Biological tissue is characterized, as any other material, with its macroscopic (bulk) physical properties (electrical conductivity, permittivity, etc.). However, the calculation of the electric field and the current density in tissue is difficult because macroscopic tissue properties show inhomogenity, dispersions, anisotropy, nonlinearity, as well as temperature dependence and changes over time.

INTRODUCTION

Biological tissue is a structure made up of cells that perform similar function. Organs are the next level of organization in the body. Accordingly, the interaction between electromagnetic field and biological systems is analyzed at different levels. The first is the coupling between external field and inside of the body, the second is the coupling between inside of the body and target structure (cellular or subcellular) and the third is the kinetics of response, if any, of target structure to the local field [1].

Before analyzing the interaction between EM fields and biological tissue it is useful to have a look at the electromagnetic spectrum (Fig. 1). The EM spectrum can be expressed in terms of frequency, wavelength or energy. Since a wide range of man-made sources of extremely low-frequency (ELF) fields, radio frequency (RF) fields and microwave radiation surround us in our everyday life, of particular interest is the non-ionizing radiation up to microwave frequencies. Common sources of RF and microwave radiation include radio emitters, cordless and mobile phones, etc. Power lines, electrical wiring, and electrical equipment are sources of ELF fields. All the above-mentioned cases are examples of accidental (unintended), long-term exposure to low-level electromagnetic fields.

We are more interested in intended exposure of biological tissue to electric fields, and in particular to the short-term exposure to intense electric fields. Usually, this is termed electrical stimulation and has numerous applications in biomedicine and biotechnology [2]. Examples include functional electrical stimulation (restoration of muscle function after injury, correction of foot-drop, sphincter stimulation, etc.), automatic cardiac pacing, defibrillation, reversible electroporation for electrochemotherapy and DNA electrotransfer, etc. Aside from beneficial effects of electrical stimulation on living tissue (i.e. healing, function restoration, etc.),

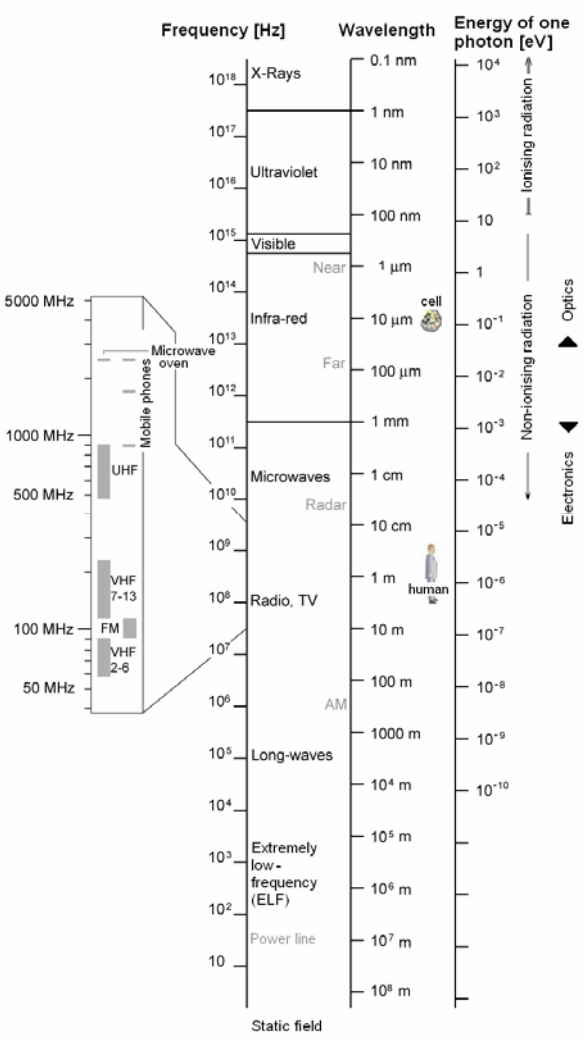

Figure 1: The electromagnetic spectrum. Size of a cell and a human is given at the wavelength scale for comparison.

flow of the electric current through tissue can cause possible hazards and irreversible damage of biological structures. Scales of short-term reactions range from sensory reactions (i.e. perception, discomfort, pain), muscle reactions (twitch, tetanus), cardiac reactions (excitation, fibrillation, defibrillation) to thermal effects (heating) and electroporation.

Due to a great variety of frequencies of EM fields used in different applications, including pulsed fields with broad spectrum and a large span of field intensities, the bulk electrical properties of biological tissues over the entire frequency range are of utmost importance when studying effects of EM fields on living bodies. We use bulk electrical properties (i.e. tissue conductivity and permittivity) presuming that tissues are (macroscopically) homogenous in spite of the fact that all biological tissues are inhomogeneous on a micro-scale (i.e. they consist of densely packed cells). From the experimental perspective this is natural, since bulk conductivity and permittivity are parameters that can be measured for different types of tissues. However, macroscopic perspective does not allow us to analyze the local fields (i.e. the field in cell membrane). Thus, if we are interested in the local effects of the electric field on a cell, we use the approach as presented in the introductory lecture. Namely, we base the analysis on simplified geometry of a cell and use the electrical conductivity and permittivity of cell membrane, intra and extracellular fluid. At tissue level, we use bulk electrical parameters.

MAXWELL'S EQUATIONS

The Maxwell's equations form the fundamental framework for EM field calculations:

$$\nabla \times \mathbf{H} = \mathbf{J} + \frac{\partial \mathbf{D}}{\partial t} \qquad \nabla \times \mathbf{E} = -\frac{\partial \mathbf{B}}{\partial t}$$

$$\nabla \cdot \mathbf{B} = 0 \qquad \nabla \cdot \mathbf{D} = \rho$$

Here **E** is the electric field intensity, **H** the magnetic field intensity, **D** the electric displacement or electric flux density, **B** the magnetic flux density, **J** the current density and ρ the electric charge density. Another fundamental equation is the equation of continuity:

$$\nabla \cdot \mathbf{J} = -\frac{\partial \rho}{\partial t}$$

In order to obtain a complete system, constitutive relations describing macroscopic properties of material are included:

$$\mathbf{D} = \varepsilon_0 \mathbf{E} + \mathbf{P} = \varepsilon_0 \varepsilon_r \mathbf{E} = \varepsilon \mathbf{E}$$

$$\mathbf{B} = \mu_0 (\mathbf{H} + \mathbf{M}) = \mu_0 \mu_r \mathbf{H} = \mu \mathbf{H}$$

$$\mathbf{J} = \sigma \mathbf{E}$$

where ε_0 is the permittivity of vacuum, μ_0 magnetic permeability of vacuum, σ the conductivity, **P** is the electric polarization vector, and **M** the magnetization vector. The parameter ε_r is relative permittivity and μ_r is relative magnetic permeability of the material. It is important to stress that the relations at the right hand side are valid only for linear materials where polarization is directly proportional to the electric field and magnetization to the magnetic field.

Maxwell's equations are valid over extremely wide range of frequencies. That is why they are so powerful and fundamental. However, they are generally very difficult to solve. The consequence of Maxwell's equations is that the fields are always retarded with respect to the change of the sources, due to the finite speed of propagation of electromagnetic waves. However, if studied geometries are much smaller than the wavelength and under the assumption that the sources generating the electromagnetic field vary slowly in time, these effects can be ignored and quasi-static approximation holds ($\nabla \times \mathbf{H} = \mathbf{J}$). Quasi-static fields have the same spatial patterns as static fields but vary with time. Quasi-static **E** and **H** fields are coupled. The least complicated is the situation when the induced currents can be neglected and the equations further reduce ($\nabla \times \mathbf{E} = 0$). This assumption is justified if the skin depth in all domains is much larger than the size of the geometry. In this case the electric field can be analyzed independently from the magnetic field since interaction between them is neglected (decoupling of **E** and **H** fields). This static field limit is useful for approximating many physical situations in which the fields rapidly settle to a local, macroscopically-static state (e.g. when a capacitor is rapidly charged from a source with a small resistor in series). Under different assumptions Maxwell's equations reduce to the well-known partial differential equations such as, wave equation, diffusion equation or Laplace's equation that are much easier to solve.

Table shown below summarizes the EM behavior according to the relationship between the wavelength of the electromagnetic fields involved (λ) and the size of the object (L) (i.e. body, organ, cell, etc.):

$\lambda >> L$	Statics & quasi-statics **E** field for electrode configurations Fields induced in objects by incident **E** fields in free space
$\lambda \sim L$	EM waves (in lossless and lossy media)
$\lambda << L$	Ray propagation effects (optics)

Evidently, static field approximation is valid for frequencies that span even to RF range for all practical electrode configurations used on tissues.

ELECTRICAL PROPERTIES OF TISSUES

Generally, materials can be inhomogeneous, anisotropic, dispersive and nonlinear[1]. Also, several of these properties can be present at the same time. Moreover, material properties can have a significant temperature dependency. Unfortunately, when exposed to electric field, biological tissues can exhibit all these characteristics.

Most tissues are neither "good" electrical conductors nor "good" dielectrics. In biological tissues, as well as in cell suspensions, charge carriers are not electrons as in metals, but ions. Living tissue is predominantly an electrolytic conductor at low frequencies since there are always free ions to migrate. At the same time, in the presence of electric field biological tissues exhibit the characteristics of dielectric materials such as polarization [1, 3].

Due to the complex structure of biological tissues several polarization mechanisms exist leading to frequency dependence of bulk tissue properties and the concept of dielectric dispersions

The bulk electrical properties of tissues can be described by two parameters: (relative) permittivity and conductivity. It is practical to describe tissue properties using complex material properties i.e. the complex permittivity $\hat{\varepsilon}$ or the complex conductivity $\hat{\sigma}$:

$$\hat{\varepsilon} = \varepsilon + \frac{\sigma}{j\omega} = \varepsilon' - j\varepsilon''$$

$$\hat{\sigma} = \sigma + j\omega\varepsilon = \sigma' + j\sigma''.$$

Since the complex permittivity (or conductivity) characterizes the response of a linear causal system to an applied stimulus, real and imaginary parts of complex permittivity (or conductivity) i.e. ε' and ε'' (or σ' and σ'') are not independent.

Extensive compilations of dielectric properties of tissues can be found in [4, 5]; for recent data see [6]. It is important to notice that measurements of dielectric properties of tissue are performed with very small sinusoidal currents (or very weak fields) with the aim of minimally disturbing the measuring sample. Thus, they are representative for linear responses i.e. for responses to not too large field intensities.

Measurement of dielectric properties requires special attention and careful calibration of measurement equipment. At low frequencies (up to a few kHz) electrode polarization is the main nuisance when measuring dielectric properties. That is due to the electric double layers which are formed at the electrode-electrolyte interface. Techniques for correcting these effects can be found in [7]. Electrochemical processes at electrodes are very complicated and depend on electrode material, state of electrode surface, current density, etc. and include both linear and non-linear effects [7, 3].

As an example to illustrate tissue electrical properties we present the conductivity and relative permittivity of liver in the frequency range from 10 Hz to 10 GHz (Fig. 2). When analyzing dielectric spectra of most biological tissues three main dispersions can be identified [3]: α-dispersion with characteristic frequency in mHz-kHz range which is attributed to the counterion effects near the membrane surfaces and ionic diffusion in the electric double layers; β-dispersion with characteristic frequency in 0.1-100 MHz range, due to the presence of cell membranes and can be explained by Maxwell-Wagner effect and γ-dispersion with characteristic frequency in 0.1-100 GHz range which is the result of dipolar mechanisms in polar media (i.e. dielectric relaxation of water).

Dispersions seen in dielectric spectra (i.e. frequency domain representation) are direct consequence of displacement of charge (i.e. relaxation process) that occurs when a voltage step is applied to a dielectric. Mathematical theory for describing dielectric relaxations is briefly outlined in [8] where more references can also be found.

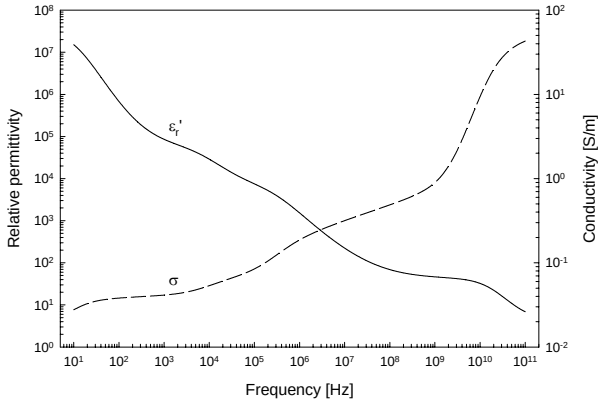

Figure 2: Conductivity and relative permittivity of liver tissue from 10 Hz to 10 GHz according to measurements performed by Gabriel *et al.* (1996) [6].

[1] An inhomogeneous material is one where its physical properties vary with space coordinates (i.e. different properties prevail at different parts of the geometry).
In anisotropic material the field relations at any point are different for different directions of propagations, meaning that physical parameters of the material cannot be defined with a single value, but 3-by-3 tensor is required.
The term dispersion is used to describe materials whose physical properties depend on the frequency. Equivalently dispersion refers to changes in the velocity of the wave with wavelength.
Nonlinearity refers to the variation in physical properties with the intensity of the field. Special case of nonlinearity is hysteresis, where not only the instantaneous field intensities influence the properties of the material, but also the history of the field distribution.

Qualitative understanding of tissue dielectric spectra can be obtained by considering microscopic tissue structure. At low frequencies, the measured conductivity is predominately the conductivity of extracellular fluid since cell membranes are poorly conducting. In spite of extremely high permittivity, conduction current dominates. As the frequency increases the conductivity increases because after β-dispersion frequency membranes do not offer significant barrier to the current flow anymore. The increase of conductivity is accompanied by the decrease of permittivity. In microwave frequency range additional dispersion occurs due to the relaxation of water molecules.

Liver is considered isotropic. Example of significant anisotropy is found in skeletal muscles at low frequencies [3], where conductivity (and permittivity) may be up to an order of magnitude larger in one direction compared to another. Consequently, it matters a lot for the electric field in tissue whether electrodes are positioned along the fibers or transverse to them. The anisotropy, if caused by cell membranes, disappears at frequencies higher than the β-dispersion frequency [3].

Nonlineary and time variance of the tissue conductivity will be illustrated in the next section.

ELECTRICAL RESPONSE OF TISSUE TO ELECTRIC FIELD

We can use the dielectric properties of liver (Fig. 2) 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 ECT). 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. Methodology and analytical solution for this was developed in [9]. Here the obtained response for the first pulse is presented (Fig. 3). At the onset of voltage pulse, capacitive transient 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. 4) [10]. For the lowest applied voltage we can see good agreement with calculated response. As the field intensity is increased, the electrical response of tissue

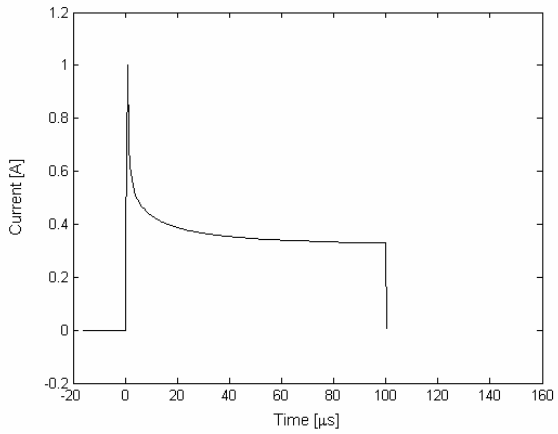

Figure 3: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 µs having the rise time of 1 µs and the amplitude of 120 V. Conductivity and permittivity for liver according to Fig. 2. Plate electrodes with 4.4 mm inteelectrode distance were assumed. Adapted from [8].

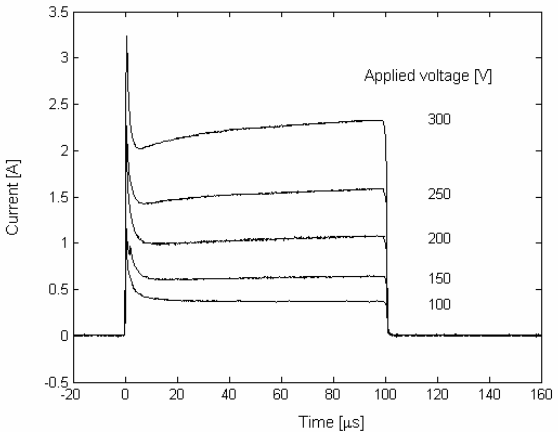

Figure 4: Measured tissue response during delivery of 100 µs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al*. [10], by permission. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm inteelectrode distance were used.

is no longer linear and increase of conductivity during the pulse is observed.

The measured response is consistent with the hypothesis that the bulk tissue conductivity should also increase measurably since on a cellular level electroporation causes the increase of membrane conductance [11].

We emphasize once again that it is possible to reconstruct the response in time domain from frequency spectra and vice versa. However, two important requirements must be satisfied: i) linearity meaning that the amplitude of the voltage applied (or current injected) must be small enough that no change of electrical properties occurs and ii) time invariance meaning that the electrical properties must not change

in time. Only under these conditions superposition principle is valid and the response to pulsed electric field can be obtained by the Fourier transform. In general, where properties depend on the amplitude of the signal or if they change in time, the equivalence of time domain response and frequency spectra does not exist. Consequently, application of literature data on dielectric properties of tissue obtained in bioimpedance measurements [4-6] underestimates the current in tissue for higher field strenghts.

THERMAL RESPONSE OF TISSUE TO A LARGE ELECTRIC FIELD

Another important aspect of application of large static electric fields to tissues is heating because the current flow through conducting medium causes the Joule effect. Consequently, temperature rise in tissue can be expected. Moreover, due to the coupling between electric and thermal variables (the increase of tissue conductivity with the rise of tissue temperature) there is a potentiation of thermal effect. Biological tissue is extremely sensitive to increase in temperature. Thermal damage begins at temperatures higher than 42ºC and the rate of damage rapidly increases with temperature rise.

Nonlinear dynamic model of tissue subjected to electric pulses was recently developed incorporating electro-thermal coupling [9]. It enables the estimation of electric field and temperature rise in tissue for different electrode geometries and parameters of pulse trains used for electrochemotherapy and electrotransfection. As an example of model performance, time course of temperature rise in bulk tissue and near the electrode edges for a train of high voltage pulses (1500 V/cm, 8×100 μs, 1 Hz) delivered through the parallel plate electrodes (Fig. 5).

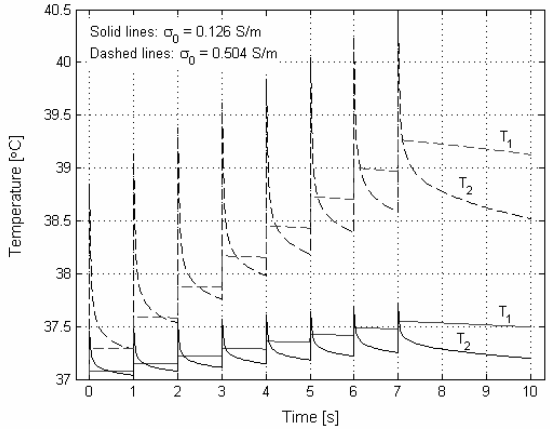

Figure 5: Time course of temperature rise in bulk tissue (T_1) and near the electrode edge (T_2) during a train of high voltage pulses (1500 V/cm, 8×100 μs, 1 Hz) delivered through the plate electrodes with 4.4 mm interelectrode distance. FEM simulations were performed for the nominal low frequency conductivity of liver and for four times higher conductivity. Adapted from [12].

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ACKNOWLEDGEMENT

This work was supported by the Ministry of Science, Eduacation and Sport of the Republic of Croatia and the Ministry of Education, Science and Sport of the Republic of Slovenia.

Igor Lacković was born in Karlovac, Croatia, in 1972. He received a Diploma, and the M.Sc. and Ph.D. degrees in Electrical Engineering, all from the University of Zagreb in 1996, 1999 and 2004, respectively. Currently he is a researcher at the Faculty of Electrical Engineering and Computing, University of Zagreb. He has concentrated on theoretical and experimental study of tissue impedance and electrical and thermal modeling of tissue during electroporation. He received Silver Josip Lončar medal for his Master Thesis in 1999.

NOTES

Physical Chemistry of Membrane Electroporation: Towards Mechanisms for Electroporative Transport Phenomena

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Abstract: The physical chemical theory of experimental membrane electroporation (MEP) focuses on the structural electric field effects, obviously apparent in cell (CEP) and skin (SEP) electroporation for drug delivery as well as in the disciplines of electrochemotherapy (ECT) and electrogenetherapy (EGT).

The primary membrane effect of electric field-pulses is rapid (ns, us, ms). It comprises both, local molecular rearrangements of membrane components and global shape changes such as particle deformations under electric Maxwell stress. MEP is chemically expressed as structural transitions of the overall type (C) = (P) between closed (C) and porous (P) membrane states, where state (P) allows transport of otherwise membrane-impermeable compounds. In more detail, MEP is a *hysteresis cycle* of the (rapid) electroporation branch and the (slow)resealing branch. Coupled to the structural hysteresis is the relatively slow (s, min, h) after-field transport such as net uptake of DNA, RNA or proteins or larger dye and drug molecules and the massive release of small ions through the slowly annealing MEP-structures.

The electrothermodynamic and kinetic analysis of experimental data, in terms of fractional changes f provides characteristic thermodynamic parameters to quantify stability of pores in terms of state distribution constants K = f/(1-f). Kinetic normal analysis yields, in principle, the kinetic coefficients for the rapid in-field processes as well as the rate and flow coefficients for the slower after-field resealing phase.

The information provided in this digression may serve as a guideline for the design of experiments and data analysis of tissue electroporation and electroporative transport processessuch as electro-insertion and electroporative membrane fusion.

INTRODUCTION

The concept and method of membrane electroporation (MEP), for the electro-release of cell-internal compounds [1] and the electrodelivery of bioactive agents such as drugs and genetic polynucleotides like DNA [2, 3], by high voltage pulses has become a familiar tool in cell biology, biotechnology and clinical medicine for tumor and gene electrotherapies [4-7]. Basic insight into the mechanism of MEP of the lipid part of cellular membranes is derived from curved lipid membranes under well-defined chemical conditions.

In particular, the powerful tool of electrooptic and conductometric relaxation spectrometry in high electric fields E ($0 \le E / kVcm^{-1} \le 100$) of the pulse duration range of 100 ns up to ms, has revealed that MEP is a *hysteresis cycle* of rapidly induced structural changes during the pulse and a much slower restructuring or resealing of the field-induced porous structures after the pulse .

It is realized that, on the pulse time scale of up to several ms, the actually (visible) transports of larger molecules like DNA or dyes like Serva blue G (model for drug molecules), respectively, are slow after-field events [2, 3, 8, 9].

WHAT IS MEP?

Practically, membrane electroporation is a powerful electric-chemical concept and technique to render lipid membranes porous, transiently and structurally reversible, by electric voltage pulses. The local porous parts are called pores. The original smaller pores may develop larger pores (at the expense of smaller ones) such as local transport passages for larger substances like DNA, oligonucleotides, dyes, drugs proteins etc.. In more complex structures like the *Stratum corneum* of skin, local transport regions (LTR) are formed due electrical and electrothermal processes [10, 11].

REACTION SCHEME FOR MEP

In the context of the first functionally effective (protein expression), direct transfer of naked foreign DNA by MEP, a simple overall scheme for MEP has been formulated in terms of an electric field-sensitive structural transition.

