Proceedings of the

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Welcome note

Dear Colleagues, Dear Students,

The idea of organizing the Workshop and Postgraduate Course on Electroporation Based Technologies and Treatments at the University of Ljubljana has been developing for several years. After many preliminary discussions and reconciliations of viewpoints, the Workshop and Course is taking place for the first time this year, with the participation of several 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 electrodes. 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 Bioelectrochemical Society and IGEA s.r.l., 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

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Abstract: This introductory lecture describes the basic interactions between biological cells and electric fields. Under physiological conditions, a resting voltage in the range of several tens of volts is continually present on the cell membrane, which also shields the cell interior from external electric fields. An exposure of the cell to an external electric field induces an additional component of transmembrane voltage, which superimposes to the resting component for the duration of the exposure. Unlike the resting transmembrane voltage, the induced transmembrane 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.

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). Namely, the specific electric conductivity of the membrane is typically some six to seven orders of magnitude lower than the conductivities of the cytoplasm and of the liquid component of the extracellular space. Due to this, when a cell is exposed to an external electric field, practically all of the electric field in the vicinity of the cell concentrates within the membrane, which thus shields the cytoplasm from the exposure. The concentration of the electric field results in a voltage (electric potential difference) on the membrane. As the electric field vanishes, so does the voltage caused by this field.

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). In the simplest case, which is an acceptable approximation for many types of cells, the body is a sphere, and the surrounding shell is also spherical. 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, owing to the negligible conductivity of the cell membrane, this complication can be disregarded in the computation of the transmembrane voltage, which will be our aim here.

[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 much easier to accomplish by hand than with advanced drawing tools.

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. 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 occurence 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 posess 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

insignificant. 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 concentrates 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],

$$\Delta\Phi_{\mathrm{m}} = \frac{3}{2} ER\cos\theta, \qquad (1)$$

where $\Delta\Phi_{\mathrm{m}}$ 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 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],

$$\Delta\Phi_{\mathrm{m}} = \frac{3}{2} ER\cos\theta\left[1 - \exp(-t/\tau_m)\right], \qquad (2)$$

where τ_{m} is the time constant of the membrane,

$$\tau_{\mathrm{m}} = \frac{R\,\varepsilon_{\mathrm{m}}}{2d\dfrac{\sigma_{\mathrm{i}}\sigma_{\mathrm{e}}}{\sigma_{\mathrm{i}} + 2\sigma_{\mathrm{e}}} + R\sigma_{\mathrm{m}}} \qquad (3)$$

with σ_{i}, σ_{m} and σ_{e} denoting the conductivities of the cytoplasm, cell membrane, and extracellular medium, respectively, R the cell radius, d the membrane thickness, and ε_{m} the dielectric permittivity of the membrane.

In certain experiments *in vitro*, the conductivity of the extracellular medium is reduced by several orders of magnitude with respect to the physiological one. The formula (3) shows that in such cases, the time constant τ_{m} increases significantly, but in addition, the factor 3/2 occuring in (1) and (2) decreases. A detailed treatment of transmembrane voltage induced in low-conductivity media is given in [5].

Under physiological conditions, however, the formula (2) can be applied with square electric pulses as short as hundreds of nanoseconds. A further generalization to the second-order equation [6] allows to deal with even shorter pulses. Similarly, Schwan's equation can be extended to the case of alternating [7] or general time-varying fields [8].

A spheroidal or an ellipsoidal cell

Another direction of generalization is to assume a cell shape more general than that of a sphere. Since all three axes of the sphere are of equal length, the most straightforward generalizations are to a spheroid (having two axes of equal length, and the third one different) and further to an ellipsoid (with each of the three radii different). To obtain the analogues of Schwan's equation for a spheroidal or an ellipsoidal cell, 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. The next two paragraphs are devoted to a description of these complications, and how one can deal with them.

As we have mentioned in the introductory section of this paper, a description of a cell is geometrically realistic if the thickness of its membrane is uniform. Two concentric spheres are equidistant, and with their radii sufficiently close together, the space between the spheres is thus an adequate model of the membrane. Unlike this, two confocal spheroids or two confocal

ellipsoids are never equidistant, and the thickness of the "membrane" in these coordinates necessarily varies with position. Due to this, the direct solution of Laplace's equation, i.e., the description of the electric potential throughout the confocal ellipsoids and their vicinity, is not applicable to "real" cells. However, we are usually only interested in the transmembrane voltage, which is the electric potential difference between the inner and the outer surface of the membrane. In this case, the knowledge of the distribution of the electric potential within the membrane is not needed. Under the assumption that the membrane conductivity is zero, the shielding of the cytoplasm is 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 filled non-conductive body of the same shape.[2] A more rigorous discussion of the validity of this approach can be found in the literature [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.

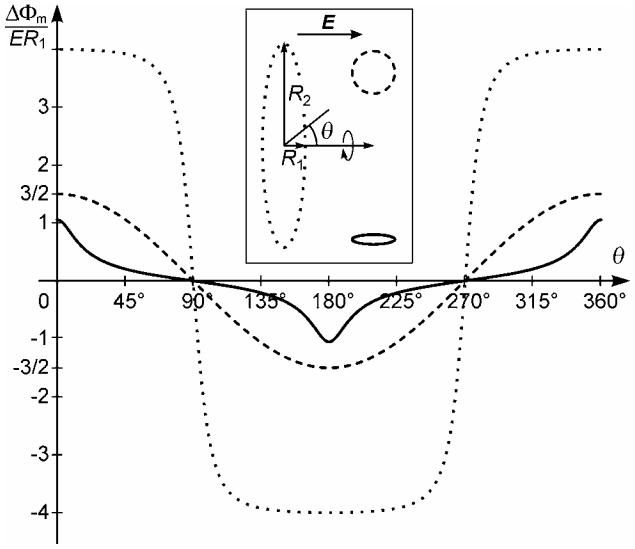

Figure 1: Normalized induced transmembrane voltage for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 1/5\ R_1$. Dashed: a spherical cell, $R_2 = R_1$. Dotted: an oblate spheroidal cell with $R_2 = 5\ R_1$. Adapted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

The second complicating consequence of the generalization of the cell shape from a sphere to a spheroid or an ellipsoid is that the formula describing the induced transmembrane voltage now also becomes dependent on the orientation of the cell with respect to the electric field. To deal with this, one decomposes the direction of the field 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.

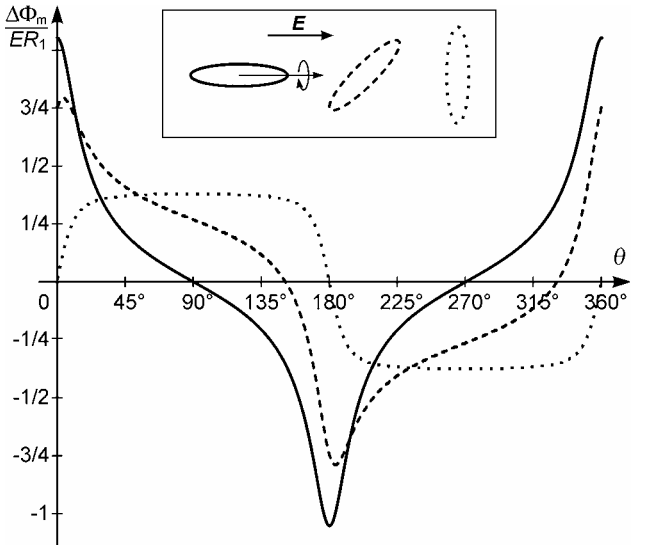

Figure 2: Normalized transmembrane voltage for a prolate spheroidal cell with $R_2 = 1/5\ R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of the authors.

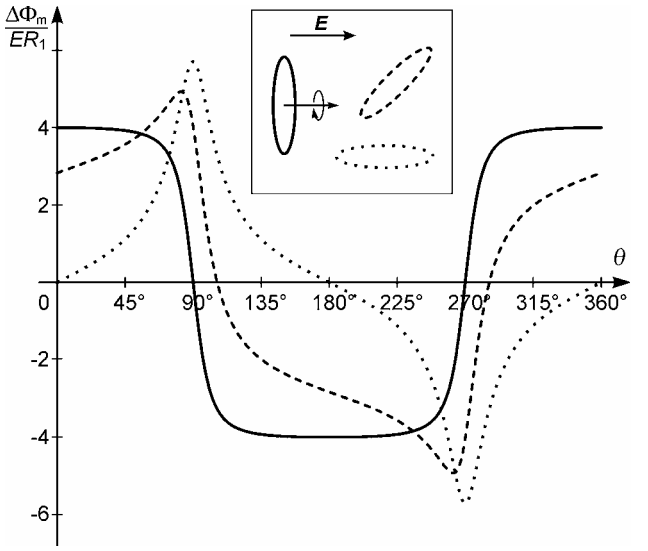

Figure 3: Normalized transmembrane voltage for an oblate spheroidal cell with $R_2 = 5\ R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field. Adapted from [11] with the permission of the authors.

Cells in suspension

When cells in suspension are exposed to an electric field, applying Schwan's equation to determine the induced transmembrane voltage is in general not valid. This is due to the fact that the local field outside a cell is distorted by the presence of other cells in the suspension. For suspensions in which the cells represent less than one percent of the total 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 the one predicted by Schwan's equation is practically negligible. As the volume fraction occupied by the cells gets larger, the prediction obtained from Schwan's equation gets less and less realistic (Fig. 4). 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 [12,13]. 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 one to be used in the analysis of the transmembrane voltage induced on cells in suspension.