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

between closed (C) and porous, permeable (P) local membrane states [2]. Eq. (1) and Eq. (3) of the Figure 1 may be chemically specified as field-induced lipid rearrangements of n dipolar (head group) lipids, to (locally) form hydrophilic pores L_n and $L_n(W)$ by water (W) entrance according to

$$nL \rightleftharpoons L_n \rightleftharpoons L_n(W). \qquad (2)$$

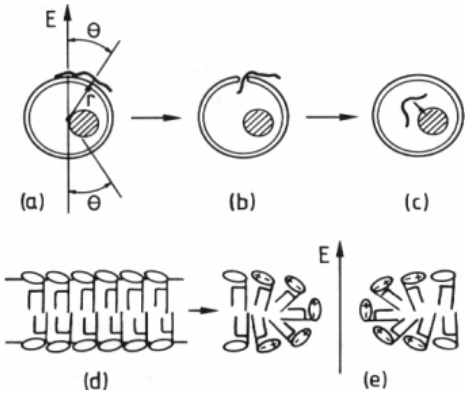


Figure 1: Diagram of the electrically induced transfer of DNA into cells by the electroporation prosess.

As early as 1982, a generalized van't Hoff relationship had been specified for the three physical poration phenomena:, for baroporation (by pressure p) or by thermoporation (by heat) and for electroporation [2]; later extended to optoporation by laser pulses which in essence is local thermoporation. The field effects of MEP are physical chemically described by the field-dependence of the overall distribution (equilibrium) constant $K = [(P)]/[(C)]$.

For isothermal (T) and isobaric (p) conditions, the van't Hoff relation for field effects reads:

$$\left(\frac{\partial \ell nK}{\partial E}\right)_{p,T} = \frac{\Delta_r M}{RT}, \qquad (3)$$

where $\Delta_r M = M(P) - M(C)$ is the reaction dipole moment for a transition like that in scheme (1), $R = N_A \bullet k_B$ the molar Boltzmann constant k_B and N_A the Loschmidt-Avogadro constant.

Integration in the boundaries K_0 at E = 0 and K(E) at E yields [11]:

$$K = K_0 \cdot e^X \qquad (4)$$

where the field factor X is given by

$$X = \frac{\int \Delta M \, dE}{RT} \qquad (5)$$

If we, as suggested by Abidor et al. [12], refer $\Delta_r M$ to the entrance of water replacing an equivalent volume υ_p of lipids, dielectric standard theory connects the volume average $\overline{\upsilon_p}$ of one pore with the ***local field*** $E = E_m$ by:

$$\Delta_t M = \frac{\overline{\upsilon}_p \, \varepsilon_0 \, (\varepsilon_w \, \varepsilon_L)}{k_B T} E_m \qquad (6)$$

where ε_o is the dielectric vacuum permittivity, ε_W and ε_L the dielectric constants of water and the lipid phase, respectively.

Substitution of Eq. (6) into Eq. (5) and integration yields:

$$X = \frac{\overline{\upsilon}_p \, \varepsilon_0 (\varepsilon_W \varepsilon_L)}{2 k_B T} E_m^2 \qquad (7)$$

Since MEP always leads to only a small surface fraction occupied by pores, the pore fraction is always very small, i.e. $f \ll 1$, hence also $K \ll 1$. Therefore, we approximate K = f/(1-f) = f and $K_0 = f_0$. Applying now Eq. (4), we obtain $K/K_0 = f/f_0 = \exp[X]$.

Since f can be obtained from a measured fractional signal S/S^{max}, we can evaluate X from the relationship

$$f - f_0 = f_0(e^X - 1) \qquad (8)$$

This method has been exemplified, for instance for fractional conductivity changes $Y = \Delta\lambda / \lambda_0$ [14, 15]. The Figure 2 summarizes the main features of the phenomenon of membrane electroporation and electroporative transport.

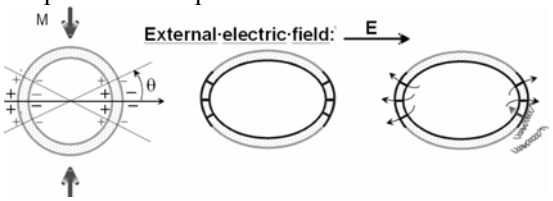


Figure 2: Interfacial polarizaiton (Maxwell-Wagner): ; Maxwell stres: Deformation; Global water; Electropores (rp = 0.4 nm); Pore enlagment (rp = 1 nm) by coalescence; Salt efflux; DNA - Uptake

From $X(E_m)$ we calculate the average pore volume $\overline{\upsilon}$. Assuming cylindrical pores, where $\overline{\upsilon}_p = \pi \cdot \overline{r_p^2} \cdot d_m$, the mean radius $r_p = \left(\overline{r_p^2}\right)^{1/2}$ is derived from the slope b of the relationship $\ell nK = \ell nK_0 + b \cdot E_m^2$, where

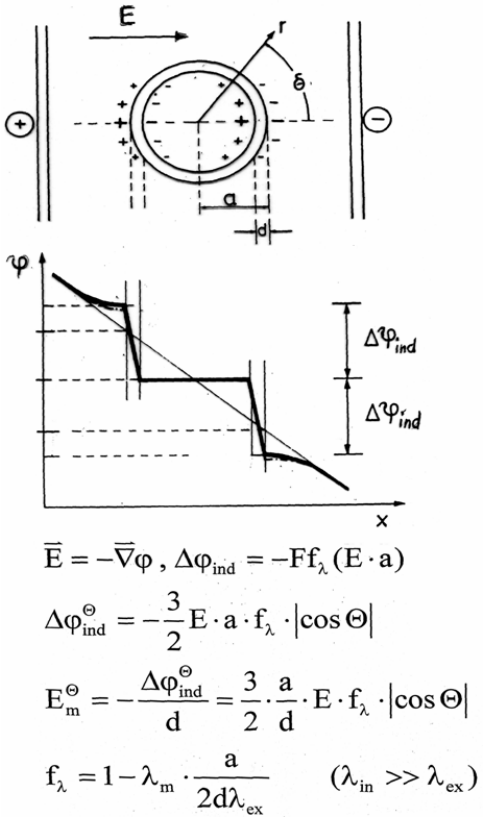
$$b = \pi \cdot r_p^2 \cdot d_m \cdot \varepsilon_0 (\varepsilon_W - \varepsilon_L)/(2 k_p T) \qquad (9)$$

Note that in the equations (4) to (7) the field factor X is specified to $X(E_m)$ by replacing E by E_m . If the data suggest entrance, for instance, of an oligonucleotide (or a part of longer DNA) the polarization volume $\overline{\upsilon}_p$ should depend on the molecular oligonucleotide volume [16].

The analytical fundamentals are summarized in the Figure 3. It is added that the very physical aspects of MEP have been treated in great detail by Chizmadzhev et al., Weaver et al., etc. [10].

EFFECTIVE MEMBRANE FIELD E_m

As compared to the high electric field strengths (10-100 kV/cm) which are required to induce conformational changes in polyelectrolytes like DNA or double-stranded poly(A) poly(U) [16], the relatively very small external fields (0.1-5 kV/cm) of cell electroporation [2] are at first very puzzling. Very fast it had been realized that the small external fields are hugely amplified by geometry, to large membrane fields E_m. The causative is the Maxwell-Wagner (ionic) polarization of the low-dielectric part of the closed membrane phase.



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

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

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

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

Figure 3: Field amplification by ionic membrane polarization (Maxwell-Wagner)

The electric potentials $\varphi(r)$ of a spherical shell, surrounded by two different environments are obtained from Maxwell's solution of the Laplace equation in spherical coordinates radius vector **a** and polar angle θ. The stationary value of the ionic field-induced electric potential drop at the membrane belt in the angular range between θ and $\theta + d\theta$ for zero membrane conductivity and a homogeneous external field **E** (Fricke, Cole) is given by (see for instance [3]):

$$\Delta\varphi = \varphi(a + \Delta a) - \varphi(a) = -(3/2) E a \cos\theta \quad (10)$$

where $\Delta a = d_m$ is the membrane thickness, $a + \Delta a$ the outer radius and a the inner radius of the spherical shell [3].

Note, for the range $0 \le \theta \le \pi/2$, $\Delta\varphi(\theta) \le 0$, because of $\cos\theta \ge 0$, whereas for the range $\pi/2 \le \theta \le \pi$, $\Delta\varphi(\theta) \ge 0$, because of $\cos\theta \le 0$. So, for the range , $\Delta\varphi(\theta) \ge 0$, the membrane field is $E_m \ge 0$ and in line with the Maxwell definition of the field vector $\mathbf{E} = -\nabla\varphi(r)$ in the direction of **E.** However, for $\Delta\varphi(\theta) \ge 0$, the calculation of E_m is more involved. Note that this picky feature is essential for the description of electrodiffusive ion flows, e.g., across membrane pores (induced by **E**)**.** The current density vector $\vec{j}_m$ is given in cartesian coordinates by:

$$\vec{j}_m = \lambda_m \cdot \vec{E}_m = \lambda_m (-\Delta\varphi_m / \Delta a)\,\mathbf{e} \quad (11)$$

where the amount e of the unit vector **e** is plus one for $\Delta\varphi(\theta) \le 0$ and minus one for $\Delta\varphi(\theta) \ge 0$ In order to avoid this potential trap, we have proposed to describe the stationary value $\Delta\varphi_{ind}(\text{for } t \gg \tau_{pol}) = \Delta\varphi(t) \cdot (1 - \exp[-t/\tau_{pol}])^{-1}$, where τ_{pol} is the Maxwell-Wagner (ionic) polarization time constant (in cartesian coordinates) [3]:

$$\Delta\varphi_{ind}^{(\theta)} = -\frac{3}{2} E a f_\lambda |\cos\theta| \quad (12)$$

In Eq. (12), $|\cos\theta| = E_C / E$ for the range $E \ge E_C$ and refers to the electroporatively affected critical area $S_C = S_0(1 - |\cos\theta|)$ of the two pole caps. The total pore fraction is given by $f_p = \Delta S_p / S_0$ [9]. For comparison with $f_{aff} = S_C / S_0 = (1 - |\cos\theta|)$,

$$f_p = \frac{\Delta S_p}{S_0} = \frac{\Delta S_p |\cos\theta|}{2 S(\theta)}, \quad (13)$$

where $S_0 = 4\pi a^2$ is the total surface area of the sphere and $\Delta S_p = N_p \pi r_p^2$ represents the outer surface area of the N_p pores. The membrane field is then given by:

$$E_m(\theta) = \frac{-\Delta\varphi_{ind}(\theta)}{d_m} = \frac{3}{2} E \cdot \frac{a}{d_m} \cdot f_\lambda |\cos\theta| \quad (14)$$

where a/d_m is the geometrical amplification factor and f_λ the conductivity factor referring to the finite membrane conductivity [3]. See the Figure 3.

KINETIC NORMAL MODE ANALYSIS

Kinetics is the very basis for the study and evaluation of molecular mechanisms in terms of intermediate states and of rate coefficients, derived from the measured normal mode time constants, and

amplitudes. The amplitudes of the relaxation modes are the targets for electrothermodynamic analysis. On the digressive level, the essential details are summarized in the Figure 4 and the Figure 5. The after-field kinetics is indicative for mechanistic details of the long-lived electroporative membrane states, where the majority of all electroporative transport events occur.

$$\left(\frac{\partial\, \delta n\, K}{\partial F}\right)_{p,T} = \frac{\Delta_r M(F)}{RT} = \frac{\langle \Delta_r m(F)\rangle}{k_B T}$$

Legendre transformed Gibbs function: $\tilde{G} = G - FM$

Moment: $M = \sum n_j M_j$

Reaction moment ($d\xi = dn_j/\nu_j$): $\Delta_r M = \left(\frac{\partial M}{\partial \xi}\right)_{p,T} = N_A\left(\langle m(B^*)\rangle - \langle m(B)\rangle\right)$

$\Delta_r M = V \cdot \Delta_r \chi \cdot F$

Reaction susceptibility (molar): $\Delta_r \chi = \chi(B^*) - \chi(B)$

Integration: $K(F) = K(0)\, e^{X}$

Field factor (energy ratio): $X = \frac{\int \Delta_r M\, dF}{RT} = \frac{V \Delta_r \chi F^2}{2RT}$

Small field approximations: $K(F) = K(0)\,(1+X)$

Figure 4: Analytical Fundamentals: Science is a differential equation. Religion is an (integration) boundary condition (Alan Turing)

Concept: Functional complexity results from structural complexity (compartimentation, interfaces), mechanistically based on coupled physical- chemical processes

Analysis: Reaction complexity = $\sum_i$ (two-state changes)$_i$

Experimental signal: S (t), kinetics

Fractional effect: $\frac{S(t)}{S_{ss}} = f(t) = \Sigma_i f_i(t)$

$f(t) = \Sigma_i \bar{f}_i (1 - \exp[-t/\tau_i])$

Two-state changes: intramolecular: $B_1 \rightleftharpoons B_2$

intermolecular: $L + B_1 \rightleftharpoons LB_1 \quad (\rightleftharpoons LB_2)$

Fractional changes: $f_2 = \frac{[B_2]}{[B_2]^{max}} = \frac{[B_2]}{[B_2]+[B_1]} = \frac{1}{1+1/K_2}$

$f_1 = \frac{[LB_1]}{[LB_1]^{max}} = \frac{1}{1+[L]/K_1}$

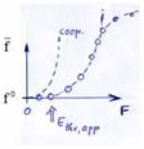


Figure 5: Electromagneto-thermo Dynamics.

The time course of the fractional after-field conductivity change, for instance, is, in the simplest case, of first order representing the resealing of the field-induced electroporative changes, and is described by [9]:

$$Y^{off}(t) = Y^{off}\left(1 - e^{(k_f^0/\tau_R)(1-\exp[-t/\tau_R])}\right) \quad (15)$$

where the experimental amplitude (stationary signal) is given by:

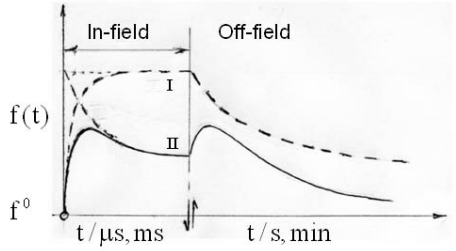
$$Y^{off} = \frac{1}{1 - e^{k_f^0/\tau_R}} \quad (16)$$

In Eq.(16), k_f^0 is the field-dependent flow coefficient and τ_R the (field-independent zero-field) time constant of resealing of the porous area in terms or the pore fraction according to

$$f(t) = f(E) \cdot e^{-t/\tau_R} \quad (17)$$

This procedure of analysis has, for instance, been applied for the resealing phase of densely-packed CHO cells [15]. See, too, the summary of Dr. Sergej Kakorin, Bielefeld.

Experiment: $\frac{S(t)}{S_{ss}} = f(t) = \Sigma_I f_I(t)$



$$\frac{df(t)}{dt} = -\frac{1}{\tau}\left(f(t) - \bar{f}\right)$$

$$f_I(t) - f_I^0 = (\bar{f}_I - f_I^0)(1 - e^{-t/\tau_I})$$

Figure 6: Kinetic normal mode analysis.

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NOTES

Analysis of Electroporative Transport: Ionic Conductivity of Membrane Electropores

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Abstract: The conductivity of membrane pores is studied in terms of electrostatic interaction of ions with a low-dielectric pore wall for the common case of unequal concentrations of electrolyte on the two membrane sides. In the case of cells and lipid vesicles, the conductivity of membrane is a non-linear, Lambert W, function of the induced potential difference across the membrane. The analysis of the conductivity data of electroporated lecithin vesicles of radius a = 90 nm yields the molar energy of interaction of monovalent ions with the pore wall: w_0 = 9 ±1 RT (or w_0 = 22 ±2 kJ mol^{-1}), corresponding to a pore radius of $\bar{r}_p$ = 0.6±0.1 nm. The lecture describes the approach to the exact determination of surface fraction and radius of ion conductive electropores from conductivity and electrooptic measurements in suspensions of cells and salt-filled vesicles, respectively.

BORN ENERGY OF SMALL IONS IN LIPID MEMBRANES

Membrane electroporation (ME) increases the membrane conductivity λ_m from the very small initial value of $\lambda_m^0 \approx 5 \cdot 10^{-13}\ \mathrm{S m^{-1}}$ for an artificial non-electroporated lipid membrane [1] up to the higher value of $\lambda_m \approx 1.1 \cdot 10^{-4}\ \mathrm{S m^{-1}}$ for the electroporated membrane [2]. Membrane electropermeabilization for small ions and larger ionic molecules can not be simply described by permeation across the densely packed lipids. Actually, a small monovalent ion, such as Na^+(aq), passing through a lipid membrane encounters the Born energy barrier of ΔG_B = 62 RT, where R is the gas constant and T = 298 K (25°C) [3]. To overcome this high barrier the transmembrane voltage $|\Delta\varphi| = \Delta G_B / |z_i \cdot e|$ has to be 1.75 V, where e is the elementary charge and z_i the charge number of the ion i (with sign). Nevertheless, the transmembrane potential required to increase conductivity of the lipid membrane usually does not exceed 0.5 V [4]. The energy barrier can be reduced in a transient aqueous electropore.

For a point charge q displaced near the boundary of two dielectrics, say of ε_w = 80 and ε_m = 2.5, respectively, the electrostatic theory yields $q' = q \cdot (\varepsilon_w - \varepsilon_m)/(\varepsilon_w + \varepsilon_m)$ for an image charge induced in the next medium (ε_m). Since $\varepsilon_w > \varepsilon_m$, q' has the same sign as q. Therefore, there is a repulsive force between q and q' tending to repel the ion from the lipid phase. Analogous to the Born energy, the interaction energy of q and q' is given by: $W_{flat} = q^2/(8\pi \cdot \varepsilon_0 \cdot \varepsilon_m^{eff} \cdot r)$, where r is the distance between q and the flat boundary and ε_m^{eff} is the effective dielectric constant: $\varepsilon_m^{eff} = \varepsilon_w \cdot (\varepsilon_w + \varepsilon_m)/(\varepsilon_w - \varepsilon_m)$. For an infinitely long cylindrical pore of radius r = r_p, the energy of a monovalent ion in the middle of the pore can be approximated by w_0 = 5RT / $|r_p|$, where $|r_p| = r_p$/nm [5]. For instance, at r_p = 0.56 nm we calculate w_0 = 9 RT.

Usually, volt – ampere characteristics of protein channels and membrane pores do not obey Ohmic law, i.e., current is not a linear function of voltage. However, the ion transport through the membrane (open passages) is commonly quantified by the membrane conductivity λ_m, calculated according to $\lambda_m = G_m \cdot d/S$, where G_m is the measured value of membrane conductance, $G_m = I_m / U_m$, U_m is the potential difference, I_m the current across pores, d the membrane thickness and S is the surface area of the lipid bilayer, see, e.g., [6].

Conductivity of planar asolectin bilayers with adsorbed UO_2^{2+} -ions has been previously described in a case of equal electrolyte contents on two membrane sides and constant U_m [6,7]. In the lecture we will generalise the previous description to a more common case of curved membrane of spherical vesicles with different intra- and extravesicular salt concentrations and account for a limiting effect of membrane conductivity on the conductance of single pores.

NERNST-PLANCK EQUATION FOR ELECTROPORATIVE MEMBRANE FLUX

Consider a small pore in a lipid membrane of thickness d separating two large compartments of aqueous solutions, external and internal, of different concentration of salt c_{ex} and c_{in}, respectively. We assume also that the amount of salt transported through the pore is small enough to consider the concentration of the electrolyte on the two pore ends constant [8]. The potential drop across the pore

produced by an external electric field is given by $\Delta\varphi = \varphi_{ex}(d) - \varphi_{ex}(0)$ (Fig. 1 a).

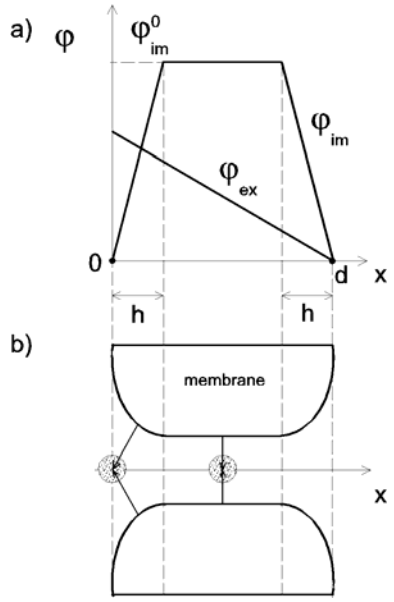

***Figure 1*:** (a) The electric potential φ_{ex} of an external transmembrane field and φ_{im} the image potential of an ion along the pore x – axis. (b) Forces on the moving ion (shown by arrows) from the induced image charge in the pore walls.

Besides of the external field potential drop $\Delta\varphi$, we include a potential φ_{im} of image charge induced by the transporting ion in the low dielectric pore wall. Note that the walls of a narrow pore have no electric double layer. Therefore the low dielectric lipids are exposed to water and ions. If the diameter of hydrophilic pore is larger at the entrances than in the middle (hydrophilic pore), there is a component of the electric force on the pore x – axis, repelling the ion out from the pore mouth (Fig. 1 b).

The concentration profile $C_i(x)$ of ions of sort i in the pore is classically described by the Nernst-Planck equation:

$$J_i = -D_i \cdot \left(\frac{dC_i}{dx} + C_i(x) \frac{z_i \cdot F}{RT} \cdot \frac{d\varphi}{dx} \right) \qquad (1)$$

where J_i is the flux of the i – ions across the pore cross section S_p , D_i the diffusion coefficient of the ion, z_i the charge number (with sign), F the Faraday constant, R the gas constant and φ is the potential in the pore. According to the principle of superposition of potentials, φ can be presented by the sum:

$$\varphi(x) = \varphi_{ex}(x) + \varphi_{im}(x) \qquad (2)$$

We assume that φ_{im} in the pore entrances, x = 0 and x = d, is zero. Therefore, the concentrations $C_i(0)$ and $C_i(d)$ of ions in the two pore ends are equal to the bulk ion concentrations $c_i(0)$ and $c_i(d)$, respectively:

$$C_i(0) = c_i(0), \; C_i(d) = c_i(d) \qquad (3)$$

Dependence $C_i(x)$ of ion concentration in the pore (Eq. 1) can be expressed now through the values of $c_i(0)$ and $c_i(d)$ by an integration of Eq. (1):

$$J_i = D_i \cdot \frac{c_i(0) \cdot e^{k_i \cdot \varphi(0)} - c_i(d) \cdot e^{k_i \cdot \varphi(d)}}{\int_0^d e^{k_i \cdot \varphi(x)} dx} \qquad (4)$$

where $k_i = z_i \cdot F/(RT)$.

Differently to traditional integration of Nernst-Planck equation, where $\varphi(x)$ is a linear function of x, in Eq.(4), $\varphi(x)$ is an arbitrary function of x.

The current density in the pore is given by the sum of fluxes of all ion sorts:

$$j = F \cdot \sum_i z_i \cdot J_i \qquad (5)$$

Ohm's law in differential form yields the conductivity λ_p of pore:

$$\lambda_p = j / E_p \qquad (6)$$

where E_p is the field strength across the pore. Usually, E_p is approximated by:

$$E_p = -\frac{d\varphi}{dx} \approx -\frac{\varphi_{ex}(d) - \varphi_{ex}(0)}{d} = -\frac{\Delta\varphi}{d} \qquad (7)$$

CONDUCTIVITY OF PORE FILLED WITH 1:1 ELECTROLYTE

In the case of 1 : 1 electrolyte, $z_+ = +1$ and $z_- = -1$, Eq. (5) reduces to $j = F \cdot (J_+ - J_-)$. Assuming that $D = D_+ = D_-$ and substituting Eq. (5) and Eq. (4) into Eq. (6), we obtain:

$$\lambda_p = \frac{F \cdot D}{E_p} \cdot \left(\frac{c_+(0) \cdot e^{k_+ \cdot \varphi(0)} - c_+(d) \cdot e^{k_+ \cdot \varphi(d)}}{\int_0^d e^{k_+ \cdot \varphi(x)} dx} - \frac{c_-(0) \cdot e^{k_- \cdot \varphi(0)} - c_-(d) \cdot e^{k_- \cdot \varphi(d)}}{\int_0^d e^{k_- \cdot \varphi(x)} dx} \right) \qquad (8)$$

For the further analysis of Eq. (8) we have to specify the function $\varphi(x)$.

Trapezium Potential of Image Charges

As the next step in our analysis and in line with Glaser et al. (1998) [6], we consider the trapezium

shape of image potential $\varphi_{im}(x)$, see Fig. 1 (a). The integration of Eq. (8) with the trapezium-shape image potential over three intervals: [0; h], [h; d-h] and [d-h; d] for the case of the small potential drop $|\Delta\varphi| < 25$ mV and the small relative size of the curved pore entrance n = h / d << 1, yields:

$$\lambda_p = \frac{F^2 \cdot D \cdot e^{-F\cdot\varphi_{im}^0/RT}}{RT} \cdot \big(c(0)+c(d)\big) \cdot \cdot \left\{ 1 + \frac{e^{-F\cdot\varphi_{im}^0/RT} \cdot (e^{F\cdot\varphi_{im}^0/RT}(F\cdot\varphi_{im}^0/RT - 1) + 1)}{\varphi_{im}^0} \cdot \right\} \cdot \left\{ \cdot \frac{(e^{F\cdot\Delta\varphi/RT}+1)}{(e^{F\cdot\Delta\varphi/RT}-1)} \cdot \Delta\varphi \cdot \frac{h}{d} \right\} \quad (9)$$

According to Eq. (9), the conductivity of a pore is a linear function of h.

In the case when $|\Delta\varphi| >> 25$ mV, approximation $(e^{F\cdot\Delta\varphi/RT}+1)\cdot\Delta\varphi/(e^{F\cdot\Delta\varphi/RT}-1) \approx |\Delta\varphi|$ applies and the integration of the Eq. (8) yields:

$$\lambda_p = \frac{F^2 \cdot D \cdot \big(c(0)+c(d)\big)}{RT} \cdot \cdot \exp\left[\left((1 - \frac{RT}{F\cdot\varphi_{im}^0}) \cdot \frac{h}{d} \cdot |\Delta\varphi| - \varphi_{im}^0 \right) \cdot \frac{F}{RT} \right] \quad (10)$$

It is noted, that up to the term $((1 - RT/(F\varphi_{im}^0))$, Eq.(10) is in line with the analogous approximation of Glaser et al. (1988) applied to one pore [6]. However, the term $((1 - RT/(F\varphi_{im}^0))$ in Eq. (10) can not be left without loss of accuracy.

Conductivity of Electroporated Membrane

If we consider the membrane pore as a conductive channel, the membrane conductivity can be expressed through the fraction f_p of membrane surface area of pores $f_p = \overline{r}_p^2 \cdot N_p / 4 \cdot a^2$ and the conductivity λ_p of pore by $\lambda_m = \lambda_p \cdot f_p + \lambda_m^0 \cdot (1 - f_p)$, where $\overline{r}_p$ is the mean pore radius and N_p the pore number per vesicle. In the most practical cases, λ_p and f_p are in the ranges of $10^{-4} \le \lambda_p / S\ m^{-1} \le 1$ and $10^{-4} \le f_p \le 10^{-2}$, respectively [3]. Therefore, the inequality $\lambda_p \cdot f_p >> \lambda_m^0 \cdot (1 - f_p)$ holds and the approximation $\lambda_m \approx \lambda_p \cdot f_p$ applies. Substitution of the Eq. (10) into $\lambda_m = \lambda_p \cdot f_p$ yields for the membrane conductivity:

$$\lambda_m = \lambda^0 \cdot f_p \cdot \exp[(\frac{\alpha \cdot h \cdot |\Delta\varphi|}{d} - \varphi_{im}^0) \cdot \frac{F}{RT}] \quad (11)$$

where $\lambda^0 = F^2 \cdot D \cdot \big(c(0)+c(d)\big)/RT$ and $\alpha = (1 - RT/(F\varphi_{im}^0))$.

Note that Eq. (11) applies only to conductivity of voltage-clamped membranes, where the transmembrane potential difference $\Delta\varphi$ can be maintained constant during the electroporation. For vesicles and cells suspended in electrolytes, $\Delta\varphi$ is a function of λ_m.

Membrane conductivity of spherical cells and vesicles

Increase in the membrane conductivity λ_m leads to a depolarization of membrane and levels off $\Delta\varphi$. In the particular case of salt filled vesicles, the conductivity of intravesicular medium λ_{in} is much larger than that of external one λ_{ex}, $\lambda_{in} >> \lambda_{ex}$. If the inequality $\lambda_m << 2 \cdot d \cdot \lambda_{ex} / a$ holds, $\Delta\varphi$ can be presented by $\Delta\varphi = \Delta\varphi_0 \cdot f(\lambda_m)$, where $f(\lambda_m)$ is the conductivity function, given by $f(\lambda_m) = 1 - \lambda_m \cdot a/(2 \cdot d \cdot \lambda_{ex})$, $\Delta\varphi_0 =$ -1.5 a E $\sqrt{\langle \cos^2\theta \rangle} = \sqrt{3} \cdot a \cdot E / 2$ is the θ - average transmembrane potential difference at $\lambda_m = 0$, E is the external field strength. Substitution of the $\Delta\varphi = \Delta\varphi_0 \cdot f(\lambda_m)$ into Eq. (11) and solution in respect to λ_m yields:

$$\lambda_m = \beta \cdot \lambda_{ex} \cdot \cdot \text{LambertW}\left(\frac{f_p \cdot \lambda^0}{\beta \cdot \lambda_{ex}} \cdot \exp\left[\frac{F \cdot (\frac{\sqrt{3} \cdot \alpha \cdot a \cdot E \cdot h}{2 \cdot d} - \varphi_{im}^0)}{RT} \right] \right) \quad (12)$$

where $\beta = 4 \cdot d^2 \cdot RT/(F \cdot \sqrt{3} \cdot \alpha \cdot a \cdot E \cdot h)$ is the dimensionless factor, and LambertW is the special function [9].

Data Analysis

Analysis of the data on f_p and λ_m (Fig. 2) from the conductometrical and electrooptical studies of the salt filled lipid vesicles [10] with Eq. (12) yields the relative size of the pore entrance n = h / d = 0.12

($h = 0.63 \pm 0.03$ nm), and the value of the image potential of monovalent ion $\varphi_{im}^0 = 0.23 \pm 0.03$ V, corresponding to the molar energy of interaction of ion with the pore wall: $w_0 = F \cdot \varphi_{im}^0 = 9 \pm 1$ RT. According to Parsegian (1975) [5], the energy $w_0 = 9$ RT refers to the mean pore radius of $\overline{r}_p = 5$ RT / w_0 = 0.56 nm. Total electrical energy w_{el} of an ion in a pore is given by $w_{el} = F \cdot (\sqrt{3} \cdot \alpha \cdot a \cdot E \cdot h / (2 \cdot d) - \varphi_{im}^0)$. For the field in the range $0 \le E / MV\ m^{-1} \le 7.5$, w_{el} increases in the range $-9 \le w_{el} / RT \le -7$.

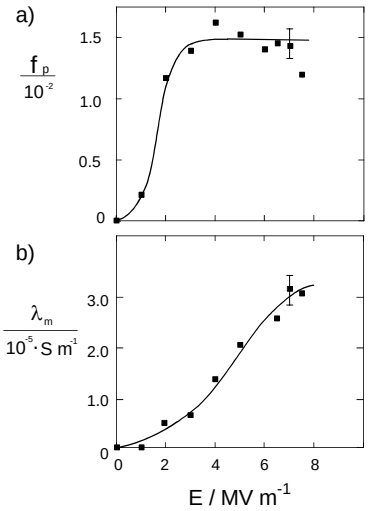

Figure 2: (a) The surface fraction f_p of membrane pores and (b) the membrane conductivity λ_m as a function of the electric field strength E, respectively. Squares in (a) and (b) represent the experimental data, the solid lines refer to the theoretical predictions. Experimental conditions: one rectangular electric pulse of the field strength E and the pulse duration t_E = 10 at T = 293 K (20° C); salt-filled unilamellar lipid vesicles (lecithin 20%) of mean radius a = 90 (±10) nm, internal NaCl content c_{in} = 0.2 M, total lipid concentration [Lip_t] = 1.0 mM, suspended in isotonic 0.33 M sucrose and 0.2 mM NaCl solution. Data are from ref. [10].

Diffusion of Ions through Annealing Pores

The diffusion of ions through the annealing pores after the termination of the field pulse is described by the Fick's first law [11]. Assuming a monoexponential resealing of the pore area with the rate coefficient k_R, the inner concentration c_{in} of electrolyte in the vesicle interior is given by [12]:

$$\frac{c_{in}(t)}{c_{in}^0} = \exp\left(-\left(k_f^0 / k_R\right) \cdot \left(1 - e^{-k_R \cdot t}\right)\right) \qquad (13)$$

where c_{in}^0 is the concentration of electrolyte in the vesicle at the end of the pulse and k_f^0 is the efflux coefficient given by:

$$k_f^0 = \frac{P_p \cdot S_p^0}{V^{in}} = \frac{3 \cdot P_p \cdot f_p^0}{a} \qquad (14)$$

where $f_p^0 = N \cdot r_p^2 / (4 \cdot a^2)$ is the fraction of the porated area at the end t_E of the electric pulse, $S_p^0 = N_p^0 \cdot \pi \cdot r_p^2$ is the maximum area of pore surfaces, $P_p = \gamma_p \cdot D / d$ is the pore permeability coefficient, and γ_p is the pore partition coefficient, given by $\gamma_p = c_p / c_{in}$, where c_p and c_{in} are the concentrations of ions in the pore water and within the vesicle, respectively.

CONCLUSION

The integrated Nernst-Planck equation combined with Lambert W functions consistently describes the ionic conductivity of vesicle membranes as a function of field strength. The theoretical approach has been shown to be useful for the exact determination of the fraction f_p of pores and of the membrane conductivities of vesicles and cells [8,12]. For instance, analysis of the electrooptical and conductometrical data yields the pore radius and the membrane conductivity [10,13].

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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. 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 [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. 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, which is 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]. 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, 7].

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) must be applied to the cell suspension. From Eq. (1), permeabilization is first obtained for θ close to 0 or π. Ep is such that:

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

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

$$A_{perm} = A_{tot} \frac{\left(1 - \frac{E_p}{E}\right)}{2} \qquad (3)$$

where Atot 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 supported on cell suspension by measuring the leakage of metabolites (ATP) [8] or at the single cell level by digitised fluorescence microscopy [9, 10]. 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 [11].

Experimental results obtained either by monitoring conductance changes on cell suspension [12] or by fluorescence observation at the single cell level microscopy [9, 10] 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 a 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 membrane organisation is long-lived in cells. Diffusion is observed during the seconds and minutes following the ms pulse. Most of the exchange takes place after the pulse [9, 10]. Resealing of the membrane defects and of the induced permeabilization is a first order process, which appears to be controlled by protein 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 contribution during the pulse remains negligible [9]. Free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its electropermeabilized part [8]. This gives the following expression for a given molecule S and a cell with a radius r:

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

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 gradient 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 [8]. A strong control by the temperature is observed. The cytoskeletal integrity should be preserved [15]. 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 [16]. 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 [17].

There is a loss of the bilayer membrane asymmetry of the phospholipids [18].

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. Structural changes in the membrane organization supporting permeabilization remains poorly characterized. 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.

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ACKNOWLEDGEMENT

Supports from the CNRS, the AFM (Association française pour les myopathies), the UE "Cliniporator" project and the "ligue départementale de lutte contre le cancer (Haute Garonne)"must be acknowledged.

This state of the art in Electropermeabilization is mostly due to the collective work of scientists and students in my group of "Cell Biophysics" in Toulouse.

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NOTES

Electroporation of cells in tissues
Methods for detecting cell electropermeabilisation *in vivo*

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Abstract: In this chapter only the analysis of the delivery of electropermeabilizing pulses to tissues (for biomedical applications) will be presented. It is worth to recall that the injuries resulting form the accidental exposure to electric fields are often related to the electropermeabilisation of the tissues crossed by these electric fields.

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 heterogeneous tissues, not limited by a physiological physical barrier (like the fascia in muscle or the capsule in liver) and only intravenously injected markers can be used, their intratumoral distribution being of course dependant on the very irregular vasculature of the tumors.

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

METHODS FOR DETECTING *IN VIVO* CELL ELECTROPORATION

As *in vitro*, to detect cell electropermeabilisation it is necessary to use a non permeant marker that will only enter and label the permeabilized cells. If this marker molecule does not bind to (or interact with) intracellular targets, then it allows to simultaneously check cell electropermeabilisation 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 elctropermeabilisation 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 either their fragility or their compactness.

Thus, for an efficient distribution of the marker, as much homogeneous as possible, it is necessary to inject it *in vivo*, intravenously if possible. Then it is necessary to wait for the redistribution of the marker from the vascular compartment to the tissue compartment, that is until the marker will be actually in the vicinity of the cells of the tissue. Depending on the marker, optimal conditions for electric pulses delivery are thus in a time window comprised between the end of the 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, 51Cr-EDTA, Propidium Iodide and (99m) Tc-DTPA.

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 ^{57}Cobalt-bleomycin is a very stable complex [4] that allows to follow bleomycin distribution in the body using e.g. gamma cameras; ^{118}Indium-bleomycin has also been used, with the interest that half life of ^{118}Indium is short allowing to inject higher specific activities than using ^{57}Cobalt; however, stability of ^{118}Indium-bleomycin is lower than that of the ^{57}Cobalt-bleomycin. In the case of the ^{57}Cobalt-bleomycin, strict experimental precautions must be taken for animal handling because of the long half-life of the ^{57}Cobalt gamma emitters (270 days).

Using ^{57}Cobalt-bleomycin, Belehradek and colleagues showed a 4 times increased retention of radioactive bleomycin in tumors exposed to permeabilizing electric pulses as compared to unexposed tumors [5]. This factor was equivalent to the one observed *in vitro* [6] using cells in suspension exposed to external concentrations of radioactive bleomycin similar to those measured in mice blood at the time of 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.

51Cr-EDTA

51Cr-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 51Cr-EDTA. One hour after the pulses delivery, a difference in the retention of the radioactivity between the muscle exposed to reversibly permeabilizing electric pulses and the contralateral non exposed muscle can already be observed in the skeletal muscle [7]. If the mouse is sacrificed 24 hours after the electric pulses delivery, the control unpulsed muscles do not contain any radioactivity and less animals can be used to have the same number of experimental samples (exposure to the electric pulses of the two contralateral muscles) [8]. The quantitative 51Cr-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 (D. Batiuskaite et al. in preparation)
- to show differences between pulses of different durations thresholds [7,9]
- to show similarities between the same tissue in different species thresholds [7,9, and D. Batiuskaite et al. in preparation]
- to show differences between different tissues (D. Batiuskaite et al. in preparation).

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 [10].

(99m)Tc-DTPA

Radiolabelled diethylenetriaminepentaacetic acid (DTPA) was used to trace the distribution and internalisation of a hydrophilic drug after *in vivo* electropermeabilization [11]. 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 a 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 1200 V/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.
- that the duration of the permeabilized state is longer 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].
- that 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 [12], 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 [13], 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.
- that, 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 [7] was used in 1999 to compare two types of electrodes: plate electrodes and rows of needle electrodes (two kind of electrodes largely used in ulterior experiments). A good fit was found between the percentages of tissue exposed to fields of a strength above a given value and the 51Cr-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* (D.Sel; Ph.D. thesis and manuscript submitted). 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.

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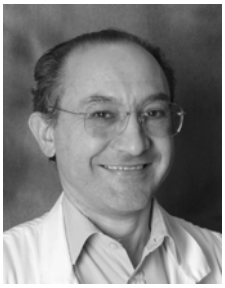

Lluis M. Mir was born in Barcelona, Spain, in 1954. He received a Masters in Biochemistry in 1976 from Ecole Normale Supérieure, Paris, and a Doctorate (D.Sc.) in Cell Biology in 1983. In 1978 he entered CNRS as Attaché de Recherches in the Laboratory of Basic Pharmacology and Toxicology, Toulouse. In 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) are used, 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 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.

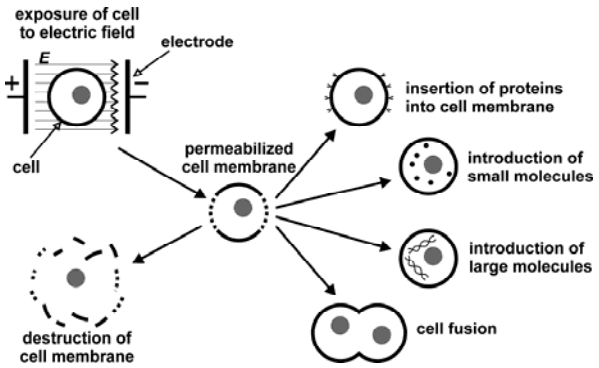

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications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

ELECTRO GENE TRANSFECTION

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; 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 electroporation 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.

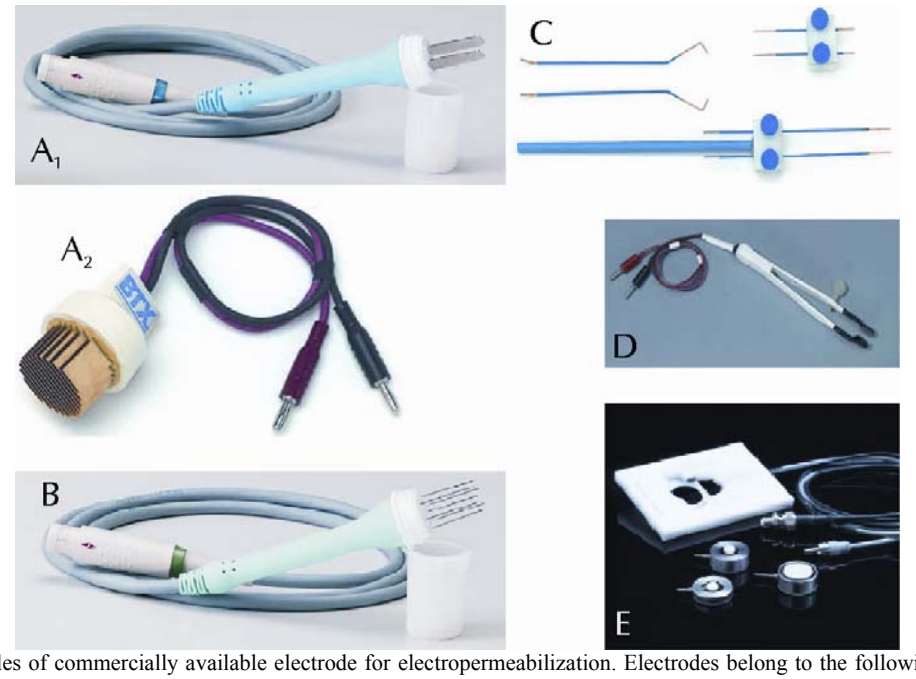

Figure 2: Examples of commercially available electrode for electropermeabilization. Electrodes belong to the following group: A_1 and A_2 – to parallel plate electrodes, B – needle arrays, C – wire electrodes, D – tweezers electrodes and E – coaxial electrodes. Electrodes A_1 and B are produced by IGEA, 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 his 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 these types of pulses 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. This general overview of electrical parameters should only be a starting point for a design of experiments or treatments with electroporation. Optimal values of parameters 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, it has to be linked also to the electrodes choice.

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ACKNOWLEDGEMENT

This research was in part supported by Ministry of Sports, Education and Science of the Republic of Slovenia, by Slovenian Research Agency, and by Framework Programs of European Commission through various grants.

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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.

NOTES

Application of 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. 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 combined modality treatment using 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; some of them are daunorubicin, doxorubicin, etoposide, paclitaxel, actinomycin D, adriamycin, mitomycin C, 5-fluorouracil, vinblastine, vincristine, gemcitabine, cyclophosphamide, carboplatin, cisplatin and bleomycin. Electroporation of cells increased cytotoxicity of some of these drugs ranging from 1.1 to up to several hundred folds. However, only two of these drugs have been 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. Only several hundred molecules of bleomycin inside the cells are needed to kill the cells. The second being cisplatin that has also hampered transport through the cell membrane. Only 50% of cisplatin is transported through the plasma membrane by the passive diffusion, the rest is by carrier molecules. Nevertheless, electroporation of the cells increases cisplatin cytotoxicity by up to 80-fold. These preclinical data *in vitro* have paved the way for testing

of these two drugs in electrochemotherapy *in vivo* on different tumor models [1-3].

In vivo studies

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

Results of these studies demonstrated that antitumor effectiveness depends on different factors:

- The drugs can be given by different *routes of administration*, injected either intramuscularly, intraperitoneally, intravenously or intratumorally. The prerequisite step is that at the time of the application of electric pulses to the tumors sufficient amount of drug is present in the tumors. Therefore after intravenous drug administration a few minutes interval is needed in animals for maximal drug concentration in the tumors. After intratumoral administration this interval is shorter and application of electric pulses has to follow administration of the drug as soon as possible (immediately) [1-4].
- The antitumor effectiveness is dependent on the *amplitude and the number of electric pulses applied*. Several studies showed that for tumor electroporation, amplitude above 1000 V/cm is needed, and that above 1500 V/cm (electrode over distance ratio) irreversible changes in normal tissues adjacent to the tumor occurred, so the window for effective and safe electrochemotherapy is between 1000 -1500 V/cm. Most studies used 1300 V/cm that induced good antitumor effectiveness, without sub optimal electroporation of the tissue or damage to the tissue due to irreversible cell permeabilization. The minimal number of the pulses used was 4, but most studies used 8 electric pulses. The studies on different *frequencies of the pulses* for electrochemotherapy showed that can be used ether 1 Hz or 5 kHz. [1-5].
- For good antitumor effectiveness optimal tissue electroporation has to be obtained. This is dependent on *electric field distribution of the tissue* that can be improved by rotation of the electric field, and by distribution of the electric field in deeper parts of the tumor, as in the case of the needle electrodes [6].

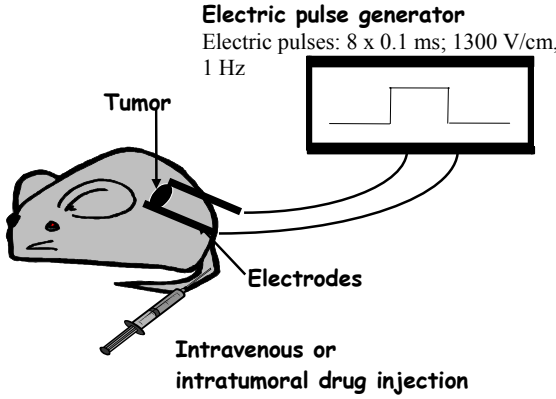

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumorally, at the doses that usually do no exert antitumor effect. After the interval that allows sufficient drug accumulation in the tumors, electric pulses are 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.

All the experiments provided sufficient data to demonstrate that electrochemotherapy with either bleomycin or cisplatin is effective in treatment of solid tumors, with drug concentrations that without application of electric pulses had none or minor antitumor effect. In addition, application of electric pulses alone had minor or no effect on tumor growth (Figure 1,2).

Mechanisms of action

Principal mechanism of action of electrochemotherapy is *electroporation* of cells in tumors, which increases drug effectiveness by enabling the drugs to reach intracellular targets. This was demonstrated in studies that measured intratumoral drug accumulation and the amount of the drug bound to DNA. Basically up to 2-4 fold higher amounts of bleomycin and cisplatin was measured in the electroporated tumors, compared to those without application of electric pulses [2,3].

Besides this principal one, other mechanisms involved in antitumor effectiveness of electrochemotherapy were described. Application of electric pulses to the tissues induces transient but reversible *reduction of blood flow*. Restoration of blood flow in normal tissue is much faster than in tumors. The decrease in tumor blood flow induces *drug entrapment* in the tissue, providing more time for

drug action [7]. Besides, this phenomenon prevents bleeding from the tissue. The effect of electrochemotherapy is not only on tumor cells in the tumors, but also on stromal cells, including endothelial cells in the lining of tumor blood vessels [8]. Therefore, another mechanism involved in antitumor effectiveness of electrochemotherapy is its *vascular disrupting effect* [9]. Due to the massive tumor antigen shedding in the organisms after electrochemotherapy, *systemic immunity* can be induced, that can be up-regulated by additional treatment with biological response modifiers like IL-2, IL-12 and TNF-α [1-3].