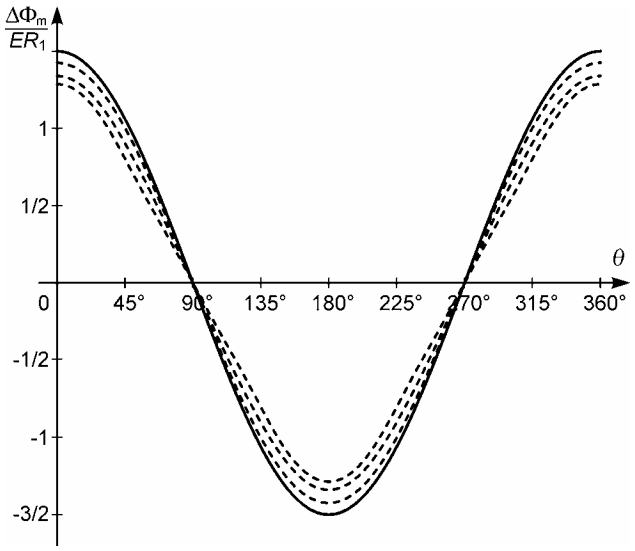

Figure 4: Induced transmembrane voltage normalized to electric field and cell radius. Solid: prediction of Schwan's equation, i.e., of formula (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (from top to bottom) 10%, 30%, and 50% of the total suspension volume. Adapted from [13] with the permission of the authors.

As the volume fraction of the cells in suspension gets larger, 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.

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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 interests lie in the fields of membrane electrostatics and electrodynamics, as well as in both theoretical and experimental study of related biophysical phenomena, especially cell membrane electropermeabilization.

Tadej Kotnik is the author of 16 articles in peer-reviewed journals. In 2001 he received the Galvani Prize of the Bioelectrochemical Society.

NOTES

Biological Tissues in Static and Dynamic Electric Fields

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Abstract: Either to induce certain biological response, or without that intention, in different situations biological tissues are exposed to static or dynamic electric fields of various intensities. The purpose of this paper is to point out to the important issues when modeling the electric field in tissues and when measuring the electrical properties of tissues. Our approach here is to treat the tissue as a material described by its macroscopic (bulk) properties to which we apply the classical theory of electromagnetic fields. Therefore, we briefly reviewed the fundamentals of electromagnetic theory, we introduced properties of materials and explained terms such as inhomogeneous, anisotropic, dispersive and nonlinear. We illustrated the introduced concepts on the electrical conductivity and permittivity of biological tissues. We discussed the equivalence of response in frequency and time domain and the necessary assumptions. Then we presented the results of measuring tissue properties that involve nonlinear effects. Finally, we summarized the most important factors affecting electrical properties of tissues, as well as factors affecting the accuracy of measurement methods.

INTRODUCTION

Exposure of biological tissues to static electric field or electromagnetic (EM) radiation keeps receiving great attention over the years due to various effects EM fields can produce and extensive application of different electric devices used either for patient treatment (defibrillators, hyperthermia apparatus, electrosurgical units, etc.) or in our everyday life (power transmission lines, cellular phones, microwave ovens, etc.). It is well known that the effects of electric fields on living tissue can be beneficial, but can also cause possible hazards and irreversible damage of biological structures [1]. The mechanisms of the interaction between EM fields and biological systems can be thermal, but also nonthermal [1, 2].

All biological tissues are inhomogeneous on a micro-scale in view of the fact that they consist of more or less densely packed cells which size and shape can considerably vary even for the same type of tissue. In spite of this, we use bulk electrical properties (tissue conductivity and permittivity) presuming that tissues are (macroscopically) homogenous. 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 renders us incapable of analyzing 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 paper. 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. On the other hand, at tissue level, we use bulk electrical parameters.

Therefore, the interaction between EM fields and biological systems is analyzed at different levels (Fig. 1). The first is the coupling between external field and inside of body, the second is the coupling between inside of body and target structure (cellular or subcellular) and the third is the kinetics of response, if any, of target structure to the field i.e. the biological response to the local field [2].

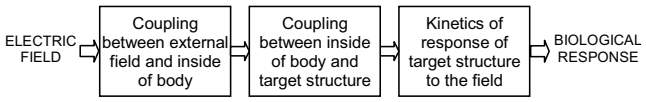

Figure 1: Different levels of interaction between EM field and tissue. Redrawn from Foster (2000) [2].

Many applications of electric fields to biological systems like the electrical stimulation of nerves and muscles do not involve EM radiation and can be analyzed using quasi-static or static theory. There are also some important applications of very large static electric fields including cardiac defibrillation and tissue electroporation for electrochemotherapy and *in vivo* gene transfection that are processes in non-linear region [3, 4].

Due to a great variety of frequencies of EM radiation used in different applications, including pulsed fields with broad spectrum that may also contain DC component and the large span of field intensities, the bulk electrical properties of biological tissues over the entire frequency range are of utmost importance when studying the effects of EM fields to living bodies.

The aim of this work is to briefly present the fundamentals of electromagnetic theory, point out the difficulties when applying it to biological systems, to review the electrical properties of biological tissues,

emphasize some measurement related problems and to analyze some difficulties when modeling biological tissues exposed to large electric field.

MAXWELL'S EQUATIONS

The fundamental equations for EM field calculations are the Maxwell's equations formulated in integral or more compactly in differential form:

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

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

Here **E** [V/m] is the electric field intensity, **H** [A/m] the magnetic field intensity, **D** [C/m^2] the electric displacement or electric flux density, **B** [T] the magnetic flux density, **J** [A/m^2] the current density and ρ [C/m^3] the electric space charge density. Another fundamental equation is the equation of continuity:

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

To obtain a complete system constitutive relations, describing the 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 [F/m] is the permittivity of vacuum, μ_0 [H/m] magnetic permeability of vacuum, σ [S/m] the conductivity, **P** [C/m^2] is the electric polarization vector, and **M** [A/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 of the equations above are valid only for linear materials where polarization is directly proportional to the electric field and magnetization to the magnetic field.

These equations form a complete set of equations needed for solution of various problems in electromagnetism. Usually, Maxwell's equations are not solved in terms of the field quantities, but are transformed into partial differential equations using potential formulations (electric scalar potential V, and magnetic vector potential **A** are introduced). To obtain unique solution appropriate gauge transforms (Coulomb gauge, Lorenz gauge) are used that lead to further simplifications. Under different assumptions Maxwell's equations reduce to well-known partial differential equations such as, for example, wave equation, diffusion equation or Laplace's equation.

The consequence of Maxwell's equations is that the fields are always retarded with the 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. The least complicated is the situation when the time derivative terms can be neglected and the equations further reduce to static situation. In this case the electric and magnetic field can be analyzed independently since there is no interaction between them.

When applying Maxwell's equations to practical problems in electrical engineering, simplifications of these equations are based on the fact that materials are either conductors or insulators. The consequence is that in electric conductors no static electric field can exist since electrons are free to move and there is no displacement current. Similarly, in insulators, as no free charge exists, in electric field electric polarization occurs and no conduction current can flow. As will become clearer later, these assumptions cannot be generally applied for biomaterials. Therefore, the application of Maxwell's equations to problems in biolectromagnetism is not straightforward. In order to correctly describe the conditions that arise in bioelectromagnetism modifications of Maxwell's equations must be introduced [5].

MATERIAL PROPERTIES

Constitutive relations that at the first glance may seem very simple can be quite complicated for some materials. Generally, materials can be inhomogeneous, anisotropic, dispersive and nonlinear. 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.

An inhomogeneous material is one where its physical properties vary with space coordinates, so that 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 will be further elaborated in the following sections, but simply dispersive material means that physical properties depend on the frequency, or 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. The consequence of nonlinearity is that the principle of superposition is no longer valid which considerably

complicates the analysis of the fields. 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.

ELECTRICAL PROPERTIES OF TISSUES

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 [3, 6].

Due to the complex structure of biological tissues several polarization mechanisms exists that lead to frequency dependence of bulk tissue properties and the concept of dielectric dispersions which is closely related to the concept of relaxation that occurs in time domain.

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 $\hat{\sigma}$. This concept is easily introduced by a capacitor having dielectric with losses or by considering conductor having some capacitive properties:

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

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

Both parameters are related by $\hat{\sigma} = j\omega\hat{\varepsilon}$. Besides, 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 what is formalized in the form of Kramers-Kronig relations [3]. Practical implication is that Kramers-Kronig relations can be used to check whether measurements of dielectric spectra were performed in the linear range.

Extensive compilations of dielectric properties of tissues can be found in [7, 8]; for recent data see [9].

Relaxation theory and polarization mechanisms

When a voltage step is applied to a dielectric, displacement of charge occurs and in the simplest case the polarization of material will relax towards the steady state as first order process characterized by a single time constant τ:

$$D(t) = D_\infty + (D_0 - D_\infty)(1 - e^{-t/\tau})$$

where D_∞ is instantaneous and D_0 steady-state intensity of electric displacement. In the frequency domain this corresponds to complex permittivity, which after the static conductivity term is added can be written as:

$$\hat{\varepsilon} = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + j\omega\tau} - j\frac{\sigma_s}{\omega}$$

where ε_∞ refers to permittivity at "infinite" frequencies and ε_s to "static" permittivity. This is the well-known Debye model [6].

However, in biological tissues dielectric relaxation mechanisms are more complicated. In frequency domain broader dispersions are observed, what in time domain corresponds to multiple relaxation processes since distribution of relaxation times is present [6]. To account for this effect, an empirical model of complex permittivity known as Cole-Cole is widely used:

$$\hat{\varepsilon} = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + (j\omega\tau)^{1-\alpha}} - j\frac{\sigma_s}{\omega}$$

where α is a positive parameter smaller than 1, and can be interpreted as a measure of a distribution of relaxation times [3].

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 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). Later on also δ-dispersion was found [3].

Dispersions

As an example to illustrate dispersions in biological tissues we present the conductivity and relative permittivity of liver tissue in the frequency range from 10 Hz to 10 GHz (Fig. 2). At low frequencies, in spite of extremely high permittivity, conduction current dominates and the measured conductivity is predominately the conductivity of extracellular fluid since cell membranes are poorly conducting. 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 the highest frequency range we can see a dispersion due to relaxation of water.