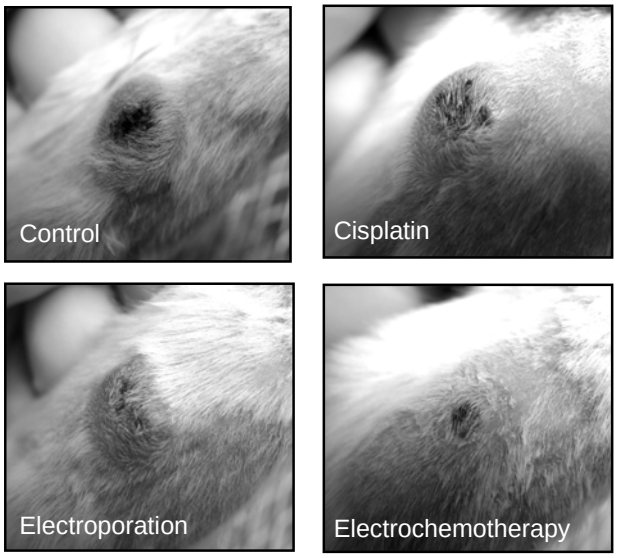

Figure 2: Example of good antitumor effectiveness on SA-1 tumors after electrochemotherapy with cisplatin. Cisplatin vas given intravenously (4 mg/kg), 3 min thereafter 8 electric pulses were applied to the tumor with plate electrodes. Electric pulses were applied in two directions; 4 pulses in one and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

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

PERSPECTIVES

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

Some chemotherapeutic drugs interact with radiation therapy. Among the radiosensitizing drugs are also bleomycin and cisplatin. As already indicated in our recent studies combined modality therapy with cisplatin or bleomycin and radiation can be improved by using electroporation of tumors [10,11].

Application of electric pulses was shown to modulate tumor blood flow. Reduced tumor blood flow and partial oxygen pressure (pO_2) are both consequences of applied electric pulses [9]. The reduction of pO_2 can selectively activate bioreductive drugs that exhibit cytotoxic effect in hypoxic conditions [12]. In addition, tumor hypoxia induced by application of electric pulses can provide improved therapeutic conditions for the use of hyperthermia, since tumor cells are more sensitive to heat in sub-optimal physiological conditions.

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, adenocarcinoma glandulae paranalis, neurofibroma and sarcoids in dogs, cats, hamsters, rabbits and horses. Recent reports demonstrated successful treatment of different neoplasms in companion animals, sarcoids in horses and perianal tumors in dogs. It is foreseen that electrochemotherapy will be broadly used in veterinary medicine for treatment of different malignancies, both primary and metastatic disease [13-16].

Electrochemotherapy is effective cytoreductive treatment, however its curative effect is dependent on the permebilization of possibly all cells in the tumors. Since this is theoretically impossible, as already mentioned, electrochemotherapy could be combined with other cytoreductive treatments. Another approach is combination of electrochemotherapy with electrogene therapy. The first promising reports and data are already available, supporting antitumor effectiveness of this concept [17,18].

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

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ACKNOWLEDGEMENT

This research was funded by research grant from the Research Agency of the Republic of Slovenia.

Gregor Serša, graduated from Biotechnical Faculty, at the University of Ljubljana in the year 1978, where he is currently full professor for molecular biology. He is employed at the Institute of Oncology in Ljubljana as Head of the Department of Experimental oncology and as Assistant Medical Director for Research and Education. His specific field of interest is the effect of electric field on tumor cells and tumors as a drug delivery system in different therapeutic approaches. Besides experimental work, he is actively involved in the education of undergraduate and postgraduate students at the University of Ljubljana.

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 [1,2]. Results of this first study of the group from Institute Gustave Roussy, has stimulated other groups to launch their own clinical studies. The pioneers of electrochemotherapy were centers in Villejuif and Toulouse in France, the group in Tampa in USA and our group at the Institute of Oncology Ljubljana in Slovenia. In the last period of time clinical experience on electrochemotherapy reported also new centers; 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 [2-30].

So far in clinical studies were included 96 patients with 411 tumor nodules treated by electrochemotherapy with bleomycin and 85 patients with 418 tumor nodules that were treated by electrochemotherapy with cisplatin. The majority of patients were with malignant melanoma but treated were also patients with metastases in head and neck region, mammary carcinoma, skin cancer, ovarian cancer and Kaposi sarcoma. The results of the studies can be summarized that electrochemotherapy has good antitumor effectiveness either using bleomycin or cisplatin, resulting in ~80% objective responses of the treated tumor nodules.

Based on these results European project was launched which had its goal to develop and produce electric pulses generator that would be on the market for electrochemotherapy. In the CLINIPORATOR project this electric pulses generator was developed and is now commercially available for those that want to perform electrochemotherapy. It is named after the project - CLINIPORATOR™. The generator is certified for clinical use. Along with the development of the generator were developed also plate and needle electrodes.

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. Along with gaining more clinical experience we have gathered a vast amount of clinical data that are being evaluated right now and will be shortly published.

STANDARD TREATMENT PROCEDURES FOR ELECTROCHEMOTHERAPY

Electrochemotherapy is used for treatment of cutaneous and subcutaneous tumor nodules of different malignancies. The indications 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 tumor nodules can be treated by electrochemotherapy with injection of bleomycin intravenously or intratumorally and by electrochemotherapy with cisplatin given intratumorally. The choice of the cytostatic in not based on tumor histology but depends on number and the size of the nodules. After drug injection 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 intratumoral injection as soon as possible. For application are available different sets of electrodes; plate electrodes for smaller tumor nodules and needle electrodes for treatment of bigger (3 cm) and thicker tumor nodules. The treatment can be performed in one session or treatment can me repeated on multiple nodules or those that recur after the treatment.

Electrochemotherapy does not have side effects due to cytostatics, since the drug dosages are very low. However application of electric pulses to the tumors induces contraction of the underlying muscles. For electroporation are used square wave electric pulses of the amplitude over distance ration of 1000-1300 V/cm, duration of 100μs, frequency 1 Hz or 5 kHz. These muscle contractions are painful, bur dissipate immediately after electric pulses application. Therefore in SOP are described also procedures for local alleviation of the pain by local anesthesia, or by general anesthesia in the case of treatment of multiple nodules.

The treatment after single electrochemotherapy session results in most cases in complete tumor eradication. When necessary, treatment can be repeated in 4-8 week interval with equal antitumor effectiveness. The results of the treatment have good cosmetic effect without scaring of 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.

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ACKNOWLEDGEMENT

This research was funded by research grant from Research Agency of the Republic of Slovenia and ESOPE project.

Zvonimir Rudolf has been employed at the Institute since 1977. This is now his third term of office. He held the post of director general in the periods between 1986-1991 and between 1996-2000. In the period between 1991-1996, he was appointed Assistant Director for Research. He started the present term of office in July 2003. Professor Rudolf is a university teacher and a renowned investigator with rich production of works published also in medical literature abroad. From 1996, he has been also chairing the Chair of Oncology and Radiotherapy at the Medical Faculty of the University of Ljubljana.

NOTES

In vitro gene transfection: what can we learn from cell imaging?

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Abstract: Electropermeabilization is a physical method that allows to transiently permeabilize cell plasma membrane. Although this approach shows promise in field of gene therapy to transfer plasmid DNA into cells in culture but also in tissues, the basic processes supporting the mechanism are still to be elucidated. The purpose of this lecture is to give some insights into them. The early events of membrane permeabilization and associated DNA transfer can be visualized by fluorescence microscopy. Permeabilization sites can be detected at the sides of the cell membrane facing the two electrodes. DNA interaction with the electropermeabilized membrane can be detected facing the cathode. Gene expression is affected by applying electric pulses in opposite directions and/or at various frequencies. These results confirm the multi-step process involved in gene electrotransfer across a membrane, i.e. accumulation of DNA towards the electropermeabilized membrane, insertion and its passage and put forward the existence of different classes of DNA interacting with the membrane based on the stability or not of the interaction.

INTRODUCTION

Cell membrane acts as a barrier that hinders the free diffusion of molecules between cell cytoplasm and external medium. However, the permeability of membranes can be transiently increased when external electric field pulses are applied, a method called electropermeabilization [1-3]. Electropermeabilization can be used to deliver potentially therapeutic molecules such as cytotoxic drugs, proteins, RNA and DNA *in vitro* as well *as in vivo* [4-7]. But the safe and efficient use of this physical method requires the knowledge of the mechanism underlying that phenomenon of electropermeabilization.

Up to these last years, almost all investigations related to the membrane electropermeabilization process, have been performed on cell populations. No clear evidence of the molecular mechanism has been obtained. Gene transfer was always evaluated by the associated gene expression. Therefore, there is a general agreement that very little is known at the molecular level about what is really occurring during membrane electropermeabilization [3]. With the development of cell imaging, it becomes now possible to visualize the membrane regions where the transfer of external molecules takes place.

The focus of this article is to make a short report on what is known on the processes supporting the electrically mediated membrane permeabilization and the DNA transfer in mammalian cells.

MATERIAL AND METHODS

Experimental procedures have been described elsewhere [8].

Briefly, Chinese hamster ovary (CHO) cells were used. They were grown in petri dishes or in suspension in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % foetal calf serum.

Electropulsation was operated by using a CNRS cell electropulsator (Jouan, France) which delivered square-wave electric pulses. A uniform electric field was generated between the electrodes connected to the voltage generator. By using a generator prototype developed at the University of Ljubljana, it became possible to change the electric field polarity between pulses on a relatively large range of pulse repetition frequencies.

Cells in suspension were centrifuged and resuspended in a low ionic content isoosmotic buffer. For plated cells, culture medium was removed and replaced by the same buffer described above. Pulses with controlled duration were applied at various frequencies at a given electric field intensity. Penetration of propidium iodide into cells was used to monitor permeabilization. Gene transfer could be visualized by using plasmids labelled with a fluorescent dye (Toto-1). Gene expression could be visualized by using a plasmid coding for a reporter gene (Green Fluorescent Protein).

For microscopic observations, electropulsation chambers were designed using two stainless-steel parallel rods put on a microscope glass coverslip chamber. The chamber was placed on the stage of an inverted digitized videomicroscope (Leica DMIRB, Germany).

RESULTS

Membrane permeabilization

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.

The field induced potential difference is added to the resting potential [9, 10]. 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. This theoretical prediction has been experimentally verified by using a voltage sensitive fluorescent dye [11].

Electropermeabilization can be described as a 3-step-process by respect with Electro-Pulsation:
1) before EP: membrane acts as a barrier that prevents the exchange of hydrophilic molecules between cell cytoplasm and external medium.
2) during EP: the transmembrane potential increase induces the formation of local Transient Permeable Structures that allow the exchange of molecules.
3) after EP: resealing is occurring. Membrane permeability to small molecules is present with a lifetime ranging from sec to min [12, 13]. Permeabilization indeed occurs only on the part of the membrane where potential difference has been brought at its critical value.

The use of videomicroscopy allows to analyse the permeabilization phenomenon at the single cell level. Propidium iodide can be used as a probe for small molecules [8]. Its uptake in the cytoplasm is a fast process that can be detected during the seconds following Electro-Pulsation. Less than one minute later, it appears at the nuclei level. Moreover, exchange across the pulsed cell membrane is not homogeneous on the whole cell membrane. It occurs at the sides of the cells facing the electrodes on an asymmetrical way (Figure 1). 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.

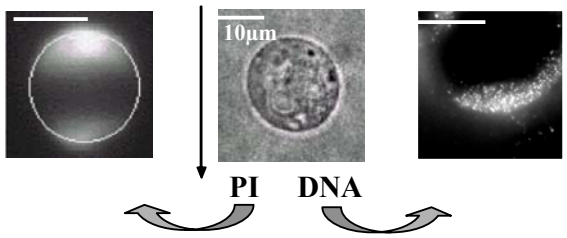

Figure 1: Visualization of the electro-mediated membrane permeabilization and gene transfer. Micrographs of a CHO cell pulsed in a buffer containing propidium iodide or a fluorescent-labelled plasmid. 8 pulses of 5 ms at 0.8 kV/cm were applied (from top to bottom). Center, phase contrast imaging. Left : entrance of propidium iodide observed 1 s following electropulsation. Right : imaging of fluorescent DNA transfer observed 1 min following pulsation.

Gene transfer

Direct transfer of large molecules to the cytoplasm is observed but only if macromolecules (proteins, DNA) are present during the permeabilizing electric pulses. Gene expression is obtained after applying electric pulses to a mixture of cell and DNA. No transfected cells can be detected in the absence of electric field, in absence of DNA or when DNA is added after the pulses. Therefore, clear differences of processes by which molecules of different sizes translocate across the electropermeabilized membrane have been observed. While small soluble molecules can rather freely cross the permeabilized membrane for a time much longer than the duration of the electric pulse application, DNA transfer involves complex steps including interaction with the membrane and no free diffusion after pulse application. If the effects of the electric field parameters are about to be elucidated (pulse strength higher than a threshold value, long pulse duration), the associated destabilisation of the membrane has still to be clearly described.

The use of fluorescent plasmids allowed to monitor the interaction of nucleic acids with membrane at the single cell level. No free diffusion of plasmid into the cytoplasm is detected. DNA molecules, negatively charged, migrate towards the anode when submitted to an electric field. Plasmids interact with the cell surface side facing the cathode where it is accumulated by the field associated electrophoretic drag for electric field values leading the membrane to be permeabilized [8]. The DNA/membrane interaction is not homogeneously distributed in the permeabilized areas facing the cathode but is present into “membrane domains» which size ranges from 0.1 to 0.5 µm (Figure 1).

These observations are consistent with a process where plasmids interact with electropermeabilized part of the cell surface due to their interfacial electrophoretic accumulation. This result is consistent with a multi step process of DNA transfer [8,14]:
1) during EP, plasma membrane is permeabilized facing the two electrodes and DNA migrates electrophoretically towards the plasma membrane facing the cathode side where it interacts,
2) after EP, a translocation of the plasmid to the cytoplasm follows. Expression is detected after hours over several days.

Such kind of localized process led to the development of experimental strategies aimed to increase amount of DNA interacting with the permeabilized membrane and therefore gene expression. While cell permeabilization is only slightly affected by reversing the polarity of the electric pulses or by changing the orientation of

pulses, transfection level increases are observed. These last effects are due to an increase in the cell membrane area where DNA interacts (figure 2). Plasmids only interact with the electropermeabilized side of the cell facing the cathode. When changing both the pulse polarity and their direction, by a 90° rotation of the electrodes, DNA interacts with the whole membrane cell surface. This is associated with an increase in gene expression [15]. Such kinds of experimental protocols have also been successfully used *in vivo* to increase electropermeabilization in the case of electrochemotherapy and gene expression [16, 17].

A new electric pulse generator prototype has been developed at the University of Ljubljana. The main advantage of this pulse generator is the ability to change the electric field polarity between pulses during the application of pulses on a relatively large range of pulse repetition frequencies. The efficiency of gene delivery was measured by GFP expression 24 hours after electric pulses application. DNA was added to CHO cells that were exposed to pulses of at various frequencies from 0.1 Hz to 1000 Hz. The electric field was applied: i) in one direction and ii) in two opposite directions. Gene expression increased with the pulse frequency when the electric field orientation was applied in one direction; it decreased for pulse frequency above 2 Hz for pulses with reverse polarities. These results correspond well to the observation that the electrophoretic accumulation of plasmid DNA from the bulk to the membrane increases with higher pulse frequencies when electric pulses are applied in the same direction, while it decreases when electric pulses are applied in opposite directions. These results 1) confirm the multi-step process involved in gene electrotransfer across a membrane, i.e. accumulation and insertion and 2) give some evidence for different classes of DNA interacting with the membrane based on the stability or not of their interaction. It can therefore be proposed that only a few DNA molecules inserted into the electropermeabilized cell plasma membrane will enter the cell and lead to gene expression.

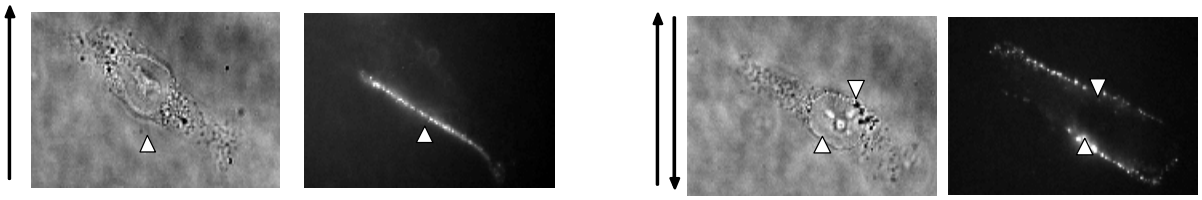

Figure 2: Correlation between electric field orientation and DNA/membrane interaction. Imaging of fluorescent DNA transfer observed 1 min following pulsation of CHO cells pulsed in a buffer containing a fluorescent-labelled plasmid. 8 pulses of 5 ms at 0.8 kV/cm were applied left : in one direction, right : in two opposite directions. The white arrows indicate the zone where DNA is interacting with the electropermeabilized cell membrane [15].

CONCLUSIONS

DNA electrotransfer is, because of its simplicity, a powerful laboratory tool to study gene expression and function. DNA transfer occurs through *microdomains* present in the electropermeabilized cell membrane. New directions of research are needed to caracterize these domains.

The use of electric pulses to transfect cells is nowadays extended *in vivo* on several tissue types, the most widely targeted tissue being skeletal muscle [7, 18]. In addition to its potential use in gene therapy, *in vivo* DNA electrotransfer is also a powerful laboratory tool to study *in vivo* gene function in a given tissue [19]. But, other studies will also be necessary to understand the cascade of events triggered by electropermeabilization at the cell and tissue levels where new constraints coming from tissues organisation are present, such as the inhomogeneity of the electric field strength and the intercellular distribution of DNA [20].

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ACKNOWLEDGEMENT

This work was supported by the CNRS, the AFM and FRM associations. This state of the art in the electrotransfer of DNA is mostly due to the works of the " GFP PhD students" I had the pleasure to supervise in the group of Justin Teissié: **G**olzio Muriel, **F**aurie Cécile and **P**hez Emilie.

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 University of Toulouse in 1984, 1989 and 1995, respectively. She is currently a Researcher at the IPBS-CNRS laboratory in Toulouse.

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 *in vitro* DNA electrotransfer. Marie-Pierre Rols is the author of 56 articles in peer-reviewed journals. In 1993 she received the Galvani Prize of the Bioelectrochemical Society.

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 one side, on the general developments of the finding that E. Neumann published in 1982 (successful DNA electrotransfer in cells *in vitro*) [1] and, from the other side, on 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 (electrogenetherapy).

INTRODUCTION

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 selectively induce the expression of the endogenous gene coding for the metallothionein I, that opens 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 51Cr EDTA uptake test [13]. The electric pulses have a second role: to electrophoretically move the DNA towards or across the "electropermeabilized" membrane. This electrophoretic component is responsible for the efficacy of the DNA transfer in tissues like the skeletal muscle [11, and S. Satkauskas et al. in preparation].

DNA ELECTROTRANSFER IN LIVER

DNA transfer in liver, using short pulses, was described in 1996 [14] (this was the second paper relating DNA electrotransfer *in vivo*, after the article by Titomirov et al in 1991 [15], 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 [16]. However appropriate combinations of HV and LV pulses also largely increase DNA uptake and expression of a reporter gene in mice liver (F. André et al, in preparation)

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) [17]. 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 in 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) [18] or using combinations of HV and LV (unpublished data).

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 with 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 [4]. No beneficial effect of the combination was found if bleomycin and these genes were transferred simultaneously.

Painless approaches or methods to control the sensations

Animals are treated after inducing general anesthesia 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 anesthesia. The sensations caused by HV pulses alone are known since they are used to treat solid tumors in patients (electrochemotherapy). It has been reported that electrochemotherapy provokes disagreeable sensations linked to the passage of the electrical current. There is "immediate" pain if these sensations are too intense, but there is never long term pain since sensations stop immediately when current passage ceases. However, the sensations (pain?) linked to the LV delivery are not yet known. LV, in laboratory animals, causes muscle contraction. It is anticipated thus that they will also produce sensations. However, on the one hand, because voltages are lower than those of the HV, but, on the other hand, pulse durations are three orders of magnitude larger, it is difficult to anticipate what will be the level of the sensations.

The ESOPE project

The Cliniporator project (QLK3-1999-00484) has allowed to develop a device appropriate for the DNA electrotransfer to various normal and malignant tissues. However this is not sufficient for the clinical use of the electrogenetherapy because it is important first to evaluate the sensations linked to the delivery of the electric pulses. The Esope Project (QLK3-2002-02003) is intended to establish the Standard Operating Procedures of the Electrochemotherapy and the Electrogenetherapy. Based on the clinical experience acquired in the treatment of cancer patients by the delivery of short pulses (HV pulses), four teams of physicians will test the transfer of a plasmid coding for a reporter protein and then they will assess the sensations felt by the patients due not only to the HV pulses but also to the LV pulses. More details on these

two projects funded by the EU commission can be found at the website: www.cliniporator.com.

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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), has been widely used for post-transcriptional gene silencing in C.elegans and Drosophila. Studies on RNAi have shown that this RNA-mediated mRNA cleavage may be part of overlapping processes leading to gene silencing in a variety of organisms. The application of RNAi to mammalian cells through the use of siRNAs indicates that it represents a powerful tool for reverse genetics in mammalians and possibly for gene therapy in humans. This is supported by the development of plasmid shRNA expression vectors able to induce RNAi in vivo as well as by accumulating data from the literature describing the use of RNAi in the treatments against various diseases. Electroporation can provide siRNA delivery in various tissues

Since its discovery [1], RNA interference has been described and extensively characterised 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]

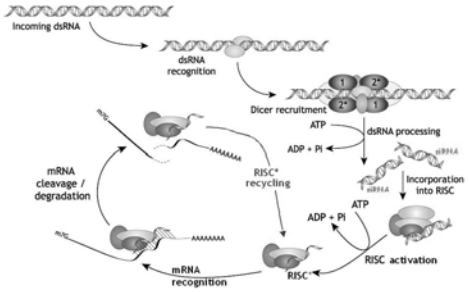

Figure 1: Current model for RNAi-induced mRNA degradation

This development requires, however, new safe and efficient *in vivo* siRNA delivery methods [7]. SiRNAs appear as a very promising new therapeutical agent but besides the problem of delivery, an unanswered problem is to know how long its effect lasts after a single dose delivery [8]. Most recently published *in vivo* results were obtained by « hydrodynamic transfection ». This stringent approach (injection within a few seconds in the tail vein of a volume one tenth the mass of the animal) appears to bring siRNA (and DNA) delivery mainly targeted to the liver [9-13]. Other methods were described where a systemic or a localised (portal vein injection) delivery was obtained by adding different chemical compounds to the siRNA solution [14-17]. SiRNA gene silencing could be obtained *in vivo* on reporter as well as endogenous genes. It was concluded that « better delivery methods … are clearly required before siRNA can be used in therapy, especially to suppress gene expression in tissues other than the liver» [7]. This remains a critical issue for the development of siRNA as an effective therapeutic. More recently, fluorescently labelled siRNA were injected IV or IP in mice being complexed with cationic lipid liposomes [16]. Their uptake in different tissues was assayed by fluorescence on cryosections. They were observed to be localized in the spleen and kidney after IV injection and to remain in the peritonea after their IP delivery. Rather high amounts (100 μg) were injected in this localisation experiment. Clearly the need for a delivery method suitable for targeting a broad range of tissues remains required. The demonstration in 1998 of drug and plasmid electrotransfer and gene expression in tumours [18,19] led to the proposal that *in vivo* electropulsation was a promising tool for exogenous agents delivery [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 electrode localisation. 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 therapeutical 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 of the same animal as a function of time with no adverse effects either on the reporter gene product or on the animal itself. 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 .

More and more studies investigated the effectiveness of electropulsation for the localized delivery of siRNA in cells and in adult mice (muscle, tumour, testis…) to obtain protein knock down [29-33].

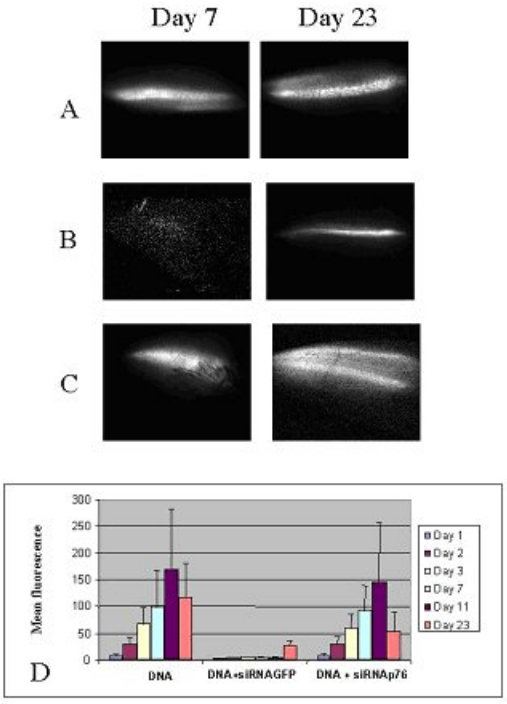

Figure 2: RNA interference in 9 weeks old C57Bl/6 mouse leg muscle. Representative images of the GFP fluorescence from the mouse leg. (Each image is 1 cm wide).
A- GFP expression resulting from plasmid alone electrotransfer as observed on days 7 and 23 in the same leg.
B- GFP expression silencing as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

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NOTES

Transdermal delivery and topical drug delivery by electroporation

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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.

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. Hence, it has been suggested that application of high voltage pulses might permeabilize the stratum corneum and enhance drug transport.

TRANSDERMAL DRUG DELIVERY BY ELECTROPORATION

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 enhanced. Few in vivo studies confirm the enhanced 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.

Light sensation and muscle contraction can be reduced by developing better electrode design.

TOPICAL DRUG DELIVERY BY ELECTROPORATION

Besides the permeabilization of the stratum corneum and the subsequent corneum and the subsequent increased skin permeability, electroporation alsa 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.

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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 L 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 number 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 Tres 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 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.

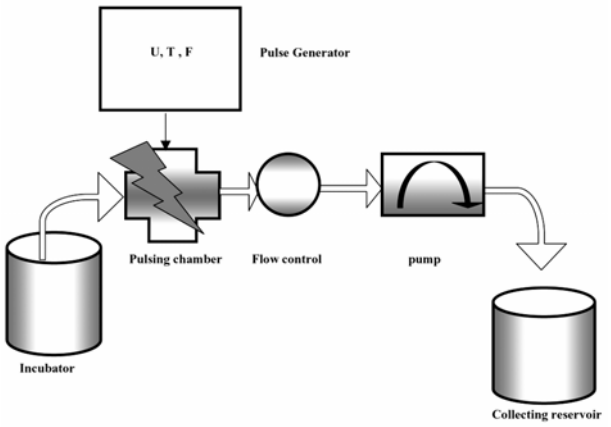

Figure 1: Flow electropulsation; A- Cells are taken from the incubator where they are growing. B- They flow through the pulsing chamber where a controlled number of calibrated pulses is applied. The pulsing chamber is connected to the high power pulse electropulsator where the voltage U, the pulse duration 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. 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.

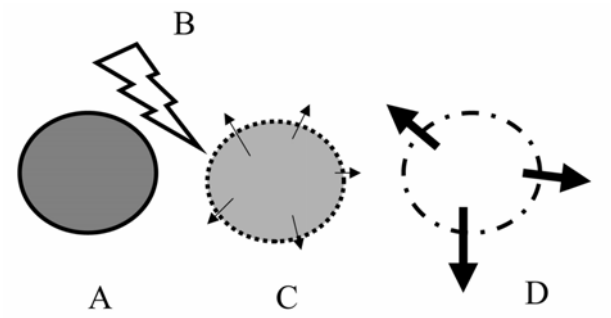

Figure 2: Irreversible Electropermeabilization; A- Intact cells. Their cytoplasm content is pictured in dark grey. B- Electropulsation. C- Cell membranes are permeabilized. The cytoplasm content leaks out as shown by the light grey colour and the small arrows. D-The cell membrane is irreversibly permeabilized 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.

Financial supports from EDF and NATO should be acknowledged. Thanks are due to my coworkers N. Eynard, M.P. Rols, V. Ganeva, B. Galutzov

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INVITED LECTURERS

Effects of Ultra-Short Electric Pulses on Cells

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INTRODUCTION

It is generally assumed that an increase in the transmembrane voltage of about 1 V will eventually lead to the "breakdown" of the lipid bilayer and pores will form that allow for the transfer of ions or even DNA while subcellular structures are not affected by the applied electric field. For pulsed electric fields of several hundred kilovolts per centimeter that are short compared to the charging time of the cell membrane, the effect on cells is different. Before the accumulation of charges along the outer membrane can shield the interior of the cell, all organelles will be exposed to an electric field. Since the charging time of the smaller substructures is, in general, shorter than for the outer membrane, organelle membranes may reach the breakdown threshold first. Although the mechanisms are not completely understood yet, the charging of membranes is likely the underlying cause of biological phenomena that are observed after exposure [1]. Since cells are often exposed using standard electroporation cuvettes with electrode distances from 1-4 mm, it requires voltages of up to 40 kV to achieve the required field strength [2]. Using smaller gaps, e.g. 100 μm between two electrodes under a microscope, allows us to reduce the output voltage of the pulse generator to values on the order of 1 kV or less [2].

INTRACELLULAR ELECTROEFFECTS

Better understanding of the effects of pulsed electric fields on membranes requires the measurement of transmembrane voltages in real time, i.e. with a temporal resolution short compared to the charging time of the membrane. When exposing Jurkat cells to a 60 ns, 100 kV/cm pulse, we observed changes in the transmembrane potential up to 1.6 V at 15-20 ns after the electric field was applied. Within several tens of nanoseconds after the pulse, the transmembrane voltage resumed resting potential values, indicating that in spite of the extremely high transient electric fields in the membrane (3.2 MV/cm), the cell membrane is not permanently damaged [3].

The possibility of a lasting effect on organelles while the plasma membrane is not noticeably compromised is seen in fluorescence images where plasma membrane stains, such as propidium iodide are compared to the response of nuclear stains such as acridine orange. Results suggest that intense ultra-short pulses target the nucleus and can modify cellular functions while plasma membrane effects are delayed [4]. Besides primary effects, an increasing number of secondary effects, including changes in cell functions, has been observed. The various secondary effects are dependent on pulse duration, pulse amplitude, and on the number of pulses. The observation of induced apoptosis [5] has led to experiments investigating the possibility to specifically target tumor cells. Fibrosarcoma tumors grown in mice exhibited growth regression resulting in a 60% reduction in size and weight as compared to the contralateral control tumor when treated with multiple pulses of 300 ns and 75 kV/cm [6]. Recent experiments, with similar exposure parameters, on melanoma tumors in mice show a mean tumor size regression of 90% within two weeks.

ACKNOWLEDGEMENT

This work has been supported by an AFOSR/DOD MURI grant on the Subcellular Response to Narrow Band and Wide Band Radio Frequency Radiation.

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NOTES

Numerical methods in biotechnology

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Abstract: Finite element method is a very flexible and powerful numerical method for solving differential equations, which can describe most of engineering applications. However the mathematical details of the method are complex. This lecture describes its basic idea and definitions.

INTRODUCTION

Every phenomenon in nature, whether mechanical, electromagnetical, geological or biological, can be described by the laws of physics, in terms of algebraic, differential, or integral equations relating the quantities of interest. Solving the equations derived for practical problems by exact methods of analysis is often impossible. In such cases approximate methods provide alternative means of finding solutions. Among them various numerical solution techniques have been developed and applied.

Whenever flexibility in the geometry is important the finite element method is a good choice. From a mathematical point of view, the method is an extension of the Rayleigh-Ritz-Galerkin technique. It therefore applies to a wide class of partial differential equations. One of the major advantages of the finite element method is that a universal computer program can be easily developed to analyse various kinds of problems.

BASIC IDEA OF THE FINITE-ELEMENT METHOD

The basic idea of the finite element method is the decomposition of a domain with a complicated geometry into geometrically simple elements, such that the governing differential equation can be approximately solved for these elements. The single element solutions are then assembled to obtain the complete system solution using given boundary conditions. In this way solving the differential equation or the system of differential equations is reduced to an algebraic problem.

THE STEPS

Discretization

The process of subdividing a domain into a finite number of elements is referred to as discretization. In reality, the elements are connected to each other along their boundaries but in a finite element analysis the assumption is made that the elements are connected only at specific points, called nodes. Nodes serve at the same time for the definition of the elements. A wide variety of element types are documented. It is up to the analyst to determine which type of elements are appropriate for the problem in hand, how distorted the elements can be not to spoil the solution and the density of the finite element mesh required to sufficiently approximate the solution.

The number of nodes assigned to an element dictates the order of the interpolation function which can be used to determine how the variation of the unknown quantity across the element is to be approximated. In most cases, a polynomial interpolation functions are used.

Set-up of element matrices

The unknown quantity in the domain of the element is approximated in terms of discrete values at the nodes. Consequently, a system of equations is formed which relates the material properties of the elements to the known and unknown quantities at the nodes.

Assembly of the system matrix

The assembly procedure combines each element approximation of the unknown quantity into a piecewise approximation over the entire solution domain. Basic rule of compatibility should be followed: the value of the unknown quantity at a node must be the same for each element that shares that node. In this way the continuity of the unknown quantity along the boundary is guaranted.

Apply the boundary conditions

Before applying the boundary conditions, the system of equations defined in the previous step is indeterminate and does not have a unique solution. Boundary conditions include essential as well as natural boundary conditions. In finite element terminology, a node is prescribed if the value of unknown quantity is already known (essential boundary condition). Usually these nodes lie on the boundaries of the finite element mesh.

Solution

The finite element method leads finally to a system of algebraic equations. Standard numerical techniques can be used in order to calculate the unknown quantity at each node.

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NOTES

Tumour Biology

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Abstract:. Cancer arises within a single cell as a result of accumulation of mutations within the DNA of that cell. When the mutations occur within key genes *e.g.* those involved in control of the cell cycle, apoptosis, DNA repair etc., it can lead to uncontrolled cell growth and eventually to a cell type that has acquired the ability to invade and metastasise. Due to the high variability of cancer types, the treatments given for cancer are highly variable and dependent on a number of factors, including the type, location, amount of disease and the health status of the patients. A short overview of carcinogenesis, tumour progression, types of cancer and treatment options is presented.

INTRODUCTION

Cancer is a class of diseases characterized by uncontrolled cell division and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (invasion) or by migration of cells to distant sites (metastasis). This unregulated growth is caused by damage to DNA, resulting in mutations to vital genes that control cell division, among other functions. One or more of these mutations, which can be inherited or acquired, can lead to uncontrolled cell division and tumour formation. Tumour ("swelling" in Latin) refers to any abnormal mass of tissue, but may be either malignant (cancerous) or benign (noncancerous). Only malignant tumours are capable of invading other tissues or metastasizing.

CARCINOGENESIS

Carcinogenesis (meaning literally, the creation of cancer) is the process by which normal cells are transformed into cancer cells.

Cell division (proliferation) is a physiological process that occurs in almost all tissues and under many circumstances. Normally homeostasis, the balance between proliferation and programmed cell death, usually in the form of apoptosis, is maintained by tightly regulating both processes to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer disrupt these orderly processes by disrupting the programming regulating the processes of cell division and cell death.

Carcinogenesis is caused by mutation of the genetic material of normal cells, which upsets the normal balance between proliferation and cell death. This results in uncontrolled cell division and tumour formation.

Carcinogenesis is divided into two steps: initiation and promotion. Initiation is rapid and irreversible and affects DNA directly (mutation). Promotion can be reversible, it's not necessary that it affects DNA directly (mutation), in this case the development of cancer requires prolonged exposure to the promoting agent.

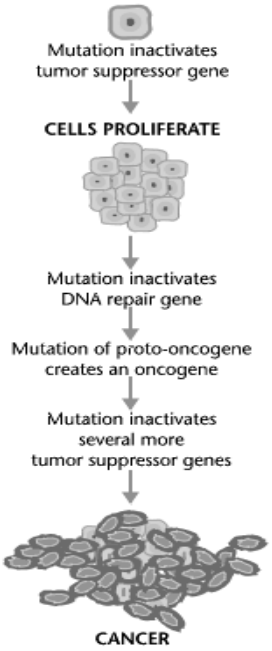

Figure 1. Development of cancer requires more than one mutation.

In most cases, more than one mutation is necessary for carcinogenesis. In fact, a series of several mutations to certain classes of genes is usually required before a normal cell will transform into a cancer cell. Only mutations in those certain types of genes, which play vital roles in cell division, cell death, and DNA repair, will cause a cell to lose control of its proliferation.

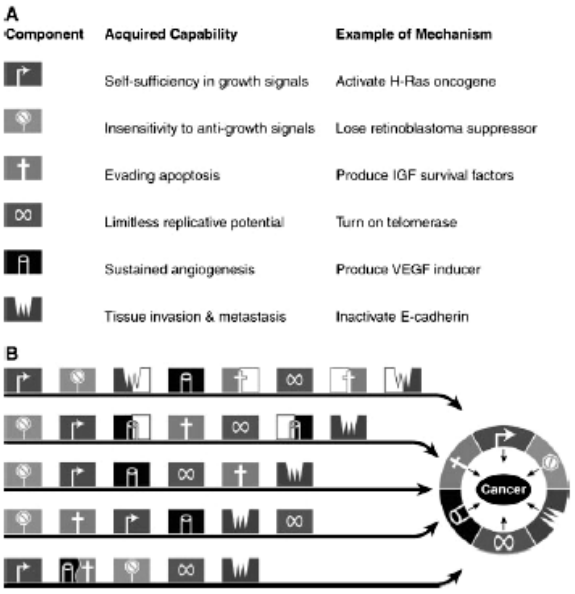

Figure 2. Parallel pathways of carcinogenesis.

MECHANISMS OF CARCINOGENESIS

Cancer is, ultimately, a disease of genes. Typically, a series of several mutations is required before a normal cell transforms into a cancer cell. The process involves both proto-oncogenes and tumour suppressor genes.

Proto-oncogenes are involved in signal transduction by coding for a chemical "messenger", produced when a cell undergoes protein synthesis. These messengers send signals based on the amount of them present to the cell or other cells, telling them to undergo mitosis in order divide and reproduce. When mutated, they become oncogenes and overexpress the signals to divide, giving cells a higher chance to divide excessively. The chance of cancer cannot be reduced by removing proto-oncogenes from the human genome as they are critical for growth, repair and homeostasis of the body. It is only when they become mutated that the signals for growth become excessive.

Protooncogenes can be thus growth factors or growth factors receptors, such as Her2/neu (erbB2), PDGF, signal transduction molecules, such as ras and src, transcription factors, such asmyc, fos and jun and others coding for antiapoptotic proteins bcl2 or mdm2.

Tumour suppressor genes code for chemical messengers that command cells to slow or stop mitosis in order to allow DNA repair. This is done by special enzymes, which detect any mutation or damage to DNA, such that the mistake is not carried on to the next generation. Tumour suppressor genes are usually triggered by signals that DNA damage has occurred. In addition, they can code for the enzymes themselves that repair DNA, or code for signals that activate such enzymes. However, a mutation can damage the tumour suppressor gene itself or the signal pathway, which activates it, "switching it off". The invariable consequence is that DNA repair is hindered or inhibited by every such event. Damage is originally checked by the tumour suppressor genes, but accumulates and becomes more abundant as more tumour suppressor genes succumb to mutation. With repair functions disabled, this inevitably leads to cancer. Examples of tumour suppressor genes are p53, Rb1, APC and BRCA1.

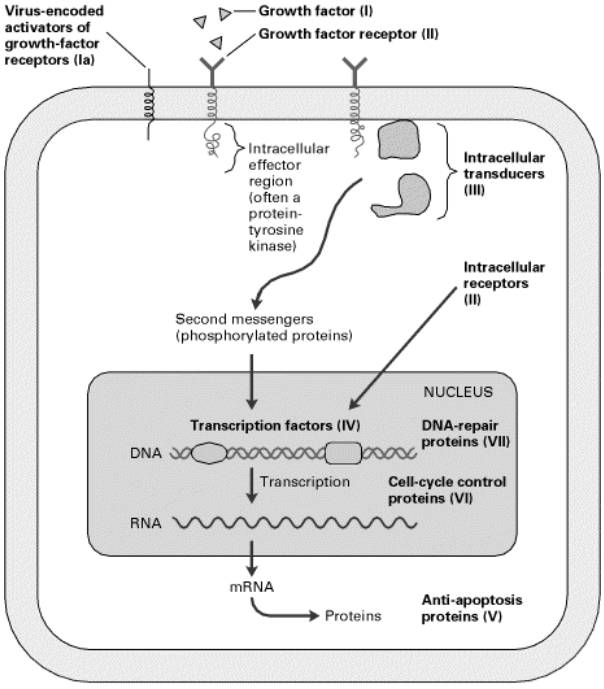

Figure 3. Mutation in the genes coding for proteins shown on the figure can lead to cancer development.

In general, mutations in both types of genes are required for cancer to occur. For example, a mutation limited to one oncogene would be suppressed by normal mitosis control (the Knudson or 1-2-hit hypothesis) and tumour suppressor genes. A mutation to only one tumour suppressor gene would not cause cancer either, due to the presence of many "backup" genes that duplicate its functions. It is only when enough proto-oncogenes have mutated into oncogenes, and enough tumour suppressor genes deactivated or damaged, that the signals for cell growth overwhelm the signals to regulate it and cell growth quickly spirals out of control.

Accumulated damage is generally theorised by most cancer researchers to build up exponentially later in life. Originally at youth defences against DNA damage are strong, but as more mutations to tumour suppressor genes occur, the rate of damage accumulation rises, causing exponential accumulation, a "death spiral" of sorts. This is further supported by the fact that the chance of acquiring cancer increases exponentially with age, rather than linearly. The average accumulated damage sampled from cancer cells tend to be immense - nearly all of the chromosomes have been mutated in some way, such as four copies of the same chromosome, trisomy, monosomy, or even completely missing chromosomes in the cell.

Mutations can be caused by various factors. In principle they can be divided into physical, such as UV and ionizing radiation, chemical, such as free radical and benzene and biological factors, such as viruses. Particular causes have been linked to specific types of cancer. Tobacco smoking is associated with lung cancer. Prolonged exposure to radiation, particularly ultraviolet radiation from the sun, leads to melanoma and other skin malignancies. Breathing asbestos fibres is associated with mesothelioma. In more general terms, chemicals called mutagens and free radicals are known to cause mutations. Other types of mutations can be caused by chronic inflammation, as neutrophil granulocytes secrete free radicals that damage DNA. Chromosomal translocations, such as the Philadelphia chromosome, are a special type of mutation that involve exchanges between different chromosomes. Among biological factors, viruses play the biggest role as some types of viruses can cause mutations. They play a role in about 15% of all cancers. Tumour viruses, such as some retroviruses, herpesviruses and papillomaviruses, usually carry an oncogene, or a gene inhibits normal tumour suppression in their genome. Examples are papilloma viruses, which are involved in development of cervical cancer, hepatitis B and C viruses in hepatic cancer and HIV-1, which is involved in development of Kaposi's sarcoma.

Many mutagens are also carcinogens, but some carcinogens are not mutagens and act as promoting agents. Examples of carcinogens that are not mutagens include alcohol and estrogen. These are thought to promote cancers through their stimulating effect on the rate of cellular mitosis (promoting phase of carcinogenesis). Faster rates of mitosis increasingly leave less window space for repair enzymes to repair damaged DNA during DNA replication, increasing the likelihood of a genetic mistake. A mistake made during mitosis can lead to the daughter cells receiving the wrong number of chromosomes, which leads to aneuploidy and may lead to cancer.

Mutations can also be inherited. Inheriting certain mutations in the BRCA1 gene, a tumour suppressor gene, renders a woman much more likely to develop breast cancer and ovarian cancer. Mutations in the Rb1 gene predispose to retinoblastoma, and those in the APC gene lead to colon cancer.

It is impossible to tell the initial cause for any specific cancer. However, with the help of molecular biological techniques, it is possible to characterize the mutations or chromosomal aberrations within a tumour, and rapid progress is being made in the field of predicting prognosis based on the spectrum of mutations in some cases. For example, up to half of all tumours have a defective p53 gene, a tumour suppressor gene also known as "the guardian of the genome". This mutation is associated with poor prognosis, since those tumour cells are less likely to go into apoptosis (programmed cell death) when damaged by therapy. Telomerase mutations remove additional barriers, extending the number of times a cell can divide. Other mutations enable the tumour to grow new blood vessels to provide more nutrients, or to metastasize, spreading to other parts of the body.

PROPERTIES OF MALIGNANT CELLS

Cells capable of forming malignant tumours exhibit many properties, which distinguish them from the cells of healthy tissue.

- They have an uncontrolled ability to divide (or, they are immortal), and they often divide at an increased rate.
- They evade apoptosis ("programmed" cell death).
- These cells are self-sufficient with respect to growth factors.
- They are insensitive to antigrowth signals, and contact inhibition is suppressed.
- These cells may exhibit altered differentiation.
- More aggressive malignant cells may also show additional abilities.

They have the ability to invade neighbouring tissues, usually through the secretion of metalloproteinases that can digest extracellular matrix material. They can form new tumours (metastases) at distant sites. They secrete chemical signals that stimulate the growth of new blood vessels (angiogenesis).

Nearly all cancers originate from a single cell, but a cell that degenerates into a tumour cell does not usually acquire all these properties at once. With each carcinogenic mutation, a cell gains a slight selective

advantage over its neighbours, resulting in a process known as *clonal evolution*. This leads to an increased chance that the descendents of the original mutant cell will acquire extra mutations, giving them even more selective advantage. Cells, which acquire only some of the mutations necessary to become malignant, are thought to be the source of benign tumours. However, when enough mutations accumulate, the mutant cells will become a malignant tumour.

STAGES OF TUMOUR PROGRESSION

Cancer has different morphology when investigated under the microscope. Cancer cells/tissue differ from normal in many features including variation in nuclear size and shape, variation in cell size and shape, loss of specialized cell features, loss of normal tissue organization, increase in cell division and a poorly defined tumour boundary. On the basis of patohistological examination cancer can be distinguished from hyperplasia, mild dysplasia, carcinoma *in situ* and invasive cancer. Imunnohistochemistry and other molecular methods are used to determine specific tumour markers, which may aid to diagnosis and prognosis of the disease.

Hyperplasia can be described by altered cell division in uncontrolled manner; but cell have a normal appearance and the process is considered to be reversible.

Dysplasia occurs when additional genetic changes in the hyperplastic cells lead to the even more abnormal growth. Cell no longer look normal, tissue may became dis-organised (loss of normal tissue organisation and cell structure). Very often cells regain their normal behaviour, but in some cases, they revert to malignant

Severe dysplasia is considered as *carcinoma in situ*. (Latin "in situ" means "in place"). Cells become more dedifferentiated or anaplastic. However, cells are still contained within the initial location and have not yet crossed the basal lamina.

Cancer is characterised by invasion into surrounding tissues and/or spread (metastasis) to areas outside the local tissue.