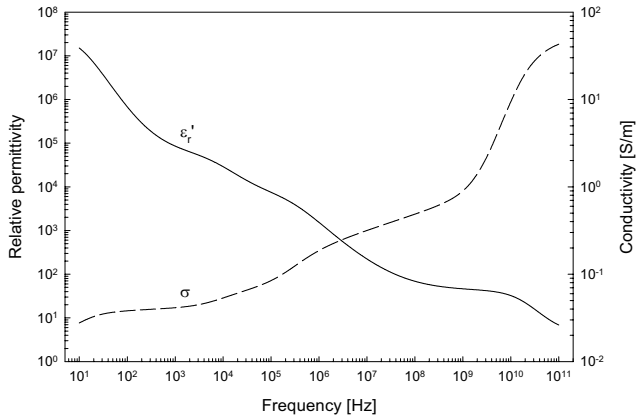

Figure 2: Conductivity and relative permittivity of liver tissue from 10 Hz to 10 GHz according to measurements performed by Gabriel *et al.* (1996) [9]. The data show presence of dispersions.

Frequency spectra and time domain response

It is possible to reconstruct the response in time domain from frequency spectra and vice versa. However, two important requirements must be satisfied: linearity meaning that the amplitude of the voltage applied must be small enough that no change of electrical properties occurs and time invariance meaning that the electrical properties must not change in time. 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. Moreover, additional precautions must be taken concerning the measurement aspects [11].

Anisotropy

Tissue conductivity (and permittivity) may be up to an order of magnitude larger in one direction compared to another. Significant anisotropy is found in skeletal muscles at low frequencies [6]. The anisotropy, if caused by cell membranes, disappears at frequencies higher than the β-dispersion frequency [3].

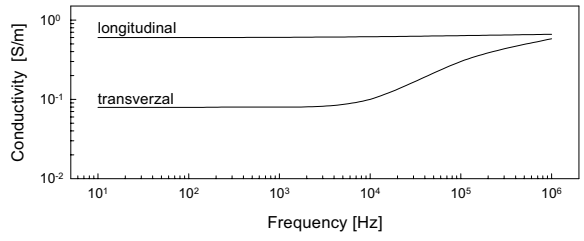

Figure 3: Conductivity of skeletal muscle tissue in perpendicular (transversal) and parallel (longitudinal) orientations exhibits strong anisotropy in low frequency range. Redrawn from Grimnes and Martinsen (2000) [3].

Nonlinearity

The dielectric relaxation theory summarized above apply to linear region i.e. it presumes responses to weak fields. As the field intensity is increased, the response will no longer be linear. As an example, we can analyze current measured during the delivery of rectangular voltage pulses to rat liver in electroporation experiments (Fig. 4 A).

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. As the applied voltage is increased, nonlinearity is observed, which can more clearly be seen if current-voltage characteristic is plotted (Fig. 4 B).

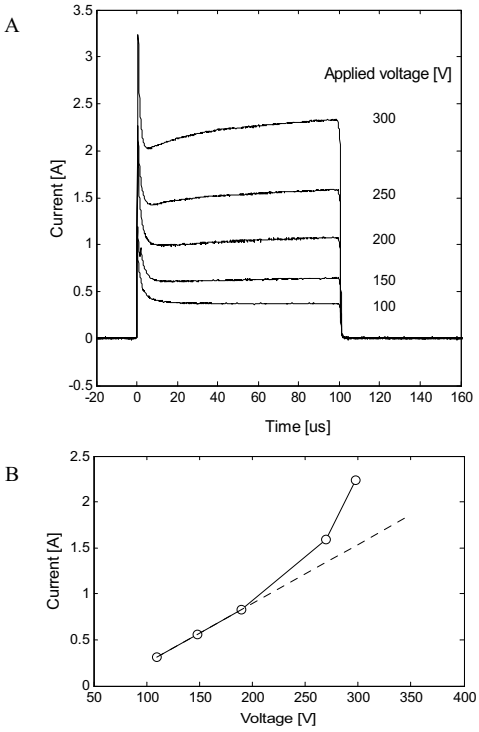

Figure 4: A: Current measured during delivery of 100 µs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al.* [10], by permission. Pulses were generated using Jouan GHT1287B; plate electrodes (distance 4.4 mm) were used. B: Current-voltage characteristics constructed based on measured voltage and measured current at the end of the pulse.

Factors affecting accuracy of measurement

Measurement of dielectric properties requires special attention and careful calibration of measurement equipment which ranges from impedance bridges (LCR-meters) that are used in low frequency range, over impedance analyzers, which cover large frequency range usually from tens of Hz to tens of MHz, up to network analyzers that are used for frequencies up to 100 GHz.

To interface the instrument to biological sample various configurations of electrodes, impedance cells and open end coaxial probes are used. At low frequencies (up to a few kHz) electrode polarization is

the main nuisance when measuring dielectric properties since electric double layers that form at the electrode-electrolyte interface cause large capacitance. Techniques for correcting these effects can be found in [12]. 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 [12, 3].

Factors affecting electrical properties of tissues

The most important factors affecting electrical properties of tissues, as we have demonstrated, are: frequency and amplitude of the applied field, anisotropy, temperature, but also water content, local tissue variability and tissue pathology (tumors). Moreover, as measurements cannot always be performed *in vivo* tissue changes following death should also be considered. These factors can explain discrepancies found in literature when comparing the results from different studies.

MODELING OF THE ELECTRIC FIELD IN TISSUES FOR ELECTROCHEMOTHERAPY AND *IN VIVO* ELECTROTRANSFECTION

It was demonstrated recently that by 3D FEM modeling of the distribution of electric field in tissue reversible and irreversible threshold for electroporation can be estimated [13, 14]. Since on a cellular level electroporation causes increase of membrane conductance, it is hypothesized that bulk tissue conductivity should also increase measurably [15]. The next step in modeling the electric field in tissues subjected to large electric field is to include the effect of increased conductivity due to electroporation. However, literature data on tissue conductivity when subjected to a large electric field are extremely sparse.

Another important aspect of application of large static electric fields to tissues is heating since current flow through conducting medium causes Joule effect and consequently temperature rise in tissue can be expected. Moreover, due to the coupling between electric and thermal variables (increase of tissue conductivity with temperature) potentiation of thermal effect can be expected. Joule heating can become critical parameter especially for *in vivo* electrotransfection since thermal damage begins at temperatures higher than 42ºC and the rate of damage rapidly increases with temperature rise.

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NOTES

Membrane Electroporation: Mechanism from Kinetics and Thermodynamics

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Abstract: The topic membrane electroporation (MEP) is introduced with a brief history of MEP of cells, organelles and lipid vesicles for the purpose of controlled release and uptake of substances.

Exploration and discussions of the concepts and theory are organized along the experimental data base. Particular emphasis is on relaxation kinetics of MEP in high-electric-fields, using electrooptic and conductometric monitoring techniques. Theoretically, both field-induced structural changes of the lipid part of the membrane phase and the subsequent changes in the crossmembrane transport are covered by classical physical models for membrane processes and shape changes of cellular objects.

The kinetics of electrooptic turbidity and absorbance changes, using optical lipid probes, indicates, that there is a whole sequence of different structural transition steps, preceding and accompanying transport processes such as small-ion conductance, uptake of dyes, oligonucleotides and DNA as well as surface insertions of peptides and larger surface-adsorbed proteins like the human annexin V.

The various discernable steps are characterized in terms of the classical chemical normal mode parameters, i. e., by the respective time constants and relaxation amplitudes. The kinetic and thermodynamic analysis of the field dependences yields polarization volumes (thus the radius of the passage or pore), the field-reducing conductance factor (thus membrane conductivity and pore conductance), the reaction dipole moments of dipolar structural transitions like lipid head-group rearrangements, as well as mechano-structural key-parameters like bending rigidity, line tension and general curvature energy terms.

The degree f_p of electroporation may be derived from the actual surface increase due to the formation of aqueous pores, allowing deformation of the cellular objects under Maxwell stress. Since electroporative transport flow is proportional to the actual transport surface $S = f_p S_0$ as the fractional surface of the total compartment surface S_0, f_p can be derived from the molar flow as a function of the concentration difference and the electric gradient driving the flow (Nernst-Planck equation).

Since usually, even at high degree of poration is not larger than $f_p \approx 10^{-2}$, the structure distribution constant K_p = (pore)/(closed membrane) = $f_p/(1- f_p)$ is very simply related to f_p. The fraction f_p is exponentially connected to the sum of all types of free energy contributions: chemical transition energy, line tension energy, curvature energy, surface tension energy and, most importantly, electric polarization energy of permanent and induced dipole moment changes being characteristically dependent on the external electric field strengths.

It is quite rewarding that the general analytical concept of field-induced shifts in the structural distribution constants, first developed for cells (1982) and then exemplified and specified for lipid vesicles, can be readily extended to cover quantitatively the behaviour of aggregated cells (pellets) and mouse tumor tissue.

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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 ΔΨ (i.e. the sum between the resting value of cell membrane ΔΨo and the electroinduced value ΔΨi) reaches locally 250 mV, that part of the membrane becomes 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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Teissié Justin was born 24 March 1947 in Poitiers, France. Got a degree in Physics at the Ecole superieure de Physique et de Chimie Industrielles de Paris (ESPCI) in 1970. Got a PhD in Macromolecular Chemistry on a project on fluorescence detection of action potential under the supervision of Prof. Monnerie (ESPCI) and Changeux (Institut Pasteur) in 1973. Got a DSC in Biophysics on a project on fluorescence characterisation of Langmuir Blodgett films in Toulouse in 1979. Was a Post Doc at the Medical School of the John Hopkins University in Baltimore in 1979-81. Present position: Directeur de recherches au CNRS. Author of more than 200 papers

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.