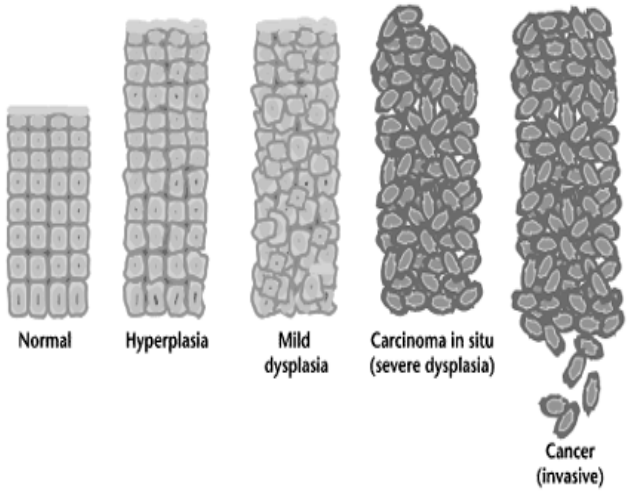

Figure 4. Stages of tumour progression.

TYPES OF CANCER

The uncontrolled and often rapid proliferation of cells can lead to benign tumours; some types of these may turn into malignant tumours (cancer). *Benign tumours* do not spread to other parts of the body or invade other tissues, and they are rarely a threat to life unless they compress vital structures or are physiologically active (for instance, producing a hormone). *Malignant tumours* (cancer) can invade other organs, spread to distant locations (metastasize) and become life threatening.

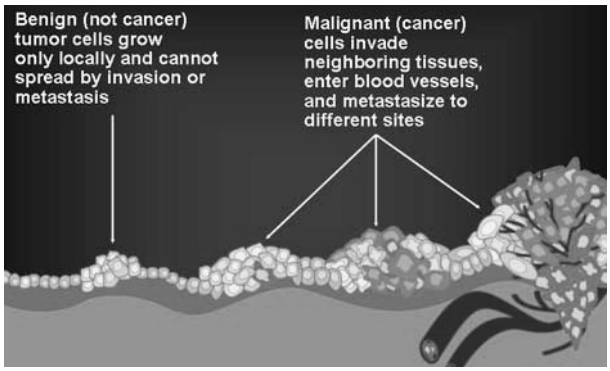

Figure 5. Difference between benign and malignant tumours.

Cancer cells within a tumour are the descendants of a single cell, even after it has metastasized. Hence, a cancer can be classified by the type of cell in which it originates and by the location of the cell.

Carcinomas originate in epithelial cells (e.g. the digestive tract or glands). *Haematological malignancies*, such as leukaemia and lymphoma, arise from blood and bone marrow. *Sarcoma* arises from connective tissue, bone or muscle. *Melanoma* arises in melanocytes. *Teratoma* begins within germ cells.

TREATMENT OF CANCER

Although extensive cancer research led to development of many new drugs and treatments for cancer there are still only three major cancer therapies; two being local: surgery and radiotherapy and a systemic chemotherapy. The choice of therapy depends upon the location and grade of the tumour and the stage of the disease, as well as the general state of the patient (performance status). A number of experimental cancer treatments are also under development.

Complete removal of the cancer without damage to the rest of the body is the goal of treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness.

Radiotherapy is the use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their DNA through direct action on DNA or indirect action via free radiacal that atack DNA., making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, spine, stomach, uterus, or soft tissue sarcomas. Radiation can also be used to treat leukemia and lymphoma (cancers of the blood-forming cells and lymphatic system, respectively). Radiation dose to each site depends on a number of factors, including the type of cancer and whether there are tissues and organs nearby that may be damaged by radiation.

Chemotherapy is the systemic treatment of cancer with drugs (chemotherapeutic or anticancer drugs) that can destroy cancer cells. The drug interferes with cell division in various possible ways, e.g. with the duplication of DNA or affects the mitotic spindle of the cell. Most forms of chemotherapy target all rapidly dividing cells and are therefore not specific for cancer cells. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy. Because some drugs work better together than alone, two or more drugs are often given at the same time. This is called "combination chemotherapy"; most chemotherapy regimens are given in a combination.

New, so-called biological treatment strategies include hormonal therapy, immunotherapy, gene therapy and others. The most promising drugs so far are either small drugs, such as tyrosine kinase inhibitor imatinib, which is used for treatment of chronic myeloid leukaemia and monoclonal antibodies such bevacizumab and cetuximab, which are used for treatment of colon cancer, transtuzmab for treatment of breast cancer, rituximab for treatment of non-Hodgkin's lymphoma, and others.

Because "cancer" refers to a class of diseases, it is unlikely that there will ever be a single "cure for cancer".

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NOTES

Design of plasmids for gene therapy clinical trials

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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). 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 currently being developed.

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.

For a specific application, such as a clinical trial several plasmids used to be tested before selecting the best one.

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 desirable for many gene therapy applications. The design and engineering of plasmids to obtain maximum transfection has been extensively researched. In addition to the transgene of interest, plasmid DNA molecules typically contain several regulatory signals such as promoter and enhancer sequences that play an important role in regulating gene expression. 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.

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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NOTES

Ethical issues in clinical research in oncology

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Discussion on ethics of clinical research in oncology may be summarized under five chapters:

1. ETHICAL DEMAND FOR RESEARCH.

Considering enormous burden imposed by cancer to every patient and to the society as a whole, it would be unethical not to search for new effective and safer approaches to treatment. During the past five decades, clinical research led to an improved outlook for virtually every cancer. Systematic clinical research is not only ethically acceptable but indeed an obligation for everybody in the field of oncology.

2. SCIENTIFIC AND PROFESSIONAL EXCELLENCE.

Compliance with high scientific and professional standards is an essential basis for any discussion on ethics of clinical research. Scientific excellence includes unbiased presentation of current knowledge, clear formulation of the hypothesis and of objectives of reearch and presentation of methods and of analysis of results. Professional excellence includes access to the medical equipment and drugs needed for the trial, personnel with proper education and experience, and adequate number of patients.

3. ETHICAL OBLIGATION OF PATIENTS.

From the legal standpoint, a patient with cancer is absolutely free to accept or to refuse participation in clinical research. From the ethical standpoint, however, a patient has a moral duty to participate in a clinical trial which has been approved by an independent scientific and ethical review and which does not constitute an unproportionate burden or risk. We base this moral duty on a fact that patients of today expect to be treated according to the best current knowledge. This knowledge increases continuously and is derived from the past clinical research. Patients of today therefore benefit from the fact that patients of yesterday participated in clinical trials – and have a moral duty to contribute to the medical knowledge which will benefit themselves, future generations of patients and society as a whole.

4. THE »ALARA« PRINCIPLE AND A POSITIVE ETHICAL BALANCE.

In the practice of clinical research, ethical puritanism is rarely achievable. In spite of meticulous plan and execution of a trial, some »ethical costs« are inevitable. Instead of speaking of zero ethical costs, we follow the philosophy of ALARA – *as low as reasonably achievable*. We also speak about a positive balance between the benefits of a trial (as applicable to patients in the trial and to society as a whole) and between ethical costs.

Here is a list of practical possibilities for lowering ethical costs and for a positive balance between benefits and ethical costs in clinical research:

- Eligibility criteria should be broad. Especially for Phase 3. and 4. trial, it is essential that the experience is applicable to a broad population of patients. Elderly patients and those with common co-morbidity (hypertension, diabetes) should not be excluded from research but rather offered an adjusted treatment schedule.
- Acceptance of real patient autonomy. Among patients with cancer, we can rarely speak about full autonomy. Patients are not only medically uneducated, but also under serious physical and emotional burden of the disease. Information for patient should be adjusted to his/her real condition: brief, understandable, with realistic presentation of the problem and also offering some hope. Very extensive information in a complicated medical and legal jargon with detailed description of all possible and rare complications of the treatment does not add to patient's autonomy. Rather, many patients will sign consent form without an attempt read such an extensive text, leading to a diminished autonomy. Even from a pure legal standpoint, it should not be too difficult to prove that many patients with cancer sign consent without being fully competent and/or do not have a free choice. If we accept limited patient's autonomy as a reality, then we have to accept also the fact that many patients do not rely on rational considerations but simply trust their doctor. Thus, while we need patient's consent, the responsibility stays with the physician. A certain degree of physician's paternalism is therefore a reality. A physician who is also a clinical researcher should be aware of the delicacy of his/her role. He should be aware that his relation to the patient does not derive from a contract, but rather from patient's trust. As he cannot get rid of a certain degree of paternalism, he has to strive constantly to push his role of a physician ahead of his role of a researcher.

- Patient's entry into a clinical trial should be formally registered with a person who is not under physician's authority. From this moment on, the patient is included in the trial, regardless of the actual course of disease or treatment. In final reports, many single-institution trials include only patients who have completed a certain number of treatment cycles and exclude those with early withdrawal due to complications, disease progression or patient's refusal. In randomized trials, it is crucial to leave registration and randomization to a person not involved in patient's care. This should eliminate a possibility for a biased randomisation.
- Experienced personnel, diagnostic equipment and supportive treatment should be available for the treatment itself and for management of complications.
- Conduct of a clinical trial should not affect other patients. Patients who are not eligible to participate in a certain trial due to a different diagnosis, age group or concomittant diseases and patients who declined participation in a trial should have equal access to diagnostics and standard treatment.
- Phase 1. clinical trials test new drugs and usually recruit patients resistant to one or several standard combinations of drugs. In such a case, the chances for an objective response after monotherapy with a new drug are quite small. These chances are even smaller for the initial sub-groups of patients who are offered only very small doses of the new drug. A reasonable compromise should seek a balance between patient's safety which demands only very gradual increases of the dose of the drug, and between patient's expectations to derive benefit from the new drug, a possibility which can only be expected with higher doses of the drug.
- Ethics of randomised clinical trials has its basis in the uncertainty principle. To maintain the uncertainty throughout the trial, it is essential to keep the recruitment period as short as possible. We have to avoid a possibility that an interim analysis would show a clear, yet statistically nonsignificant superiority of one treatment over the other. As a rule of the thumb, we propose that the recruitment period should not be longer than a double of the expected median time to event (e.g., with 12 months as expected median survival, recruitment period should not extend beyond 24 months).

Quick and unbiased publication of results is a key element for a positive balance between benefits for the society and inevitable ethical costs of medical research. Publication of negative trials is essential for futher research and for preparation of meta-analyses. Publication of clinical research should not be left to commercial sponsors. Surveys on publication bias have pointed to investigators as those who are to be blamed for delayed or missing publication of negative trials. Editors and reviewers of most medical journals do not select manuscripts for publication on the basis of positive or negative results.

5. ETHICS OF RESEARCH WITHOUT DIRECT INVOLVEMENT OF A PATIENT

In epidemiological research which includes personal questionnaires, most patients will consent to the survey. Problems with consent rarely arise.

In other epidemiological trials, in retrospective clinical surveys and in research on past bioptic material, patients are not directly involved. Quite often, such research includes data or biopsies from patients who are already dead. Such research – often performed in academic institutions and without commercial sponsors – benefits future generations of patients and is essential for better understanding of neoplastic diseases. Still, such research does not to any significant degree affect the interests of the patient as the primary source of this information or bioptic material. A proposal for such research should be submitted to independent scientific review and approved by appropriate ethical committee. However, there is no uniform opinion regarding patient's consent. In recent years, there is a trend in many legislations to extend to this type of research the demand for individual patient's consent, or (in case of death) consent of his/her heirs. This is legal puritanism. Human rights should not be understood as synonymous to individual autonomy, disregarding other principles such as human solidarity and public interest. Patients are not only individual human beings with their right of autonomy but also members of society. They expect to benefit from the society and have an obligation to offer their contribution. In my view, demands for individual consent present an unproportionate burden for this type of research and are therefore not justified.

STUDENTS' ABSTRACTS

The combination of electrochemotherapy and immunotherapy with CpG oligodeoxynucleotides in the treatment of metastatic cutaneous cancers: preliminary results.

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Cell electroporation is nowadays a well-known and controlled technique for the introduction of drugs, DNA and other non-permeant molecules into living cells, both in vitro and in vivo. The electrochemotherapy (ECT) is a solid tumor local treatment combining the injection of small amounts of bleomycin or cisplatin with trains of 8 short (100µs) square-wave electric pulses. In the mouse experimental models of subcutaneous tumors, pulses are delivered transcutaneously, at an applied voltage to distance between the electrodes ratio of 1300 V/cm.

The aim of the present study was to evaluate the efficacy of a new anti-tumor strategy consisting in the combination of the ECT with the administration of short oligodeoxynucleotides containing the non-methylated CpG DNA sequence (ODN-CpG). The ECT using bleomycin leads to cell death in the treated tumors, with the concomitant infiltration of the treated tumor by immune system cells. Tumor cell death can result in a consequent liberation of tumor antigens, which can be taken up and presented by antigen-presenting cells, specially the dendritic cells. We wondered whether this step would be potentiated by the action of ODN-CpG, a potent stimulator of both innate (direct) and acquired (indirect) immunity, which helps in better dendritic cell recruitment and stimulation via the Toll-like receptor9.

The antitumor effects, both local and systemic of this combination, were tested on two models of murine cutaneous tumors: a fibrosarcoma (LPB) and a melanoma (B16). The same tumor (either the LPB or the B16) was implanted on both flanks of the mice. The left-sided tumors implanted three days before the right-sided tumors. ECT treated the left-sided tumors in a single session, at day 8 after left side tumors implantation (day 0 ECT). The injection of the ODN-CpG into the treated tumor at day 1,8,15, and 22 for LPB tumors or day 2 and 9 for B16 tumors following ECT. The contralateral right-sided tumors were left untreated.

This combined therapy led to the complete local regression (100% at day 28) of the left-sided (treated tumors) and to the stabilization and/or regression of the right-sided (non-treated) tumors in the LPB model. Tumor regression obtained by the combined therapy was statistically different from the regression caused by either of the two single treatments alone ($P \leq 0.05$). Therefore consistent systemic effects were obtained by the combination of two local treatments. Furthermore, there was no tumor recurrence in the combined group even at day 60 after the treatment.

We are now looking for the bases of the efficacy of such combined treatment. The ECT alone results not only in the infiltration of the treated tumor by immune system cells but also on the increase in the global expression of the Toll-like receptor9. These results demonstrate the interest of the injections of ODN-CpG after the treatment by ECT.

The combination of ECT and an immunological adjuvant, like ODN-CpG, could thus be a reliable new strategy in the treatment of metastatic skin cancers.

ACKNOWLEDGEMENTS

The authors acknowledge Veronique Scott and Stephanie Esselin for their technical assistance in the development of RT-PCRs.

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The Effect of Electroporation on Treatment with NAMI-A

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INTRODUCTION

NAMI-A. Ruthenium complex NAMI-A, [ImH] [*trans*-$RuCl_4$(DMSO-S)Im] (Im=imidazole) is one of potential chemotherapeutics in cancer treatment.

Some authors believe that NAMI-A enters tumor cells by passive diffusion and by active transport [1], but certain difficulties exist for these complexes to penetrate cell membrane [2]. We wanted to examine the effect of antitumor ruthenium complex NAMI-A *in vitro* in combination with electroporation where uptake of NAMI-A present in medium is enhanced.

Electroporation. Efficiency of electroporation depends on parameters of external electric field (pulse duration, shape and number of pulses, electric field strength) [3]. Firstly, we determined the toxicity of NAMI-A at different concentrations. Secondly, we evaluated cell survival under different voltage applied during electroporation. Finally, we combined treatment with concentrations of NAMI-A that were lower than the toxic ones and electroporation under conditions that do not essentially reduce cell survival.

MATERIALS AND METHODS

Compound. NAMI-A was dissolved in PBS and sterilized by filtration with 0,22 μm filter.

Tumor Cell Line. An established B16F1 cell line was cultured according to standard procedure.

Cytotoxicity of NAMI-A on cell culture. The cell suspension of confluent culture was exposed to different concentrations of NAMI-A for 1h at 37 °C. After the incubation cells were diluted in growth medium and plated in Petri dish for clonogenic test (200 cells per Petri dish). Control cell suspension was handled in the same way without NAMI-A.

Electroporation. To determine the voltage that would not essentially reduce cell survival a 50 μl droplet of cell suspension containing 10^6 cells was placed between stainless steel electrodes and exposed to a train of 8 square electric pulses. The voltage 0 V (control), following 80 V to 240 V in 40 V steps was applied. The cells were then incubated at room temperature for 30 min and plated in Petri dish for clonogenic test.

To determine the survival of cells to a combined treatment with NAMI-A and electroporation the cell suspension was mixed with different concentrations of NAMI-A. Voltage applied during electroporation was 160 V. Other steps in protocol were the same as described above.

RESULTS AND FIGURES

The survival of cells treated with different treatment combinations was determined in comparison to the appropriate controls, in order to demonstrate the interaction between the treatments on cell survival. When the cells were treated with combination of NAMI-A and electroporation the highest cytotoxicity was observed.

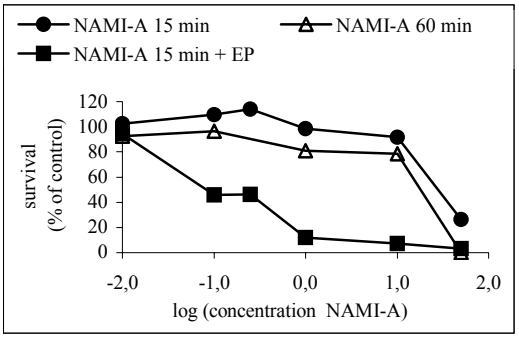

Figure 1: Survival curves of B16F1 cells after combined treatment with different NAMI-A concentrations and electroporation (EP) (8 pulses, duration 100 μs, repetition frequency 1 Hz), and after incubation in NAMI-A for 15 min or 60 min.

CONCLUSION

We have shown that the NAMI-A cytotoxicity is increased by electroporation. Based on these results we can conclude that NAMI-A has at least partially hindered transport through the cell membrane. Thus, electroporation increases cytotoxic effect of NAMI-A *in vitro*.

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Electrode Optimization by Visualizing the Spatial Resolution of Gene Expression for Efficient EGT

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INTRODUCTION

Membrane electroporation (MEP) is a powerful technique with increasing importance in clinical applications like electrochemotherapy of skin tumors [1] and gene therapy [2]. The small range of the applied electric field and pulse duration [3] and the presence of irreversible cell damages near electrode surfaces [4] show the necessity of methods to quantify them. Knowledge of the spatial resolution of gene expression between the electrodes is the basis to improve not only electric gene transfer parameters but also the design of optimized electrodes minimizing unavoidable cell damages required for efficient electrochemotherapy and electrogenetherapy.

METHODS

As cell system an oligolayer of adherently grown CHO–K1 (*Chinese hamster ovary*) cells is used in NUNC culture dishes with Nunclon$^{TM}\Delta$ surface processing cultured three days with fetal calf serum containing DMEM/F12 culture medium. The confluent cell layer is washed with a 1:1 mixture of 290 mM sucrose and CMF–PBS solution (S-PBS). Then the cells are incubated and pulsed with S-PBS containing 56 ng/µl naked GFP plasmid. After MEP, the cell layer is washed again with S-PBS and cultivated for another 3 days with cell culture medium. After this incubation, the used medium is removed and washed again with S-PBS. The fluorescent microscopic images are computed with a MATLAB program to get the mean relative spatial intensity of the expressed GFP [5].

RESULTS

The mean relative spatial resolution visualizes the transfection success of vital CHO cells after pulse application versus the distance. Using different types of electrode materials show different curve progressions: Over all, Aluminium shows a more homogeneous curve, but a lower amount of GFP expressed cells (shown in Figure 1).

CONCLUSION

The proposed method is useful to determine the success of gene expression after its electrotransfection. Varying the parameters, e.g. the electrode material, the optimal setup for the electrode system is found. In combination with other electric measurements [6], the most efficient setup is determined.

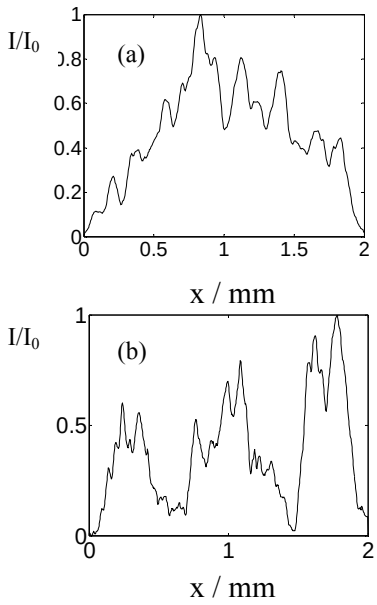

Figure 1: Mean relative spatial intensity versus the distance between the electrodes, visualized 3 days after transfection with plasmid (electrode distance = 2 mm, 12 pulses, E_{ext} = 1750 V/cm, t_E = 83 µs). As electrode material it was used (a) stainless steel, (b) aluminium.

ACKNOWLEDGEMENT

We gratefully acknowledge technical help from Ina Ehring and Thomas Seipp and financial support of the DFG, grant EN 227/9-5 and the Fonds der Chemie.

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An Update on Clinical Trials with Electrochemotherapy using Bleomycin

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INTRODUCTION

For more than 20 years electroporation has been a means to facilitate transport of normally nonpermeant molecules over the cell membrane. By applying an electrical field to the cell which just surpasses the capacitance of the cell membrane, the cell membrane becomes permeable and consequently allows different types of molecules to enter [1]. This technique has *in vitro* been used to load ions, dyes, proteins, RNA and DNA into cells. *In vivo* oligonucleotides, radiotracers and drugs have been electroporated into cells and research is proceeding regarding electroporation as a non-viral method for gene-therapy [2].

ELECTROCHEMOTHERAPY

Since electroporation creates a direct passage through the cell membrane, the transport and the efficacy of different types of chemotherapeutic drugs have been investigated. Drugs that under normal circumstances pass slowly or difficultly over the cell membrane can due to electroporation increase their intracellular concentration and hence the toxicity.

The combination of electroporation and chemotherapy is designated *Electrochemotherapy*, or ECT, and it has been studied *in vitro*, *in vivo* and in clinical trials. An interesting drug used in ECT is the hydrophilic charged antibiotic, Bleomycin. Bleomycin is a potent cytotoxic agent and is widely used in the treatment of e.g. testicular cancer and lymphoma, but the toxicity is restricted by the inability of bleomycin to freely diffuse through the plasma membrane. Preclinical data has shown that the toxicity of bleomycin can be increased 300-700 fold at the level of 50% cell kill by addition of electroporation as reviewed in Gothelf [3], and in clinical trials bleomycin is the most common drug used in ECT.

Another cytotoxic agent, which has shown promising results, is cisplatin [4], but the present study is focussed on bleomycin based ECT.

CLINICAL TRIALS

In the literature, from 1993 to August 2005, 125 patients with 486 malignant tumours have been treated with ECT [3;5-8]. The major part were patients with metastatic malignant melanoma (43 patients/254 tumours), head and neck cancer (*primary* 13 patients/13 tumours, *recurrent* 13 patients/13 tumours or *metastatic* 15 patients/86 tumours), or basal cell carcinoma (31 patients/69 tumours).

Four patients with breast cancer had 23 tumours treated with ECT, and six patients with lung cancer, Kaposi's sarcoma, bladder cancer, hypernephroma, metastatic squamous cell carcinoma and chondrosarcoma had 28 tumours (range 1-17) treated with ECT.

The rates for complete response (CR) vary between 0-100% depending on the technique used but overall the CR was 65% and PR (partial response) 25%.

Apart from the patient with digital chondrosarcoma [6], and the 26 patients with primary or recurrent head and neck cancer the rest of the treated tumours were cutaneous or subcutaneous nodules.

CONCLUSION

Electrochemotherapy is an efficient treatment of cutaneous or subcutaneous tumours, and to date mainly data from treatment of basal cell carcinoma, metastatic squamous cell carcinoma and metastatic malignant melanoma has been reported in the literature.