NOTES

Electrodes and corresponding electric field distribution for effective *in vivo* electroporation

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Abstract: Permeabilizing electric pulses can be advantageously used for DNA electrotransfer *in vivo* for gene therapy, as well as for drug delivery. In both cases it is essential to know the electric field distribution in the tissues: the targeted tissue must be submitted to electric field intensities above the reversible permeabilization threshold and below the irreversible permeabilization threshold (to avoid cytotoxic effects of the electric pulses). A three-dimensional finite element model was built for different electrodes used in various *in vivo* experiments. Electrodes of different geometries were modelled by applying appropriate boundary conditions in corresponding grid points of the model. The anatomically based model was previously validated by comparing our calculations with magnetic resonance current density imaging and electrochemotherapy for specific electrodes. The observations resulting from the numerical calculations, like mean electric field magnitude within the tumors are in good correlation with the effectivenes of electrochemotherapy.

INTRODUCTION

In the last two years, promising results for a new non-viral efficient gene therapy have been obtained in *in vivo* DNA electrotransfer studies [1]. It is also important to note that, recently, drug delivery using electric pulses has entered an active period of clinical trials [2,3]. These two new therapeutical approaches are based on cell electropermeabilization, also termed electroporation, a phenomenon where a transiently increased plasma membrane permeability is obtained after the cells were exposed to short and intense electrical pulses. Electropermeabilization thus allows otherwise nonpermeant molecules to enter the cytosol.

For effective drug delivery and gene transfection *in vivo*, the knowledge of electric field distribution is of utmost importance, to obtain an effective permeabilization as well as to maintain the viability of the electropermeabilized cells. Indeed, in order to achieve electropermeabilization in the tissue of interest, the magnitude of electric field intensity has to be above a critical threshold value, i.e. the reversible threshold. Furthermore, the magnitude of electric field intensity should not exceed the value which would produce irreversible damages to the plasma membrane, i.e. the irreversible threshold. Thus, the magnitude of electric field intensity should be high enough to cause reversible electropermeabilization but lower than the value causing irreversible damage [4]. The latter is the most critical for *in vivo* gene transfer but is also desirable in electrochemotherapy in order not to produce large instantaneous necrosis, which would result in massive tumor necrosis and possible exulceration and wound appearance. Moreover, for gene therapy, it has been recently reported that, under relatively homogeneous exposure conditions, the optimal conditions for gene transfer correspond to the use of long pulses (20 milliseconds) at a voltage just necessary to obtain cell electropermeabilization, i.e. just above the reversible permeabilization threshold. Above the irreversible permeabilization threshold, when permanent damages are inflicted to the plasma membrane, viability is lost and efficacy of the DNA transfer is severely impaired [1]. Therefore it is necessary to determine (i) the electric field distribution in the target tissues, and (ii) the reversible as well as (iii) the irreversible permeabilization thresholds in order to use voltages and electrode geometries resulting in optimal exposure of the targeted tissue to electric fields intensities comprised between the two thresholds. Very few studies have dealt with these questions. In ex vivo experiments, using two parallel plates separated by 2mm , that represents a rather homogeneous exposure system, a variable threshold (ranging from 300 V/cm to 500 V/cm) was found for a fibrosarcoma tumour exposed to 8 pulses of 100 microseconds at a frequency of 1 Hz. Recently, using a numerical two-dimensional model for electric field distribution, parallel plates as electrodes, and a quantitative Cr^{51}-EDTA uptake assay, threshold for reversible *in vivo* permeabilization of mouse skeletal muscle was found at 450 V/cm for the same type of pulses. Our work [4] using a three-dimensional finite element model in which needle electrodes of different diameters were modeled and compared to appropriate experiments in rabbit liver tissue showed excellent agreement between numerical predictions and experimental observations. By this approach it was possible to make the first precise determination of the magnitude of the electric field intensity for reversible (362±21 V/cm, avg±std) and for irreversible (637±43 V/cm) permeabilization thresholds of rabbit liver tissue *in vivo*.

A variety of electrodes has been used in *in vivo* electrochemotherapy and gene delivery by electroporation (Figure 1). The first electrodes and the most widely used are plate electrodes (Figure 1 A) which are used as plate electrodes of fixed distance or variable distance between the electrodes as conductive parts of electrodes are mounted on caliper. This type of electrodes is used for electrochemotherapy of cutaneous and subcutaneous experimental tumors, smaller tumors in patients and gene delivery in rat mouse and subcutaneous tumors. Other types of electrodes (Figure 1 B to E) have been used and compared in electrochemotherapy of experimental subcutaneous mice tumors with variable response [5]. Another type of electrodes – "honeycomb" has been constructed and used for treatment of larger volumes. In this electrode (Figure 1 F) a division of volume to smaller fractions is introduced as pairs of needle electrodes are sequentially fired in a way that eventually the whole (arbitrarily large) volume is being permeabilized. Since it is difficult to compare the "effectiveness" of all these electrodes due to different voltages applied by different electrodes we compared electric field distribution in the tumor obtained by numerical modeling with electrochemotherapy efficiency using different electrodes and corresponding voltages in *in vivo* electrochemotherapy experiments reported previously [5-8]. In addition to correlation between mean magnitudes of electric field in the tumor and electrochemotherapy effectiveness we also report the minimum and maximum electric field magnitudes in tumors for all electrodes analyzed. This measure of inhomogeneity of electric field distribution is important specially for gene delivery, where cell have to maintain their viability.

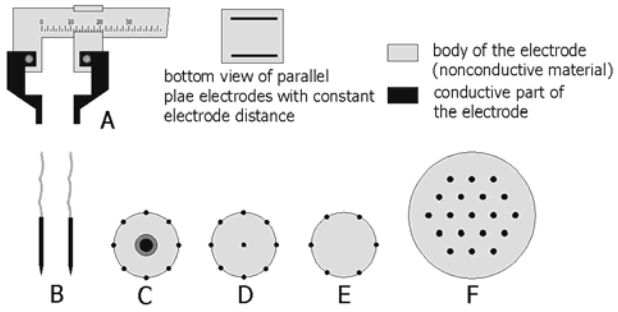

Figure 1: Various electrodes used for *in vivo* electro permeabilisation for electrochemotherapy and gene delivery.

MATERIAL AND METHODS:

Electrodes and Electrochemotherapy

The exact protocols for electrochemotherapy and electrodes used were previously reported [5,6,8]. Briefly, electrochemotherapy consisted of application of electric pulses to the tumor 3 minutes after intravenous injection of Bleomycin. The dose used was sufficient for killing the cells once being permeabilized and was well tolerated by mice. Electric pulses were delivered by either pressing electrodes on the skin or inserting the electrodes through the skin and applying voltage as reported in Table 1. Additionally to the electrodes presented in Figure 1 also specific electrodes with known distribution of electric field were used for electrochemotherapy [6].

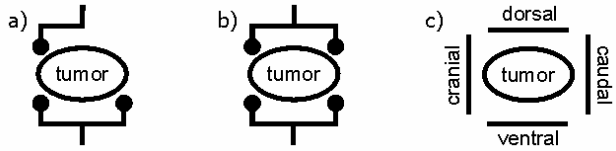

Figure 2: Electrodes used in electrochemotherapy (top view). Electrodes 2+1 (left) and 2+2 (middle) were needle electrodes which were pressed against the skin. Plate electrodes (right) were used as two plate electrodes pressed against the skin in either dorsal-ventral direction or cranial-caudal direction. In addition, plate electrodes were also used to apply four electrodes in one direction and the other four electrodes in perpendicular direction (4+4).

Eight square-wave pulses of 100 ms and repetition frequency of 1 Hz were delivered in one direction unless otherwise specified in Table 1. Current and voltage were monitored during pulse delivery. Control groups included mice without treatment, mice treated with electric pulses and bleomycin as single treatment. Electrochemotherapy was performed on tumors when they reached approximately 50 mm3 in volume (day 0). Tumor volume was estimated by measuring three main mutually perpendicular tumor diameters.

Mean tumor volume and standard error of the mean were then calculated for each experimental group and presented as a tumor growth curve. For each tumor individually tumor doubling time was determined, i.e. the time tumor needed to double its initial volume of day 0. For each experimental group mean and standard error of the mean was calculated.

Finite Element 3D

The model has been described in extenso in our previous publications [6,8]. Briefly, a three dimensional (3D) anatomically based finite element (FE) model of the mouse with injected subcutaneous solid tumor was built using MSC/EMAS (Electro-Magnetic Analysis System) software package (trademark of The MacNeal-Schwendler Corporation, USA). The geometry of the model was based on the 14 cross section scans of one typical animal with a subcutaneous tumor, obtained by magnetic resonance imaging. The geometry of the model was described with 1390 points, which defined 3859 curves/lines. A

total of 1379 3D geometric bodies were defined using those curves. The resulting three dimensional geometric structure was built of eleven different tissues (organs), i.e. skin, fat, skeletal and heart muscles, bone, connective tissue, intestine, kidney, liver, lung and tumor. Anisotropic characteristics were considered for skeletal and heart muscles, while all other tissues (organs) were modeled as isotropic. The values of the electric conductivity of tissues (organs) used in the model were collected from literature and used in one of the previous studies where similar model was verified with the measurements of electric potential in the 5 points in the tumor and surrounding tissue. In addition, the model was validated by comparing numerical results with current density magnetic resonance imaging [6].

Different electrode sets were modeled by applying appropriate boundary conditions in the grid points corresponding to each of the electrodes (Figure 1 and 2). Increased area with the same electric potential under each electrode resulting from the use of conductive gel was also taken into consideration. Fixed values of scalar electric potential, i.e. Dirichlet boundary conditions, were assigned to grid points in the regions where electrodes were placed. All electrode sets were modeled according to the position of the electrodes with respect to the tumor. Potentials of 0 V and 1300 V (or other, as specified in Table 1) for electrochemotherapy were assigned to groups of appropriate grid points of the FE mesh corresponding to each of the electrodes. On the remaining outer surfaces of the model, a Neumann boundary condition was applied. This boundary was considered as the interface between a conducting medium and air (assimilated to an ideal dielectric). Since the conductor (skin layer) was linear and isotropic, the usual Neumann condition was applied i.e. the normal derivative of the electric potential on the interface between the model and surrounding air was zero. Distribution of electric field intensity was then calculated from the values of the scalar electric potential in the grid points of the model. The distribution of the electric field was more precisely studied for 48 elements representing subcutaneous tumor since we were most interested in the electrical phenomena inside tumor tissue. Mean magnitudes of electric field inside 48 elements representing the tumor were calculated at appropriate voltages used in electrochemotherapy with different electrodes. For the electrode configurations e.g. 4+4, where for the last 4 pulses the electrodes were oriented perpendicularly with respect to the position of the electrodes for the first 4 pulses, electric field distribution was determined as a combination of the results for the cranial-caudal and dorsal-ventral electrode configurations. Since electroporation is a threshold phenomenon, it can be assumed that in the 4+4 electrode configuration the effective magnitude of electric field intensity in each finite element of the model is the highest of the magnitudes for cranial-caudal and dorsal-ventral electrode configurations in that particular element. The same approach was used also for electrodes 2x2 (Figure 1 E) where two by two opposite electrodes were connected to the opposite poles of the generator and electric pulses were delivered in six directions, and for 3x3 electrodes (Figure 1 E), where three by three electrodes were connected to the opposite poles of generator and six pulses were applied altogether by rotating the electric field in the tumor. Minimal and maximal values in the tumor are reported as well.

RESULTS

For all electrodes used in electrochemotherapy and presented in Figure 1 and 2 electric field was calculated in all 7089 elements of the mouse model corresponding to experimentally applied voltage (Table 1 and 2). The mean, standard deviation, minimum and maximum of electric field magnitudes in 48 elements representing the tumor are given in Table 1 and 2. The mean values of electric field magnitudes are presented in Figure 3 and 4 in the form correlation diagrams for each of the electrode orientations and antitumor effectiveness of electrochemotherapy. The summary of the data together with experimental results, i.e. tumor doubling times are listed in Table 1 and 2. Good agreement between the level of anti-tumor effectiveness and the mean value of electric field intensity for each particular electrochemotherapy treatment regime was obtained.

Table 1: Electrodes with resulting electric field and effectiveness of electrochemotherapy for studies reported in [6,8]

Electrode type with figure reference	Applied voltage (V)	Number of pulses	E (V/cm) mean±std	Emin (V/cm)	Emax (V/cm)	max/min	doubling time mean±std (days)
2+1 Fig 2a	1300	8	202±61	115	317	2.7	11.4±0.5
2+2 Fig 2b	1300	8	251±60	166	408	2.4	17.8±1.4
CC Fig 2c	1040	8	327±93	210	518	2.5	20.9±1.0
DV Fig 2c	1040	8	406±65	327	643	2.0	25.1±1.4
4+4	1040	8*	425±64	333	643	1.9	30.0±1.4

*pulses were delivered 4 in direction caudal-cranial and 4 in dorsal-ventral direction

Table 2: Electrodes with resulting electric field and effectiveness of electrochemotherapy for study reported in [5]

Electrode type with figure reference	Applied voltage (V)	Number of pulses	E (V/cm) avg±std	Emin (V/cm)	Emax (V/cm)	max/min	doubling time (days)
caliper Fig 1a	700	8	629±204	373	1048	2.8	19.5±1.6
s.c. needles Fig 1b	330	8	189±47	129	291	2.2	11.7±1.3
8+1 needle Fig 1d	750	8	644±362	339	1350	4.0	20.6±1.4
8+ cilinder Fig 1c	700	8	351±164	191	842	4.4	15.0±1.1
6needle 2x2 Fig 1e	1300	6*	696±107	550	909	1.6	30.9±1.5
6needle 3x3 Fig 1e	705	6*	435±61	349	542	1.5	19.9±1.2

*single pulses were delivered in six different orientations

The results of electrochemotherapy with all electrode sets are given in Figure 3 and 4. Tumor growth was most retarded in experimental groups of electrochemotherapy where the electric field magnitude in tumor was the highest. Tumor growth delay in electric pulses alone and chemotherapy alone was negligible.

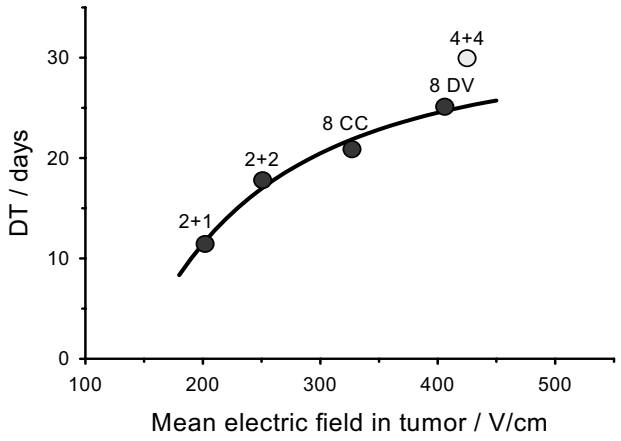

Figure 3: Correlation graph between effectiveness of electrochemotherapy (DT-tumor doubling time) and mean electric field in tumor for various electrodes used in electrochemotherapy experiments [6,8].

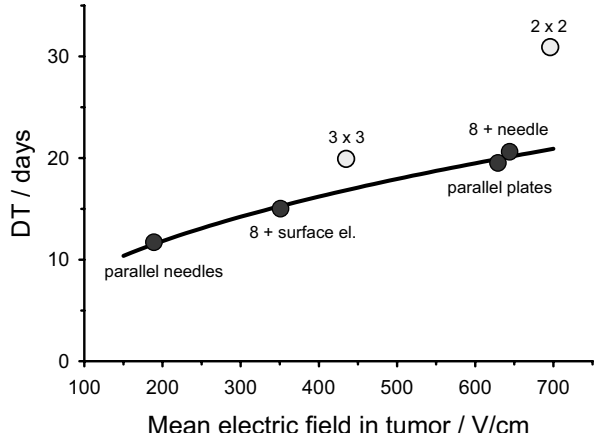

Figure 3: Correlation graph between effectiveness of electrochemotherapy (DT-tumor doubling time) and mean electric field in tumor for various electrodes used in electrochemotherapy experiments [5].

CONCLUSIONS

Mathematical modeling for *in vivo* electroporation has proven to be relatively simple and efficient tool for the analysis of electrical phenomena inside biological tissue. It is very useful for the explanation of experimental results and analysis of different electroporation regimes. We demonstrated, that better coverage of tumors with sufficiently high electric field is necessary for improved effectiveness of electrochemotherapy and so this approach can be very useful in further search for electrodes which would make electrochemotherapy and *in vivo* electroporation in general more efficient. The objective of such studies would be to optimize electrode configuration in order to obtain electric fields over threshold value in the whole selected tissue, e.g. tumor.

Mathematical modeling can thus be used in the transfer of the knowledge gained in experimental work into clinical practice. The long term perspective of mathematical modeling is to contribute to understanding and wider clinical applicability of electrochemotherapy in treatment of cancer and of electro gene transfection as a future treatment for various diseases.

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Damijan Miklavčič was born in Ljubljana, Slovenia, in 1963. He received a Masters and a Doctorate in Electrical Engineering from University of Ljubljana in 1991 and 1993, respectively. He is currently an Professor and Head of the Laboratory of Biocybernetics at the Faculty of Electrical Engineering, University of Ljubljana. His research areas are biomedical engineering and study of the interaction of electromagnetic fields with biological systems. In the last years he has focused on the engineering aspects of electropermeabilization as the basis of drug delivery into cells in tumor models *in vitro* and *in vivo*. His research includes biological experimentation, numerical modeling and hardware development for electrochemotherapy. 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 the tumors. Since drug uptake can be increased by electroporation for only those drugs that have impeded transport through the plasma membrane, among many drugs that have been tested so far, only bleomycin and cisplatin have found their way from preclinical testing to clinical trials. *In vitro* studies demonstrated several fold increase of their cytotoxicity by electroporation of cells. *In vivo*, electroporation of tumors after local or systemic administration of either of the drugs i.e. electrochemotherapy, proved to be effective antitumor treatment. Studies on several tumor models by electrochemotherapy, either with bleomycin or cisplatin, elaborated treatment parameters for effective local tumor control. In clinical studies electrochemotherapy was performed on accessible tumor nodules of different malignancies. All studies provided evidence that electrochemotherapy is effective treatment for local tumor control growth 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

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 metastasize. Due to the high variability of cancer types, the treatment given for cancer is highly variable and dependent on a number of factors, including the type, location, amount of disease and the health status of the patients. The treatments are designed to directly kill/remove the cancer cells or to lead to their eventual death by depriving them of signals needed for cell division or by stimulating the body's own defenses.

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 are some that have 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 their transport through plasma membrane towards the intracellular targets.

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 drugs 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.

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.

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).

- 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 use of different frequencies of the pulses for electrochemotherapy were also tested, but mostly it was 1Hz. The minimal number of the pulses used was 4, but most studies used 8 electric pulses.

- 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.

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).