Currently the place of ECT is primarily in the palliative setting, but future research in topics such as electroporation-mediated gene-therapy may offer new possibilities for this technique

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Electrotransfection results on Jurkat cells on a microdevice

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INTRODUCTION

This paper presents Green Fluorescence Protein (GFP) transfection results on Jurkat cells thanks to electroporation technique on a microdevice. After optimisation of the electrical fields through electrical analysis of the microdevice and biological experiments with Propidium Iodure (IP) and calceïn, transfection rates up to 70% were obtained.

MATERIALS AND METHODS

1) The microdevice used is a conventionnal interdigited 100µm wide microelectrodes devices made of titanium and gold as proposed in [1]. The device was electrically characterized as well as the electroporation medium using HP4192 and simulation were performed for electric field optimisation (Figure 1).

2) The experimental electroporation procedure was repeated at least three times for each field value and was as follows: Jurkat cells were used at 5.10^5 C/ml in a low conductivity buffer (KH_2PO_4/ K_2HPO_4 2mM, Sucrose 292 mM, 200µS/cm, 300 mOsm/L). To test the optimal electrical field we first used propidium iodide (PI): 100 µl of cell suspension with 100 µM PI were put on the chip and 10 bipolar pulses of 500 to 900 V/ cm (2 ms, 1Hz) as proposed in [2] were applied. Cells viability was monitored with calcein-AM 5 µM one hour after the pulsations. The simulated field variations were confirmed through the apparent effect on the cells after the pulses (Figure 2) and optimum applied field determined (Figure 3).

3) Electrotranfection tests were made adding 5 µg of pEGFP-C3 with the best electrical field parameters i.e. 800V/cm. After the pulsations, the microsystems was put into an incubator to culture 24h prior the fluorescence visualisation.

RESULTS AND FIGURES

With optimised parameters, transfection rates up to 70% were obtained on Jurkat cells (Figure 4) with the microdevice. These results are comparable to macroscopic data [3].

Future work will involve using the same methodology with other cell types and confine the cells with microstructures at the electrical field optimized places.

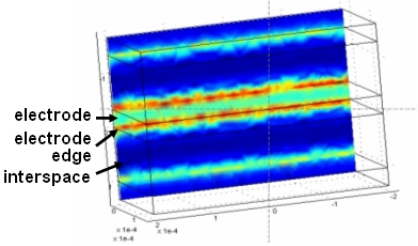

Figure 1: Field simulations. Field doubles at the edge of the microelectrodes

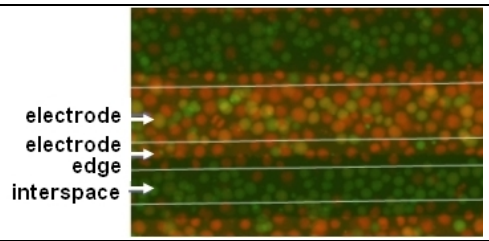

Figure 2: Picture showing the different field effect according to the place on the microdevice

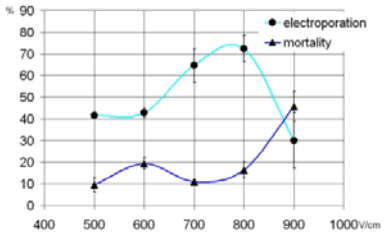

Figure 3: Electroporation and mortality rates VS V/cm

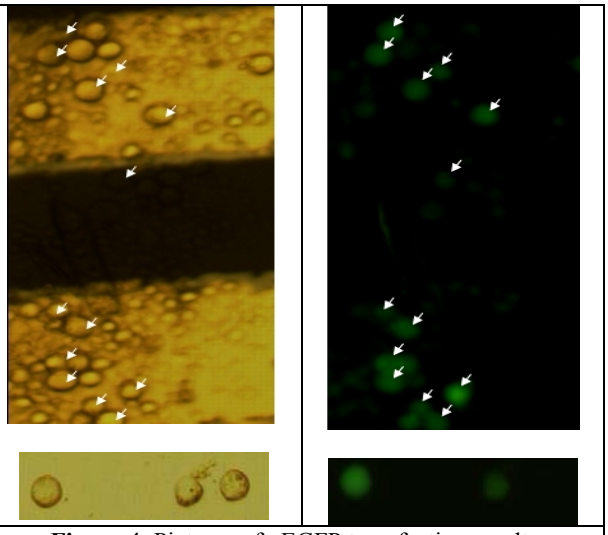

Figure 4: Pictures of pEGFP transfection results

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Nonlinear current voltage relationship of the plasma membrane of single CHO cells

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ABSTRACT

The application of electric field pulses to Chinese hamster ovary (CHO) cells causes membrane electroporation (MEP). [1,2] The increase in number of pores within the lipid bilayer part of the membrane causes changes in the electrical conductance, which are calculated from the data of voltage clamp and current clamp configurations, respectively.

If a voltage or current puls is applied, the membrane conductance nonlinearly increases (and saturates) with increasing field strength and time. Occasionally, the current/voltage characteristic displays a sharp sigmoidal increase in the conductance.

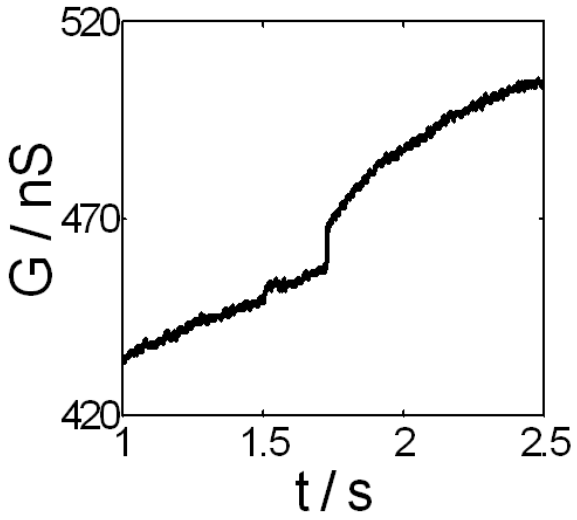

Figure 1: Sigmoidal increase of membrane conductance G(t) = I(t)/U(t) at 1.7 s.

This increase is interpreted as the formation of one or a few larger pores. In current clamp experiments pores with radii between 2.5 nm and 20 nm are formed. In voltage clamp configuration, the radii are between 2.5 nm and 55 nm. The larger pores are predominantly found during hyperpolarisation, i.e., to more negative membrane voltages at both clamp configurations. [3]

For analytical reasons, rectangular pulses are used to display the dependency on time and field strength of the appearance of these sigmoidal increases.

The single cell experiments are performed in whole cell clamp configuration, each with a single CHO cell with a radius of $5 < r_{cell} / \mu m < 8$. This technique allows to apply depolarising or hyperpolarizing electric field pulses selectively to single cellular membranes [4]. Note that this measuring configuration is different to MEP of cells in suspensions, where basically only the pole cap areas of the cells are electroporated and the anodic pole cap (hyperpolarized) is affected more strongly than the cathodic pole cap (depolarized).

It is emphasized that in single cell clamp configurations, the entire membrane is identically exposed to the same electric field directions from outside to the inside. There is no angle dependence and averaging over membranes with different orientations relative to the external field.

It is remarkable that the data reveal a great variability of the natural membrane potential $\Delta\varphi(nat) = \varphi(in) - \varphi(out)$, in the range $0 \leq \Delta\varphi(nat) / mV \leq 10$.

ACKNOWLEDGEMENTS

We thank Ina Ehring and Thomas Seipp for maintenance of CHO cell cultures.

We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft for grant Ne 227/9-3, /9-4, the Fonds Chemie and the European Union, Brussels, for grant QLK3-CT-1999-00484 to E. Neumann.

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Electrically-assisted gene delivery to tumors in mice is time dependent

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INTRODUCTION

Electrically-assisted gene delivery technique has become a powerful tool for efficient transfection of living cells with naked DNA. Many studies demonstrated the feasibility and effectiveness of this method also *in vivo*, elaborating on pulse parameters and plasmid construction. The time interval between the plasmid DNA injection and application of electric pulses to the tissue of interest may also play important role in achieving efficient transfection, however this issue has not been elaborated yet in the case of the tumors.

For this purpose we studied the optimal time interval between plasmid DNA injection and application of electric pulses on different tumor models in mice in order to improve *in vivo* electrically-assisted gene delivery.

MATERIALS AND METHODS

Plasmid. Plasmid pEGFP-N1 (encoding green fluorescent protein (GFP)) and plasmid pCMVluc (encoding luciferase) were prepared using the Endo-Free kit (Quiagen, Hilden, Germany), according to manufacturer`s instructions and diluted to concentration of 1mg/1ml.

Tumors and mice. SA-1 sarcoma syngeneic to A/J mice, LPB sarcoma and B16F1 melanoma syngeneic to C57Bl/6 mice were initiated by subcutaneous injection of cell suspension in the right flank of mice. LPB: 1.3×10^6 cells/ 0.1 ml EMEM; SA-1: 5×10^5 cells/ 0.1 ml EMEM; B16F1: 1×10^6 cells/ 0.1 ml EMEM.

Protocol. When the tumors reached approximately 6 mm in diameter, they were treated with intratumoral injection of plasmid (50 μg) and at different time points before or after injection of plasmid, tumors were exposed to 8 square wave electric pulses (600 V/cm, 5 ms, 1 Hz). Electric pulses were delivered through two parallel stainless steel plate electrodes with 6 mm distance between them and were generated by an electroporator GHT 1287 (Jouan, St. Herblain, France). Tumors were excised 48 h post-transfection.

Assessment of response. Tumors treated with plasmid pCMVluc were homogenized in 1 ml of Cell Culture Lysis Reagent (Promega, Madison, WI) using sonificator. Luciferase activity was measured in supernatants using TD-20/20 luminometer (Turner design, Maude Avenue, Sunnyvale, CA). Tumors treated with plasmid pEGFP-N1 were embedded in Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, IN, USA) and stored at -20ºC. Frozen samples were cut into 20 μm thick sections (24 sections per tumor). Transfection efficiency and spatial distribution of GFP was estimated in frozen tumor sections using fluorescence microscopy.

Statistical analysis. Significance tests were carried out using analysis of variance (ANOVA). Values of $P<0.05$ were considered as significant.

RESULTS

The results demonstrated that transfection efficiency of electrically-assisted gene delivery to tumors in mice is dependent on time interval between injection of plasmid into tumors and electroporation. In B16F1 tumor model the highest transfection efficiency was obtained when plasmid pCMV-luc or plasmid pEGFP-N1 was injected 5-15 minutes before the application of electric pulses. At these time intervals, GFP was distributed through the whole tumour sections. When plasmid was injected 1 hour, 30 and 0 minutes before electroporation of tumors, lower transfection efficiency was determined and GFP was observed only at the periphery of the tumor sections. In LPB tumor model there was no statistically significant difference in transfection efficiency when pCMV-luc or pEGFP-N1 plasmid was injected in time interval from 1 hour to 0 minute before the application of electric pulses. However, the highest level of luciferase and GFP expression was obtained at 15 and 10 minutes interval. No transfection was obtained when plasmid DNA was injected 2 hours before or 5, 10 and 30 minutes after electroporation of LPB tumors. In SA-1 tumor model the highest luciferase concentration was detected when pCMV-luc plasmid was injected 10 minutes before the application of electric pulses. In control groups (tumors injected with plasmid DNA only, without electroporation), levels of luciferase and GFP expression were at the limit of detection. Variability in the transfection efficiency was determined depending on the tumor model used. The highest expression was obtained in B16F1 melanoma (~5500 pg luc/mg tumor), less in LPB sarcoma (~ 850 pg luc/ mg tumor) and even lower in SA-1 sarcoma (~ 250 pg luc /mg tumor).

CONCLUSIONS

Our results demonstrate that transfection efficiency of electrically-assisted gene delivery to tumors in mice is time dependent. However, regardless of the tumor model, the best timing for plasmid injection into tumors is ~ 5-15 minutes before the application of electric pulses to the tumors. Within this time interval, 10 minutes seems to be optimal. Level of transgene expression is tumor type dependent.

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Induction of Immune Response using Dendritic Cells Transfected with Selected Tumor Antigens Associated with Human Breast Carcinomas

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INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells specialized in presenting foreign antigens to the immune system, and thus are central to the initiation of immunity [1].

The development of protocols to generate large numbers of DC in vitro has provided a rationale to design and develop DC-based vaccination studies for the treatment of infectious and malignant diseases. However, the efficacy of antigen loading and delivery into DC is crucial for optimal induction of T-cell-mediated immune responses [2]. The use of DC transfected with RNA encoding tumor antigen offers the prospect of antigen specific immunization without requiring prior knowledge of the immunogenic epitope in patients expressing a defined restricting allele [3], since epitopes from the translated protein are processed by the endogenous antigen-presentation machinery.

We have previously established an anti-tumor vaccine using autologous DC pulsed with p53 peptides for treatment of metastatic breast cancer [4]. Using in vitro synthesized mRNA and square-wave electroporation the object now is to improve this treatment by transfecting DC with mRNA encoding selected tumor antigens for transient expression of antigens in DC.

MATERIALS AND METHODS

DC generation: Buffy coats from healthy donors were separated by density gradient centrifugation and PBMC were exposed to plastic adherence. The adherent cells were subsequently treated with GM-CSF and IL-4 in X-VIVO medium, and incubated for 6-8 days for differentiation of DC.

Production of in vitro transcribed mRNA: The polyadenylated green fluorescence protein EGFP-pCIpA$_{102}$ plasmid (kindly provided by prof. Gustav Gaudernack, the Norwegian Radium Hospital, University of Oslo, Norway) was linearized, purified and served as DNA template for the in vitro transcription reaction. Transcription was done using Ribomax-T7 RNA production system (Promega) with the addition of m^7 cap analog (Promega) according to manufacturer's manual.

Transfection of DC: DC were washed, suspended in X-VIVO and placed on ice. DC were mixed with 20-100 µg mRNA in a 0.2 ml volume. The cells were then transferred to a 2-mm-gap cuvette and pulsed with a PA-4000 square-wave electroporator (Cytopulse). Transfection efficiency, DC maturation and cell viability were all analyzed with the FACSCalibur flow cytometer (BD Biosciences).

RESULTS AND CONCLUSION

We have made series of experiments to determine optimal conditions for transient expression of transfected antigens in DC by means of square-wave electroporation.

Using mRNA encoding EGFP, the square-wave electroporation data show high yield and viability (>90 %), and high transfection efficiency (>90 %) of DC. The flow cytometry analysis also demonstrated that the expression of EGFP peaked 48-72 h after electroporation in DC transfected either before or after maturation. However, higher levels of expression and viability were obtained when DC were electroporated after maturation. Furthermore, cryopreservation of DC before and after electroporation did not alter the level of EGFP expression. Taken together, these preliminary results show that the use of square-wave electroporation as a transfection method seems to be a useful and effective technique to charge DC with tumor antigens.

Next, in vitro analysis of anti-tumor T cell-reactivity from patients operated for primary breast cancer will be initiated using square-wave electroporation for transfection of DC with the tumor antigens p53, survivin and hTERT

ACKNOWLEDGEMENTS

This work is supported by the Aase & Ejnar Danielsens Foundation

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Programmable amplifier for measuring of bioelectric signals

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INTRODUCTION

Bioelectric signals are inherently small in potential and therefore sophisticated amplifiers are required in order to enable their measurement. Signals produced by bioelectric phenomena are relatively small in potential and therefore require sophisticated amplifiers to facilitate their measurement. ECG signals have amplitudes of 0.02 to 5mV, amplitudes of EEG signals are in range of 0.3µV to 0.3mV when using surface electrodes and of up to 50mV with nail electrodes. EGG, ERG and EOG also have amplitudes of up to few mV. Instrumentation amplifiers are suitable for this purpose for variety of reasons, important ones being high CMRR for interference downsizing and possibility of implementing high gain. Programmable instrumentation amplifier is one step better solution in terms of having A/D converter and computer interface for gain selection and digital signals processing and display.

AMPLIFIER STRUCTURE

We based the design of our high precision programmable amplifier on a low noise, precision integrated instrumentation amplifier AMP01 manufactured by Analog Devices. Some features that make this amplifier excellent for this application are its high CMRR of 130dB, 16-bit linearity at a gain of 1000, gain range 0.1 to 10,000 and output drive ±10V at ±50mA.

The gain is set in terms of equation $G=20R_S/R_G$, R_S and R_G being external resistors, selected by means of differential MUX and analog switch. The gain is set by resistor network in steps of 1, 2, 5, 10, …, 1000 and fine tuned afterwards by software. Frequency of the low pass filter between the output of the amplifier and input of the A/D converter can be set to 20Hz (appropriate for EGG, ERG and EOG, and also 50Hz interference elimination), 500Hz or some other frequency. The frequency is digitally selected. Filter is realized as a 2nd order Butterworth. The A/D converter is LTC1605 16-bit with successive approximation, produced by Linear Technology. It has bipolar input range of ±10V, integral nonlinearity of ±2LSB max, and SNR of 86dB.

DIGITAL CONTROL

All this digital circuitry needs to be operated by the microcontroller. An 8051 µC satisfies all the design requirements. It receives the result of the measuring via 16 ADC data lines and provides digital control signals to MUX (gain, attenuation and filter select), analog switches and ADC.

RESULTS

Since full scale range is 20V and ADC has 16-bit precision, absolute size of LSB is 300µV. When utilized of maximal gain of 1000, the theoretical measurement resolution is 0.3µV. When we obtain the result in memory we can remove the gain error of max 0.6% by means of smart programming so most of the error is in terms of ADC's internal reference characteristics and LP filter pass band unity gain discrepancy.

In standard commercial temperature range voltage drift is very low: 0.3µV/°C and max of 10ppm/°C gain temperature coefficient in AMP01, and also typ. ±5ppm/°C for ADC's internal reference.

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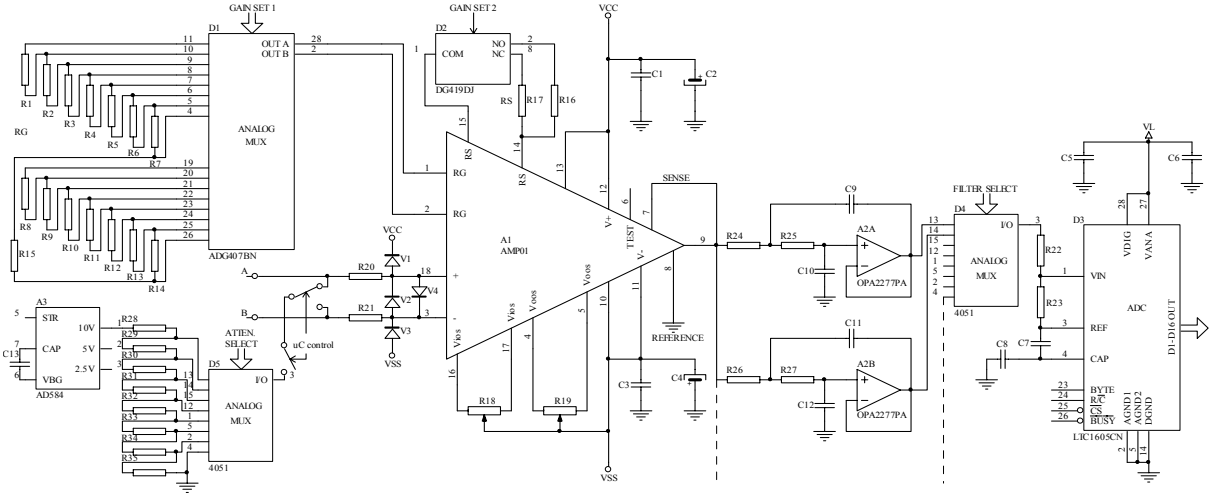

Figure 1: Diagram of the programmable amplifier.

Electrically-assisted gene delivery into canine skeletal muscle

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INTRODUCTION

Electrically-assisted gene delivery into skeletal muscle of different experimental animals has been successfully achieved using two different types of electroporation (EP) protocols. The first one utilized only low voltage electric pulses with long duration (e.g. 100-200 V/cm, 20-50 ms). Lately it has been shown, that better transfection efficiency can be achieved using combination of high voltage (HV) electric pulses (600-800 V/cm, 100 μs), which cause permeabilization of cell membrane, followed by low voltage (LV) electric pulses to enable electrophoresis of DNA across destabilized cell membrane [1].

The aim of this study was to determine optimal electroporation protocol for delivery of plasmid DNA into canine skeletal muscle. For this purpose we used reporter gene encoding green fluorescent protein (GFP), injected intramusculary into *m. semitendinosus*, followed by application of 5 different EP protocols.

MATERIALS AND METHODS

Animals. One female and 5 male beagle dogs, aged from 8-10 years were used. All procedures were preformed on animals under general anesthesia, according to the official guidelines after obtaining permission from the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia.

Plasmid. Plasmid pEGFP-N1, encoding green fluorescent protein was prepared using the Quiagen Endo-Free kit (Quiagen, Hilden, Germany), according to manufacturer's instructions and diluted to concentration of 1 mg/1 ml.

Plasmid transfection protocol. Incision of the skin and fascia was made in order to expose *m. semitendinosus*, followed by intramuscular injection of 150 μg of plasmid. Electric pulses were applied to muscles 20 minutes after plasmid injection, with electric pulses generator Cliniporator (IGEA, Carpi, Italy), using needle electrodes (4 mm). Skin incisions were closed with standard surgical procedures immediately after application of EP.

Altogether 5 different EP protocols were utilized, each applied to two muscles. The control group received only plasmid without application of electric pulses. Details of each protocol are listed in Table 1.

EP 1:	1 HV (600 V/cm, 100 μs), followed by 1 LV (80 V/cm, 400 ms, 1Hz)
EP 2:	1 HV (600 V/cm, 100 μs), followed by 4 LV (80 V/cm, 100 ms, 1Hz)
EP 3:	1 HV (600 V/cm, 100 μs), followed by 8 LV (80 V/cm, 50 ms, 1Hz)
EP 4:	8 LV pulses (200 V/cm, 20 ms, 1Hz)
EP 5:	6 LV pulses (100 V/cm, 60 ms, 1Hz)

Table 1: Details of EP protocols

Assessment of transfection efficiency. Incision biopsies of muscles were performed 2 and 7 days after electrotransfection. Samples were embedded in Tissue-Tek O.C.T. Compound (Miles Inc., Elkhart, IN, USA) at -20˚C. Frozen samples were cut into 20 μm thick sections and transfection efficiency determined using fluorescence microscope.

RESULTS

The highest level of GFP fluorescence was observed in two sets of muscle samples: samples taken from group, where EP 2 (1HV+4LV) were applied and from group, where EP 4 (8 pulses of 200 V/cm, 20 ms, 1Hz) were applied. In both protocols significant GFP fluorescence was detectable both 2 and 7 days after transfection.

EP 3 (1HV+8LV) yielded markedly lower degree of transfection, compared to EP 2 and 4. GFP fluorescence was less pronounced and it was detectable only on samples, taken at day 2 after electrotransfection. One week after transfection no GFP fluorescence was observed.

Muscle samples, taken from control group and from groups, where EP 1 (1HV+1LV) or EP 5 (6x100 V/cm, 60 ms, 1Hz) were used, showed no visible GFP fluorescence either 2 or 7 days after transfection.

The only observed side-effect was slight tissue swelling at the site of electroporation, which spontaneously resolved within 2-3 days after the procedure.

CONCLUSIONS

Based on the results of this study, we can conclude that good transfection efficiency of canine skeletal muscle can be achieved with two different electroporation protocols: either 1 HV (600 V/cm, 100 μs) followed by 4 LV (80 V/cm, 100 ms, 1Hz) or 8 pulses of 200 V/cm, 20 ms, 1Hz.