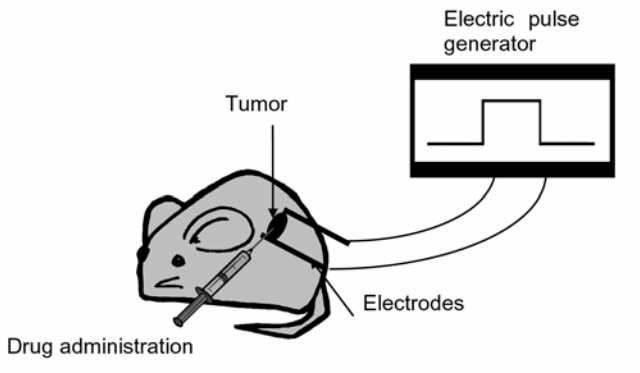

Figure 1: Protocol of electrochemotherapy of experimental tumors presented schematically. The drug is injected either intravenously or intratumorally, at the doses that usually do no exert antitumor effect. After the interval that allows sufficient drug accumulation in the tumors, electric pulses are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

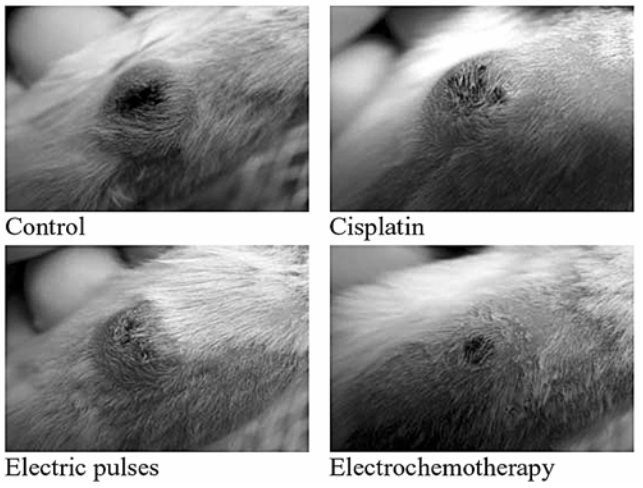

Figure 2: Example of good antitumor effectiveness of SA-1 tumors after electrochemotherapy with cisplatin. Cisplatin vas given intravenously (4 mg/kg), 3 min thereafter 8 electric pulses were applied to the tumor with plate electrodes. Electric pulses were applied in two directions; 4 pulses in one and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

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 amount of bleomycin and cisplatin was measured in the electroporated tumors, compared to those without application of electric pulses.

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. 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. Therefore, another mechanism involved in antitumor effectiveness of electrochemotherapy is its vascular targeted effect. 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 and TNF-α.

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 antitumour 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 the recent study combined modality therapy with cisplatin and radiation can be improved using electroporation of tumors.

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. The reduction of pO_2 could selectively activate bioreductive drugs that exhibit better cytotoxic effect on hypoxic cells than on the cells that are well oxygenated. 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.

CLINICAL DATA

Based on preclinical data, electrochemotherapy with bleomycin and cisplatin entered clinical trials. Cutaneous and subcutaneous tumor nodules of different malignancies, such as head and neck squamous, adeno and adenic cystic carcinoma, basal cell carcinoma, adenocarcinoma of the breast and salivary gland, hypernephroma, malignant melanoma, Kaposi sarcoma, bladder carcinoma were treated. Most of the treated nodules responded with objective responses (comprising complete and partial responses) in 60-100%.

In the protocols both intravenous and intratumoral drug administration were used. Current knowledge about antitumor effectiveness of electrochemotherapy considers electrochemotherapy as local treatment that is effective on most tumor types tested so far. Electrochemotherapy can be performed as single treatment or as an adjunct to the ongoing chemotherapy. In the latter case, antitumor effectiveness of systemically given drug is potentiated

locally on accessible tumor nodules by application of electric pulses.

Comparison between the antitumor effectiveness of electrochemotherapy with bleomycin and electrochemotherapy with cisplatin, given intravenously or intratumorally is possible in treatment of cutaneous tumor nodules of malignant melanoma (Table 1).

Table 1: Summary of electrochemotherapy trials on malignant melanoma

Treatment	No of pts.	No of nodules	OR(%)
Intravenous			
ECT-BLM	14	94	89
CDDP	9	18	22
ECT-CDDP	9	27	48
Intratumoral			
ECT-BLM	11	106	95
CDDP	10	27	38
ECT-CDDP	24	293	81

BLM- bleomycin; CDDP- cisplatin;
ECT- electrochemotherapy; OR- objective responses

The results indicate that electrochemotherapy with bleomycin is equally effective when the drug is given intravenously or intratumorally. Electrochemotherapy with cisplatin is more efficient when the drug is given intratumorally than when it is given intravenously. When given intratumorally, the results are comparable to the antitumor effectiveness of electrochemotherapy with bleomycin. The advantage of electrochemotherapy with cisplatin is that the drug itself, without application of electric pulses may exert considerable antitumor effect.

Electrochemotherapy with cisplatin 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 and horses.

It is difficult to foresee all the potential applications of electrochemotherapy. In the first step more controlled clinical trials are needed evaluating treatment responses of different tumor types. So far, only percutaneously accessible tumor nodules were treated in the clinical trials. Development of new electrodes will enable treating also tumors seeded in internal organs. In its concept electrochemotherapy is local treatment, therefore ways must be exploited to add a systemic component, either by adjuvant immunotherapy or in combination with other systemic treatments.

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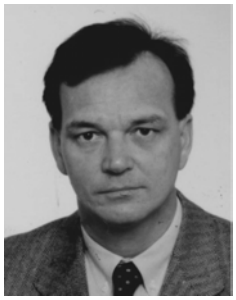

Gregor Serša, graduated from Biotechnical Faculty, at the University of Ljubljana in the year 1987, where he is currently full professor for molecular biology. He is employed at the Institute of Oncology in Ljubljana as a researcher in experimental oncology and radiation biology. His specific field of 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, and in the management of the research area of oncology at the Ministry of Education, Science and Sport of the Republic of Slovenia.

Maja Čemažar, graduated from Biotechnical Faculty, at the University of Ljubljana in the year 1992. She obtained Ph.D from Medical Faculty, University of Ljubljana in 1998. Her post doctoral studies were at Gray Cancer Institute, UK. Curently she is employed at the Institute of Oncology in Ljubljana as a researcher in experimental oncology and radiation biology. Her specific field of interest is the effect of electric field on tumor cells and tumors as a drug delivery system in different therapeutic approaches, especially in gene delivery. Besides experimental work, she is actively involved in the education of postgraduate students at the University of Ljubljana.

NOTES

In vitro electric field mediated gene transfer and expression

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Abstract: Electropulsation is one of the non-viral methods successfully used to transfer genes into living cells *in vitro* The lecture describes the processes supporting DNA transfer *in vitro*. The description of *in vitro* events brings the attention of the reader on the processes occurring before, during and after electropulsation of DNA and cells. Gene transfer across the plasma membrane results from the interaction of electrophoretically accumulated plasmid against the membrane during its electropermeabilization. DNA forms aggregates at the membrane level and then very slowly migrates across the cytoplasm towards the nucleus where protein expression starts.

INTRODUCTION

The application of electric field pulses to cells leads to the transient permeabilization of the membrane (electropermeabilization). A free access to the cytoplasm is given to small polar molecules (see the lecture on cell electropermeabilization). In 1982, E. Neumann demonstrated that it was possible to obtain by such a physical method gene transfer and expression in mammalian cells. Since it was demonstrated that gene transfer can be obtained on many different cell systems. The experimental protocol has the main advantages of being easy to use, fast, reproducible and safe. One of the limiting problems remains that as in the case of cell electropermeabilization very little is known on the physicochemical mechanisms supporting the transfer of macromolecules across the plasma membrane and targeting their movement to the nucleus where expression starts.

This lecture describes the electrical as well as more biological parameters regulating the gene transfer and expression. The events occurring before, during and after electropulsation of DNA and cells are described. A more biophysical interpretation of the processes is given as a conclusion.

A WHAT DID ELECTROPERMEABILIZATION TELL US?

Permeabilization is controlled by the field strength. Parts of the cell surface facing the electrodes are affected. The extend of the permeabilized surface of a spherical cell, Aperm, is given by:

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

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. Permeabilization, due to structural alterations of the membrane, remained restricted to a cap on the cell surface. The density of these alterations is strongly controlled by the pulse duration [1, 2]. An increase of the number of pulses leads to an increase of local permeabilization level.

Molecular transfer of small molecules (< 4 kDa) across the permeabilized area is mostly driven by the concentration gradient across the membrane. Most of the exchange took place after the pulse [2, 3]. Electrophoretic contribution during the pulse remains negligible [3]. This free diffusion of low weight polar molecules after the pulse can be described by using the Fick equation on its electropermeabilized part [1]. But the permeability coefficient appears to be strongly dependent on the molecular weight. In other words, no free diffusion is obtained with macromolecules. Nevertheless, transfer can be obtained but clearly the mechanism is different.

B DNA ELECTROTRANFER *IN VITRO*

Gene expression is indeed observed after applying electric pulses to a cell DNA mixture as successfully reported in 1982 by E. Neumann [4]. No transfected cells were detected in absence of electric field, in absence of DNA, or when DNA was added after the pulses [5, 6]. This last observation is a definitive evidence that the mechanism of plasmid transfer is different from the process of electropermeabilization where small polar molecules can cross the plasma membrane after the pulse.

DNA transfer can be monitored by observing fluorescently labelled nucleic acides or by detecting the ultimated goal: the expression of the coded protein. One should take into account that many steps follow the transmembrane transfer before the protein can be detected.

B-1 Events before electropulsation.

The temperature of incubation of DNA and cells has been reported to affect transfection [7, 8]. Low temperature prevents DNA degradation by external DNAases before pulsation and limits the deleterious consequences of joule heating during pulsation.

Videoobservation of fluorescent DNA cannot detect adsorbed plasmids on mammalian cell surfaces [9].

B-2 Events during electropulsation: Membrane – DNA interaction.

Pulsation medium is usually culture medium without serum, phosphate or HEPES buffers. Sucrose (or other carbohydrates) can replace salts of these buffers. While preserving the osmolarity of the buffer, it decreases the conductivity and limits joule effect. Osmotic pressure can influence the membrane undulations of the cell and the associated hydration forces and can therefore modulate the DNA interaction to the membrane [10].

B-2-1 Effect of the electric field parameters.

Field strength must be strong enough to permeabilize (locally) the cell membrane for plasmid-membrane interaction to occur. Plasmids interact only with the permeabilized cell surface. It is accumulated by the field associated electrophoretic drag as shown by fluorescence microscopy [9]. This videomicroscopy study showed that plasmid DNA was trapped in the electropermeabilized membrane where it formed aggregates within a few seconds after the pulses. Negatively charged DNA molecules migrate when submitted to an electric field [6, 11]. But, electrophoretic DNA accumulation by itself is not enough to bring transmembrane transfer and gene expression. The direct consequence of this electophoretic migration is that under unipolar conditions, plasmids interact only on one single cap on a given cell.