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Influence of pulse repetition frequency and electric field orientation on the efficiency of electrogene transfection

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INTRODUCTION

Electrogene transfection is a non-viral method used to transfer genes into living cells by means of high-voltage electric pulses. Electrogene transfection is therefore based on electroporation [1] and it has been proven to be successful in *in vitro* and *in vivo* conditions [2, 3]. Until recently, researchers mainly investigated the influence of amplitude, duration and number of electric pulses on the efficiency of electrogene transfection [4]. Moreover, some researches showed that change of electric field orientation increases the efficiency of electropermeabilization [5] and consequently the electrogene transfection [6]. In this study we investigated the influence of pulse repetition frequency and electric field orientation on the efficiency of electrogene transfection and electroporation.

METHODS

CHO cells were growing either in petri dishes or in suspension. Square-wave pulses generators were used. Electroporation of the cells was determined with fluorescence dye propidium iodide (PI). A plasmid DNA (pEGFP-C1), which expresses green fluorescent protein (GFP), was used for transfection. Results for electroporation and electrogene transfection were obtained by flow-cytometry. The efficiency of electrogene transfection was determined both by the fraction of transfected cells and the fluorescence intensity of transfected cells. To visualize the interaction between the plasmid DNA and cell membrane, the plasmid DNA was stained with fluorescent dye thiazol orange homodimer (TOTO-1) and observed under microscope [6]. Nine different pulse repetition frequency (0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 77 Hz) and four different electric field orientation (normal, inverted, cross and cross-inverted) were used during this study.

RESULTS

The results of electrogene transfection show that the fraction of transfected cells depends on the electric field orientation and it decreases with the increase of the frequency. The electric field orientation affects cell transfection, because it changes the surface of the electroporated membrane for DNA uptake. The decrease of cell transfection is most probably induced by the decrease of cell viability at higher frequencies.

The results of fluorescence intensity show that at a given frequency fluorescence intensity depends on electric field orientation. For normal electric field orientation the fluorescence intensity increases with frequency, while for inverted electric field orientation it decreases. We show that electroporation is not the reason for different influence of the electric field orientation on the fluorescence intensity at a given frequency. The change in intensity can be explained by the influence of polarity change on the transport of the plasmid DNA to the membrane. An energy barrier, namely, prevents the DNA to bind on the membrane without external force.

CONCLUSIONS

On the basis of our results we can conclude that for the electrogene transfection the cross-inverting electric field orientation at lower frequencies is more efficient than the other used parameters, because it results in the highest fraction of transfected cells.

ACKNOWLEGMENTS

The authors want to thank French-Slovenian Scientific Cooperation (PROTEUS Programme), Slovenian Research Agency (ARRS), Paul Sabatier University in Toulouse and French AFM association for supporting this study.

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Optimization of Electroporation with multi-field method

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INTRODUCTION

Research on electroporation(EP) modeling has been extensively improved. Bilska et al elaborated a model through Smoluchowski equation [1]. After solving the equations of the model using numerical methods, the number of pores and transmembrane potential (TMP) are obtained. The number of pores gives the EP efficiency. This study investigates the effects of triangular, exponential and rectangular pulses and determines some of the optimal electrotransfection parameters including strength and duration by simulation. Using the pulsed magnetic field to cause EP to occur easier is the subject of our new work.

MATERIALS AND METHODS

Finding the equations governing the transient current through membrane [1] and solving them, the N, V_m equations emerge as follows:

$$\frac{dN}{dt} = \alpha e^{(V_m/V_{ep})^2}(1 - \frac{N}{N_{eq}}) \quad \text{Where } N_{eq} = N_0 e^{q(V_m/V_{ep})^2}$$

In the above equations N is the number of pores, V_m is TMP, V_{ep} is characteristic voltage of EP, N_0 is the equilibrium density of the pores with C_m denoting the membrane capacitance per area. E is the intensity of the applied field, σ_e is the extracellular conductivity of the cell, θ is the angle between E vector and the normal to the surface of the cell. With the exception of N and V_m and E all the other parameters are constant quantities [1].

Since values obtained from above equations give the number of pores in a specific direction on the cell, to find the whole number of pores on a single cell, with radius a, we make use of the following equation:

$$N = 2\pi a^2 \int_0^{\pi} N(\theta)\sin(\theta)d\theta$$

We have solved the model through the second and forth order Runge- Kutta using MATLAB and SIMULINK toolbox.

RESULTS

For each of the chosen parameters in this simulation a graph is obtained. Some typical graphs are as follows:

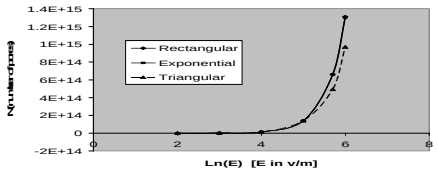

Figure 1: Effects of different pulse intensities on the number of pores. Special duration is considered (duration=0.1 s)

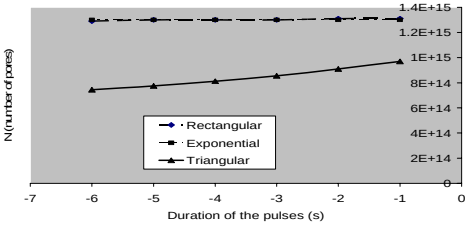

Figure 1: Effect of different pulse duration on the number of pores. Special intensity is considered (E=10^6)

In this work we have ignored the thermal effect of exponential pulse. Using exponential and square pulses with the identical intensity has no considerable difference between their EP efficiency. Using considered pulses with the same power, exponential pulse in contrast with square one produces more pores. Also results show that the efficiency of EP using triangular pulse is not better in comparison with square one. Pulses with the intensity more than 10^5 v/m and with duration or time constant of 0.1 sec are the best for producing the pores. Exponential pulses are recommended but in order to avoid thermal effects, the pulse power must be chosen identical to optimum rectangular pulse power.

In continuance we are going to use the pulsed magnetic field especially in order to changing TMP in the deep regions of the tissues. The magnetic field penetrates freely almost everywhere. From Faraday's law of induction, we know that a disappearing magnetic field leaves in its place a cyclic electric field. In this way, deeply in the tissue, electric fields are developing from the penetration and extinction of the magnetic field [2]. With high intensity short duration pulsed electromagnetic field we can obtain desirable pulse with low rising heat. We can use the synergy of this effect with usual EP (obtained in the first part of study) to cause the increase of EP efficacy and also applying this technique in the deeper regions compared to conventional Electroporation. By applying different field consequently or simultaneously, the EP can occur in lower intensity foe each field and therefore would be safer for the normal cells or tissues. The efficiency of this method can be simulated and obtained with similar method mentioned in this abstract and also in practical setups.

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Pain and Muscle Contraction during the Application of Electric Pulses in Electrochemotherapy

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INTRODUCTION

In electrochemotherapy a train of eight square electric pulses of 100 μs duration delivered at a repetition frequency of 1 Hz is used to electropermeabilize the tumor cells in order to enable chemotherapeutic agents such as bleomycin or cisplatin to cross the cell membranes. The application of pulses elicits painful sensations and also causes unwanted muscle contractions. The increase of the repetition frequency to 5 kHz reduces the number of muscle contractions from eight (1 Hz) to only one, however, the muscle contraction force increases by approximately two folds [1].

The aim of this study was to compare the intensity of pain caused by the application of electric pulses of two different repetitions frequencies (1Hz and 5 kHz).

MATERIALS AND METHODS

Patients: Skin melanomas were treated in 81 patients within the ESOPE EU program (QLK3-2002-02003) of the EU 5th FP. Patients were of different ages with tumors located in various regions of the body. Pain intensity was measured on a 100 mm visual analogues scale (VAS), while the intensity of muscle contraction was determined on a scale of 0-3 by the therapy operator.

Statistical methods: We analyzed the data using non-parametric statistical methods – the Mann-Whitney test, the Kruskal-Wallis test and the Spearman correlation coefficient. Significance level $\alpha = 0.05$ was chosen for all conducted tests. The statistical analysis was carried out with the statistical software Sigmastat 3.01.

RESULTS

Table 1: Pain intensity during the application of electric pulses of repetition frequencies 1 Hz and 5 kHz.

Repetition frequency [Hz]	Number of patients	Pain intensity [mm]	Statistical significance (p)
1	16	10	0.032
5000	41	30	

We established that electric pulses delivered at repetition frequency of 5 kHz cause pain of greater intensity than those delivered with 1 Hz (Table 1). The intensity of caused muscle contraction, however, did not differ significantly between both used repetition frequencies (Table 2).

Table 2: Intensity of muscle contractions during the application of electric pulses of repetition frequencies 1 Hz and 5 kHz.

Repetition frequency [Hz]	Number of patients	Muscle contraction intensity	Statistical significance (p)
1	20	2	0.629
5000	54	2	

CONCLUSIONS

Electric pulses of repetition frequency 5 kHz cause more intense pain than those of 1 Hz; however the difference (20 mm on a VAS) can barely be considered clinically significant [2]. The number of painful sensation was not taken into account, although it undoubtedly contributes to the patients' perception of the therapy. A two-dimensional measurement of pain with two independent VAS, one for pain intensity and one for pain unpleasantness would be more appropriate for evaluation of pain during electrochemotherapy, because the unpleasantness dimension incorporates also the number of painful sensation.

Since the intensity of muscle contraction, unlike pain intensity, did not change with the change of repetition frequency, we conclude that pain during the application of electric pulses is most probably caused by the direct stimulation of sensory skin nociceptors and not by painful muscle contractions.

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WORKBOOK

L1 - Measurements of the induced transmembrane voltage with fluorescence dye di-8-ANEPPS

THEORETICAL BACKGROUND

When a biological cell is exposed to an external electric field transmembrane voltage (ITV) is induced on its membrane. The amplitude of the ITV is proportional to the amplitude of the applied electric field, and with a sufficiently strong field, this leads to a strong increase in membrane permeability. As a result, molecules for which the membrane is otherwise impermeable can be transported across the membrane. Increased permeability is localized to the regions of the cell membrane where the ITV exceeds a certain critical threshold, which is in the range of 250 – 1000 mV. In order to obtain an efficient cell permeabilization it is therefore important to determine the distribution of the ITV on the cell membrane. By using a potentiometric fluorescence dye di-8-ANEPPS it is possible to observe the variations of the ITV on the membrane and to measure its value.

EXPERIMENT

To experimentally determine the ITV on cells, di-8-ANEPPS will be used. ANEPPS is a fast potentiometric fluorescence dye, which binds to the cell membrane, its fluorescence intensity varying linearly with the change in the ITV.

Protocol: The experiments will be performed on Chinese hamster ovary cells (CHO) grown on a cover glass in the culture medium (HAM-F12). When cells attach to the glass (usually after 2 to 3 hours), carrefully replace the culture medium with 1 ml of SMEM medium containing 30 μM of di-8-ANEPPS and 0.05% of Pluronic. After staining for 12 min at 4°C, wash the cells thoroughly with pure SMEM to remove the excess dye. Before the experiments, replace SMEM with an isoosmotic buffer (10 mM K_2HPO_4/KH_2PO_4, 250 mM sucrose, 1 mM $MgCl_2$).
Expose the cells to a 40 V of voltage applied for a duration of 150 ms on two parallel electrodes with a 4 mm distance between them. Apply five consecutive pulses of 150 ms duration with a delay of 4 s and during each pulse acquire the fluorescence image of the cell (excitation 490 nm, emission 605 nm).
From these images, subtract the control image (acquired before the pulse delivery) and average the corrected images to increase the signal-to-noise ratio. Quantify the changes in the fluorescence by measurements of gray levels of the region of interest, which is a line encircling the cell at the site of the membrane. Transform the fluorescence changes to the values of the ITV ($\Delta F/F$ = ~9% / 100 mV), and plot them on a graph as a function of the arc length.

Duration of the experiments: appr. 60 min
Max. number of participants: 4
Location: Cell laboratory

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NOTES & RESULTS

L2 - Electric field orientation and uptake of propidium iodide

THEORETICAL BACKGROUND

Biological cell exposed to external electric field is permeabilized due to induced transmembrane voltage formed on the membrane. The induced transmembrane voltage is position dependant and reaches maximal values at the regions of the cell facing the electrodes. Besides, it depends on the shape and orientation of the cell in the external electric field. Changing the orientation of the electric field increases the area of electropermeabilized membrane and enhances the uptake of the transport of molecules across the membrane.

EXPERIMENT

The students will observe the electropermeabilization of the plated CHO cells by using propidium iodide and determine the fraction of permeabilized cells for different electric field orientations.

Protocol: The experiments will be performed on Chinese hamster ovary cells (CHO) grown on multiwell in the culture medium (HAM-F12). Cells will be plated 24 hours before the experiment in concentration $5x10^4$ cells per well. On the day of experiment the culture medium is replaced with 150 μl of SMEM medium containing 1.5 mM propidium iodide. The cells are exposed to electric field of different orientations and directions and voltages. Apply 8 pulses with duration 100 μs, repetition frequency 1 Hz and pulse amplitudes: 0 V control and 200 V and 400 V treated cells. The distance between electrodes is 5 mm. Electric field orientation applied is i.) in one direction (8 pulses in one direction) and ii.) in two directions perpendicular to each other and two electric field orientations (2 pulses in each condition).
View the sample using a flourescence microscope at 20x magnification using rhodhamine filter with excitation λ = 510 nm. Determine the proportion of fluorescent to non-fluorescent cells at different orientations of the applied electric field and diffrerent applied voltages.

Duration of the experiments: appr. 45 min
Max. number of participants: 4
Location: Cell laboratory

FURTHER READING:

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NOTES & RESULTS

C1 - Numerical determination of the induced transmembrane voltage on a biological cell

THEORETICAL BACKGROUND

Exposing a cell to external electric field leads to induced transmembrane potential on the cell membrane. If the induced transmembrane voltage is high enough, the permeability of the cell membrane increases thus allowing the entrance of otherwise non-permeant molecules into the cell. Besides the experimental methods (e.g. potentiometric fluorescence dyes) the induced transmembrane voltage can also be determined numerically. This way it is possible to calculate and visualize the electric potential and field distribution around a cell. As the conductivity of the cell membrane is several orders lower than the conductivity of the extracellular medium and the cytoplasm, the cell can be represented as a simple-shaped nonconductive object for steady state (DC) calculations.

EXPERIMENT

Numerical modeling program package FEMLAB (now COMSOL Multiphysics) based on the finite element method will be used to calculate electric potential and field distribution. A simple geometry, consisting of a box with the side of 50 μm, representing the extracellular medium and a hole with the diameter of 10 μm, representing the cell, is already prepared. Representing the cell as a hole is valid, because the conductivity of the cell membrane is very low, so very little current flows through the membrane.

First, you will have to define the application mode of the model. Because we use steady state conditions, "Conductive media DC" is the appropriate application mode.

Second, based on the known values of the extracellular conductivity you define the specific conductivity of the block ($\sigma_{extracellular}$ = 0.5 S/m).

Third, to induce electric field inside the box, you apply proper boundary conditions on the borders of the box. The default value for boundary conditions in "Conductive media DC" is ground, which is not right for our case. On all boundaries except two you have to define "Electric insulation" boundary conditions. Two exceptions are two opposite boundaries, used to generate electric field. On those two boundaries you will apply "Electric potential" (V_{12}) with the value of:

$$V_{1,2} = \pm \frac{\vec{E}_{threshold} \bullet d}{2},$$

where $\vec{E}_{threshold}$ stands for the desired electric field strength and d stands for the size of the side of the box (in our case 50 μm). Example: to obtain voltage to distance ratio $\vec{E}_{threshold} = \frac{V_{12}}{d}$ (electric field) of 500 V/cm, you will have to apply ± 1.25 V.

Select one crossection through the cell and plot induced transmembrane voltage around the obtained circle.

Duration of the experiments: appr. 45 min
Max. number of participants: 2
Location: Laboratory of Biocybernetics

FURTHER READING:

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NOTES & RESULTS

C2 - Electropermeabilization with four needle electrodes and different electric field orientation

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. These pulses generate electric field in the tissue and tumor through the electrodes, inserted in the tissue. For efficient electrochemotherapy the electric field inside the whole tumor must exceed the threshold value needed for electropermeabilization (reversible electropermeabilization), while at the same time it should not damage the tissue (irreversible electropermeabilization), thus enabling nonpermeant chemotherapeutics to enter cell interior and exert their cytotoxic action. It is not necessary that the whole tumor is electropermeabilized in one pulse or pulse sequence. By combining more than two pulses (or pulse sequences) applied on a combination of electrodes it is possible to electropermeabilize the whole tumor.

Figure 1: Electrodes used for electrochemotherapy. Left: plate electrodes; middle: linear needle electrodes, arranged in two rows of four electrodes; right: an hexagonal array of seven needle electrodes, where pulses in one pulse sequence are delivered between different electrodes to permeabilize the whole area in-between the needles.

EXPERIMENT

Numerical modeling program package FEMLAB (now COMSOL Multiphysics) based on the finite element method will be used to calculate electric potential and field distribution in and around the tumor. On a prepared 3D model of the subcutaneous tumor (sphere with radius 5 mm), surrounding tissue (box with size of 5 cm) and 4 electrodes (needles are 40 mm long with diameter 1 mm) inserted into the tissue define specific conductivities as shown in table below. Apply the voltage to two opposite electrodes first and calculate electric field distribution. If you consider that the threshold value of electric field for permeabilization of tumor is 470 V/cm, determine the voltage, when more than 50 % of the tumor is permeabilized. Then apply the same voltage to the other pair of electrodes. Calculate electric field distribution in this case.

When a combination of both electric field orientations is used in one sequence of pulse delivery, permeabilized tissue is permeabilized either by first pair of the electrodes or by the second pair of electrodes. Based on the results obtained before, determine the efficiency of combined pulse delivery at the same applied voltage.

Component	**Specific conductivity [S/m]**
Muscle longitudinal	0.5
Muscle transversal	0.1
Tumor	1
Electrodes	4×10^{6}

Duration of the experiments: appr. 45 min
Max. number of participants: 2
Location: Laboratory of Biocybernetics

FURTHER READING:

1. Serša G, Čemazar M, Semrov D, Miklavčič D. Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice. Bioelectrochem. Bioenerg. 39: 61–66, 1996.
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3. Faurie C, Phez E, Golzio M, Vossen C, Lesbordes JC, Delteil C, Teissié J, Rols MP. Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells. BBA 1665: 92-100, 2004.

NOTES & RESULTS

C3 - Electric field distribution in the tumor during electrochemotherapy

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. For efficient electrochemotherapy the electric field inside the whole tumor must exceed the threshold value needed for electropermeabilization (reversible electropermeabilization), while at the same time it should not damage the tissue (irreversible electropermeabilization), thus enabling nonpermeant chemotherapeutics to enter cell interior and exert their cytotoxic action (Figure 1).

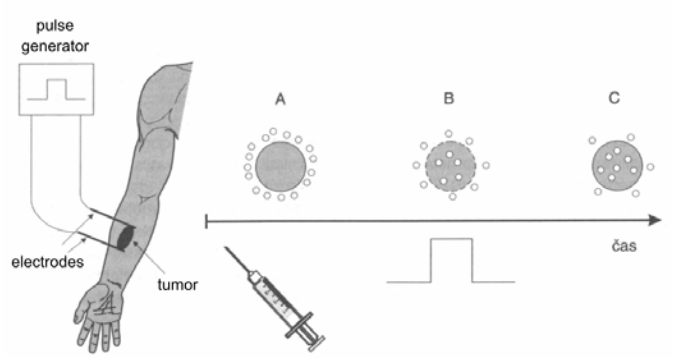

Figure 1: Electrochemotherapy. A – injected chemotherapeutic agent surrounds cells, B – electric pulses permeabilize cell membrane. Chemotherapeutic enters permeabilized cell. C - cell recovers after electropermeabilization, chemotherapeutic exerts its cytotoxic action.

The electric field is generated by applying the electric pulses to the electrodes. 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 appropriate pulse amplitude is difficult to obtain experimentally, mostly because electric field distribution between the electrodes is inhomogeneous because of the different tissue properties and the geometry of the electrodes. With numerical modeling, it is possible to calculate the electric field distribution in the tissue and estimate the pulse amplitude necessary for electropermeabilization of the whole tumor.

EXPERIMENT

In a program package for finite element modeling FEMLAB (now COMSOL Multiphysics) we have prepared a 3D model to calculate electric field distribution during electrochemotherapy of subcutaneous tumor. The geometry of the model consists of a surrounding tissue (box with size of 5 cm), subcutaneous tumor (sphere with radius 5 mm) and electrodes. There are two types of electrodes, used in this model: linear needle electrodes (8 needles arranged in two rows with row to row distance of 10 mm and needle to needle distance 2 mm, needles are 40 mm long with diameter 1 mm) inserted into the tissue and 2 plated electrodes (10 mm wide, the distance between the plates is 10 mm). Set specific conductivities for all the components of the model and the voltage applied on the electrodes according to the table below. Calculate electric field around the electrodes and determine the lowest value of electric field in the tumor for two different pulse amplitudes applied. If you consider that the threshold value of electric field for permeabilization of tumor is 470 V/cm, determine at which pulse amplitude the whole tumor is permeabilized for both type of electrodes.

Component	**Specific conductivity [S/m]**
Muscle longitudinal	0.5
Muscle transversal	0.1
Tumor	1
Electrodes	4×10^6

Pulse amplitude	Permeabilization	Lowest E
100V 8 needle 4 mm	Yes/No	
400V 8 needle 4 mm	Yes/No	
100V plate 6 mm	Yes/No	
400V plate 6 mm	Yes/No	

Duration of the experiments: appr. 45 min
Max. number of participants: 2
Location: Laboratory of Biocybernetics

FURTHER READING:

1. 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.
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3. Šel D, Mazeres S, Teissié J, Miklavčič D. Finite-element modeling of needle electrodes in tissue from the perspective of frequent model computation. IEEE Trans. Biomed. Eng. 50: 1221-1232, 2003.
4. Šemrov D, Miklavčič D. Calculation of the electrical parameters in electrochemotherapy of solid tumours in mice. Comput. Biol. Med. 28: 439–448, 1998.

NOTES & RESULTS

V1 - Electrochemotherapy - interactive learning

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. For efficient electrochemotherapy the electric field inside the whole tumor must exceed the threshold value needed for electropermeabilization (reversible electropermeabilization), while at the same time it should not damage the tissue (irreversible electropermeabilization), thus enabling nonpermeant chemotherapeutics to enter cell interior and exert their cytotoxic action (Figure 1).

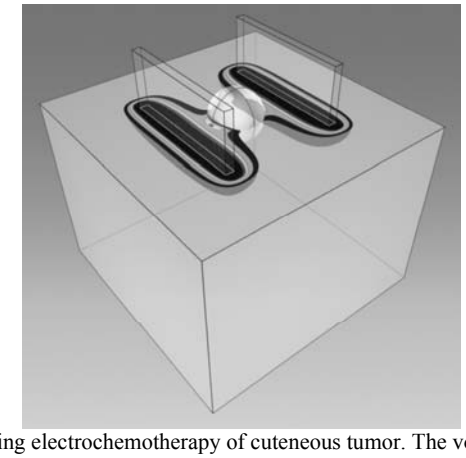

Figure 1: Electric field distribution during electrochemotherapy of cuteneous tumor. The voltage of 600 V is applied on 10 mm wide plated electrodes with the distance between the electrodes of 8 mm.

The electric field is generated by applying the electric pulses to the electrodes. 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 because of the different tissue properties and the geometry of the electrodes. With numerical modeling, it is possible to calculate the electric field distribution in the tissue and estimate the pulse amplitude necessary for electropermeabilization of the whole tumor.

EXPERIMENT

Explore the problems of electric field distribution during electrochemotherapy using oa web application developed for e-learning. You will become familiar with different definitions used in the field of electrochemotherapy as well as become more familiar with the impact of pulse intensity and electrode geometry on electric field distribution in the tissue.

Duration of the experiments: appr. 45 min
Max. number of participants: 4
Location: Laboratory of Biocybernetics

NOTES & RESULTS

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Proceedings of the

Electroporation based Technologies and Treatments

International SCIENTIFIC WORKSHOP and POSTGRADUATE COURSE

Ljubljana, Slovenia
November 14-20, 2005

ISBN 961-243-022-5

9 789612 430221

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