Pulse duration plays a critical role in the formation of the plasmid-cell complex. The complex between the plasmid and the cell surface is detected only when the pulse duration is at least 1 ms.

The intramembrane aggregates can be detected during several minutes after pulsing. Their dissociation is followed by a diffusion of the plasmid in the cytoplasm towards (and in) the nucleus.

These contributions of the field strength and of the pulse duration are experimentally proven by the observation of their control of the intramembrane DNA content, determined by the local emission of labeled DNA and the size of the fluorescent spot [9].

This contribution of the pulse duration to the plasmid-membrane interaction has already been illustrated by a complex dependence of the gene expression [6]. The associated gene expression Expr is shown to obey the following equation:

$$Expr = K \cdot N \cdot T^{2.3} \cdot \left(1 - \frac{E_P}{E}\right) \cdot f\left(ADN\right) \qquad (2)$$

as long as the cell viability is not affected to a large extent by the electrical treatment [9]. All parameters are as described above, K being a constant.

The practical conclusion is that *in vitro* an effective transfer is obtained by using long pulses in order to drive the DNA towards the permeabilized area of the membrane but with a field low enough to preserve cell viability [12, 13]. Nevertheless transfection was obtained with short strong pulses in the pioneering experiments [4].

B-2-2 Effect due to plasmid: size, form and concentration.

A linear increase of the transfection level was observed with an increase of the amount of DNA [5, 6]. Nevertheless, a toxic effect on the cell viability has been observed with high DNA concentration [6, 14].

B-3 Events after Electropulsation

The main conclusion of the observations during the pulse is that plasmids do not cross the membrane during that step, even if the membrane is permeabilized (for small molecules). They remained trapped at the permeabilized membrane level.

B-3-1 Effect of the temperature

A higher level of transfection is obtained when the post pulse incubation is run at 37°C rather than at 4°C [15]. A long life of the permeabilized state does not favour the transfer of DNA.

B-3-2 Effect of the post-pulse medium composition

A change of the osmolarity buffer just after the pulsation modulates the level of transfection. The plasmid-membrane interaction depends on the control of the undulations forces of the membrane [10].

B-3-3 DNAase effect

Cell electroassociated DNA remains accessible to DNAaseI, several seconds after pulsing CHO cells [16]. The DNA aggregates, which are anchored in the membrane after the electric field application, remain accessible to the externally post pulse added enzymes, which cannot cross the membrane.

B-3-4 Plasmid traffic into the cell towards the nucleus

Protein expression can be detected within 2 hours after the electrical treatment. These steps are under the control of the cell metabolism, which may be affected by electropermeabilization. A major problem is the nuclear envelope which must be considered as a critical barrier for gene transfection.

C How does it work?

Many theoretical models have been proposed in an attempt to explain the mechanisms of electropermeabilization and gene transfer [17-22]. It was proposed in the case of mammalian cells that plasmids cross the membrane either i) due to the existence of long-lived "electropores", which must be huge taking into account the size of DNA [4, 17, 21], or ii) after a binding step at the cell surface and then electro-diffusion through the electropores [17], or iii) during application of the electric pulses due to transmembraneous electrophoretic forces associated with the external field [5, 22] or iv) by adsorption by electrostatic interaction with the lipids, followed by an insertion bringing passage of DNA through a hydrophilic percolated porous zone [20]. Within such models, plasmid DNA could enter the cell either added after application of electric pulses (i and ii) or only during pulse application (iii and iv). Assymetry in DNA transfer is associated to an assymetry of the pores on the two sides of the cell in i).

These models are challenged by the experimental facts. No free plasmid diffusion into the cytoplasm is detected while this was proposed in i). No plasmid membrane interaction occurs if the nucleic acids are added after electropermeabilizing cells as proposed in i) and ii). Plasmid transfer is still under process after the pulse as shown by the DNAse effect in conflict with the predictions of iii) and iv). Indeed, experimental results led to the conclusion that plasmids had to be present during electropulsation but crossed the electropulsed membrane in the minute following it. These results were obtained on bacteria, yeast and mammalian cells [10, 23, 24].

We proposed a model in which Electrotransfection appears as a multistep process [6] supported by its direct experimental evidence [9] .

During the pulse,

i- electropermeabilization takes place

ii- plasmids are electrophoretically driven into contact with the cell surface

iii- a metastable complex is formed between plasmids and the localised electropermeabilized part of the cell membrane.

iv- plasmid clusters are formed at the membrane level

iv- after the pulse, plasmids slowly leave the complex and diffuse in the cytoplasm

v- a small fraction crosses the nuclear envelope to be expressed.

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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

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, b 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.

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

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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.

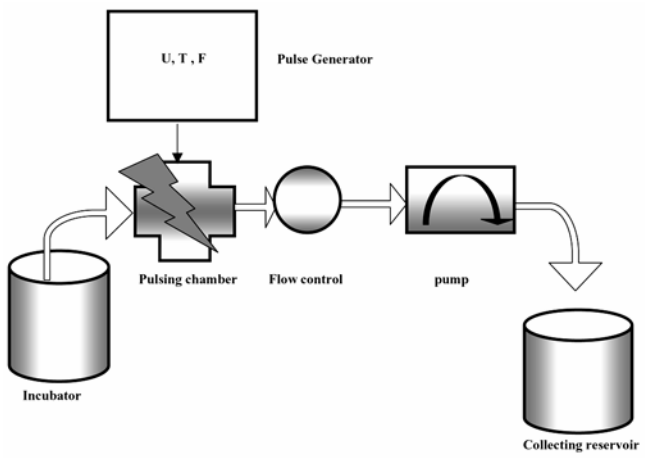

Figure 1: Flow electropulsation; A- Cells are taken from the incubator where they are growing. B- They flow through the pulsing chamber where a controlled number of calibrated pulses is applied. The pulsing chamber is connected to the high power pulse electropulsator where the voltage U, the pulse duration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

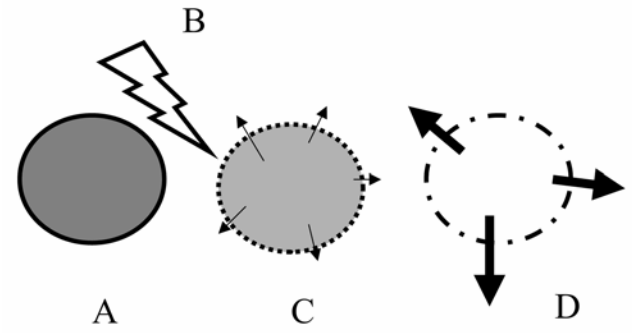

Figure 2: Irreversible Electropermeabilization; A- Intact cells. Their cytoplasm content is pictured in dark grey. B- Electropulsation. C- Cell membranes are permeabilized. The cytoplasm content leaks out as shown by the light grey colour and the small arrows. D-The cell membrane is irreversibly permeabilized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

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.

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NOTES

INVITED LECTURERS

Current status in the field of tumor vaccines

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Abstract: Among the most attractive and promising variants of cancer immunotherapy, a special place is reserved for tumor vaccines. Until now, many outstanding approaches for the creation of tumor vaccines have been developed that exploit the latest knowledge in immunology, tumor physiology, as well as in molecular biology. With regard to the basic principles of the vaccine creation and their expected activity, the tumor vaccines could be divided into two subclasses: I) first-generation vaccines that base on the principles of classical immunology – i.e. classical tumor vaccines, and II) second-generation vaccines that base on the principles of molecular immunology - genetically modified and recombinant tumor vaccines. With no intention to be comprehensive, I will try to review some of the approaches for the creation of tumor vaccines as well as to discuss their future perspectives.

INTRODUCTION

In the last few years, we have witnessed a great progress in the fields of immunology, tumor physiology and molecular biology. Namely, the basic facts about the recognition of various structures by the immune system through the cooperation of MHC have been explained. The structures of MHC class I, and MHC class II have been studied and their function analyzed quite thoroughly. The complex mechanisms of antigen presentation and the role of presenting cells have been investigated in details.1 Various cell receptors (especially T cell receptors) have been discovered, and the methods of signal transduction and the activation of T lymphocytes (the major performers of the cellular immunity) have been elucidated.2 The production of monoclonal antibodies towards different well-defined structures has become a routine procedure, which facilitates the transition of such antibodies into clinical praxis.3 Today, we are also familiar with the structure of and are equipped to produce various immunomodulatory cytokines, which in turn, assists their application in the treatment of some malignant diseases (hairy-cell leukemia, malignant melanoma, renal cell carcinoma).4 On the other hand, also the methods for precise determination of different genes and their transduction into mammalian cells have been extensively studied. Thus, the researchers created various vector systems that can be divided into two large groups: (1) viral vectors (retroviral, adenoviral, adeno-associated viral vectors, herpesviral vectors)5 and (2) nonviral vectors (calcium-phosphate precipitation, liposomes, microinjections, electroporation, poly-lysine conjugates, receptor-mediated endocytosis, gene gun).6,7 These discoveries have become the groundwork for the renewed and new biological approaches towards the treatment of malignant diseases.

TUMOR VACCINES

The first tumor vaccines were created on the principles of classical immunology and comprised irradiated tumor cells and nonspecific immunomodulators. Further approaches towards the creation of tumor vaccines base on the principles of molecular immunology and quite often it is hard to distinguish them from the classical gene therapy approaches. The newer vaccines also include autologous or allogeneic cells, with the difference that various genes coding for proteins involved in the stimulation of immune response (e.g. genes coding for growth factors and cytokines, as well as genes coding for co-stimulatory molecules) are introduced into these cells. Instead of whole cells, also certain specific structures that are responsible for the antitumor immune response can be used.

Classical tumor vaccines

It was not until recently that we gained some information about the specific antigens that are present on the surface of the tumor cells, and about the co-stimulatory molecules that are necessary for the activation of the immune system, so the first true tumor vaccines were composed of (1) autologous or allogeneic irradiated tumor cells, (2) tumor cell lysates with viral antigens or (3) tumor cells with nonspecific immunomodulators (Corynebacterium parvum, Bacillus Calmette-Guerin).10,11 These first-generation vaccines that base on the principles of classical immunology were termed classical tumor vaccines.

Genetically modified and recombinant tumor vaccines

The group of second-generation vaccines that base on the principles of molecular immunology comprises genetically modified and recombinant tumor vaccines.

Just like the first-generation vaccines also the second-generation vaccines can be divided into the ones that utilize whole autologous or allogeneic cells, and ones that utilize only certain specific structures. Yet, unlike the first-generation vaccines that are prepared strictly with autologous or allogeneic tumor cells, the second-generation vaccines employ either tumor cells or non-tumor cells (mostly autologous) as fibroblasts and dendritic cells. Regarding the approach towards a better recognition of the tumor cells by the immune system, the second-generation vaccines can be subdivided into the (1) vaccines prepared with genetically modified tumor cells, (2) vaccines prepared with genetically modified non-tumor cells (most frequently dendritic cells and fibroblasts), and (3) recombinant vaccines.

The approach towards the creation of tumor vaccines on the base of genetically modified tumor cells includes different modes of preparation. The more promising modes of preparation are the transfection of tumor cells with genes coding for antigens that are being presented through MHC class I and II, with genes coding for co-stimulatory molecules, and with genes coding for various cytokines.

CONCLUSION

Speaking about the novel systemic cancer treatments, I am convinced that, rather than approaches where the therapeutics are acting directly against tumor cells, the approaches that propose the mobilization of protective mechanisms in the host will turn out to be more effective. That implies the development of the tumor vaccines that would be capable of triggering an antitumor immune response and preparing the host for a long lasting control of tumor growth and metastasis. At the moment, it is difficult to predict what kind of vaccine is going to be the most successful. It seams that the most effective one is going to be the vaccine that includes the tumor presenting cells armed with some genes encoding tumor antigens and immunostimulatory cytokines. On the other hand, the advantage will be given to the vaccine that comprises the elements of adjuvant and standard therapy allowing the application as a single adjuvant therapy after the surgical removal of the tumor, or in combination with the therapies that aggressively act directly against the tumor cells.

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Srdjan Novaković gained the Master of Science degree in the field of Microbiology and Immunology, at the Medical Faculty, University of Ljubljana. He spent the period between August 1992 and August 1993 as the visiting scientist at the University of Texas Medical Branch at Galveston, in Texas, USA, and became the Doctor of Medical Sciences in 1994, at the Medical Faculty, University of Ljubljana. His major research interest is in the field of Biological Therapies in Oncology, and in 1995, he became the principal investigator of the research grant dealing with tumor vaccines¢ preparation. Since 2001, he is the head of the Department of Tumor Biology at the Institute of Oncology.

NOTES

E-Learning: Functions, Services and Solutions

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INTRODUCTION

Telecommunications and information technologies based e-learning shows rapid growth in number of applications and users involved. Efficient and successful e-learning solutions are present in various fields of knowledge and aim to different target groups and educational purposes, including corporate training and academic education. E-learning is being introduced for different reasons, but among the most common ones time, spatial and financial barriers can be encountered [1].

In the academic environment e-learning has multiple roles: (i) it can serve as a supplemental or even key learning method within the undergraduate, graduate or postgraduate study, and thus complements the traditional learning, (ii) and is an extremely efficient method for the project result dissemination. The later is particularly due to possible short times for course implementations, large and dispersed target audience and possibility to structure and develop the course material in several phases.

E-learning is becoming an integral part of the IT (information technology) systems, overcoming the frequently present hype of a miraculously simple and powerful solution, requiring only a minimal level of involvement by the e-learning providers. Fully functional e-learning systems are usually based on e-learning platforms - dedicated application systems enabling the management of the learning processes and users, evaluations and progress tracking, content creation and delivery and various modes of communication [2]. There are two main development directions present, first focused on the learning processes - LMS (Learning Management Systems) and second focused on the content - LCMS (Learning Content Management Systems). Both functional areas can be implemented separately or within the same application system.

E-LEARNING COMPONENTS

E-learning solutions reflect actual education process in academic environment and enhance educational possibilities in innovative way regarding the course access, contents and level, in order to meet diversified needs of target groups. The only way to accomplish this is to support all e-learning components through complete solutions.

1) Content presentation and interactivity

Content of e-learning can be developed with different commercially available tools. It is commonly presented in html format. This allows content to be portable between different e-learning platforms. It is application independent and developed with standardized developing tools. Multimedia that is supported by Internet is used to enrich the content. Real time audio or video streaming clips as well as java applets can be used to explain experiments. Content management and even higher portability is enabled with use of XML technology to specify content of e-learning.

Web-conferencing systems enable application sharing, virtual blackboard and lectures at distance without seeing a lecturer. Virtual lectures are enhanced with use of videoconferencing systems. They are used for lectures at distance in intranets and environments with fast Internet access. With the introduction of broadband technologies into residential Internet access, the use of this technology increases. These synchronous learning experiences can be accessed on demand in case the learner doesn't have the opportunity to attend the lecture at distance in the real time.

Interactivity is achieved with Internet based interactive animations and simulations developed with standardized tools that enable browser independent viewing of the contents. Automatic e-learning system response to the learner's actions is achieved with the use of appropriate e-learning platform.

2) Management of learning systems

E-learning management consists of managing students, learning staff, learning content, different learning tools and disseminating learner's statistical data.

E-learning platform has to support extensive yet easy to use management options. Several types of users should be supported in order to distinguish learners from pedagogical and administrative collaborators, thus enabling them to have completely personalized access to learning.

Manipulation of the learning material should be flexible. Individual learner should be able to adjust the pace and content of e-learning according to the learning objectives. E-learning platform should make possible for developers to form arbitrary e-learning courses from existing material.

Another aspect of successful e-learning management is tracking possibility of learner's activities that is enabled by the e-learning platform. Statistical data of users activities during learning

process, provided by the platform, should be easy to process, manage and report. This gives effective feedback on the learners activity and acquired knowledge and therefore facilitates work of pedagogues within e-learning.

3) Collaboration between learners

One of the main disadvantages of e-learning is lack of socialization and teamwork between peers because of the distant locations from which learners access the e-learning. Communication tools and other features of e-learning platform have major role in surmounting this disadvantage. E-learning platform has to include the possibility of forming different groups of learners, giving them common assignments and enabling them to have guided or non-guided discussions among themselves. Enhanced teamwork includes virtual classroom creation, where learners can use audio and video conferencing tools as well as application sharing tools.

4) Communication with tutors, mentors, professors

The e-learning flexibility can be achieved through professor - tutor - learners communications via advanced communications channels. These can be asynchronous: e - mail, discussions and notice boards, or synchronous: chats or audio & videoconferences.

5) User support

E-learning pedagogues in academic environment usually have other engagements in classical education, such as lecturing or assisting. Their e-learning activities are added as supplement. The extent of their additional engagements in e-learning can be minimized with the use of appropriate E-learning platform that should ease access to learners and information about their progress. Tutors must have direct access to content of e-learning and to communication tools that are needed.

Apart from pedagogical support, technical support is also needed. In case of previously described e-learning scenario, it is provided by the LSP.

6) Assessing knowledge

Assessing knowledge in e-learning is not only about getting feedback on the learners activities and gained knowledge, but also about acquiring complete picture of the quality of e-learning. The most common model to achieve this is through multi-level evaluation. This way we evaluate reaction of the learners, track learners progress and get the results of e-learning. The platform should support questionnaires that learners need to fill in before the start or after the completion of the e-learning, several different types of questions and methods to track transfer of the knowledge and skills gained through the e-learning to the real world.

Implementing two different kinds of knowledge evaluation increases efficiency and quality of study when using ICT. Simultaneous knowledge assessment is achieved by giving assignments to learners during e-learning. Final tests are similar to the knowledge evaluation in the classical education.

PROVIDING E-LEARNING SERVICES

Providing e-learning requires different activities to support all the components needed. Frequently a signle institution (e.g. university, department or even project group) provides all the components, but often the players tend to specialize. In this case it is of high importance, that particular responsibilities can be delegated among different institutions/persons and still enabling a transparent and uniform e-learning experience for the end-user. The Learning Service Provider (LSP) combines activities of various players and presents them to the users.

A LSP can provide the learning infrastructure to corporate, academic and other environments. LSP enable target environment to concentrate only on learning aspects such as defining learning objectives, developing learning material, pedagogical study support, etc. Technology aspect of e-learning can be left to the LSP.

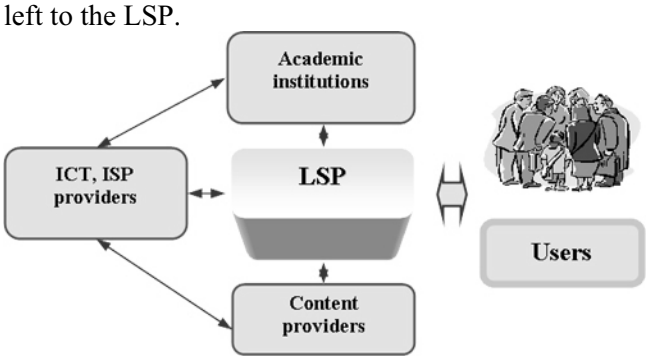

Figure 1: E-learning players

Content development is the key part of a successful e-learning implementation. There are usually three types of experts involved in the content creation: (i) the subject matter expert, (ii) the instructional designer and the (iii) implementer. The three roles can be combined in one person, but splitting them makes it possible for everyone to focus on the role he/she really masters.

E-CHO E-LEARNING PLATFORM

ECHO is an e-learning system which provides various e-learning related functionalities in an Internet based environment. It comprises of a powerful shell which stores, manages and distributes one or more e-learning courses and manages students.

Three main guidelines were set up during the functional specification of the e-learning system:

- the simplicity of use,
- system independent course content creation,
- personalization of the learning activities,
- multiligualism.

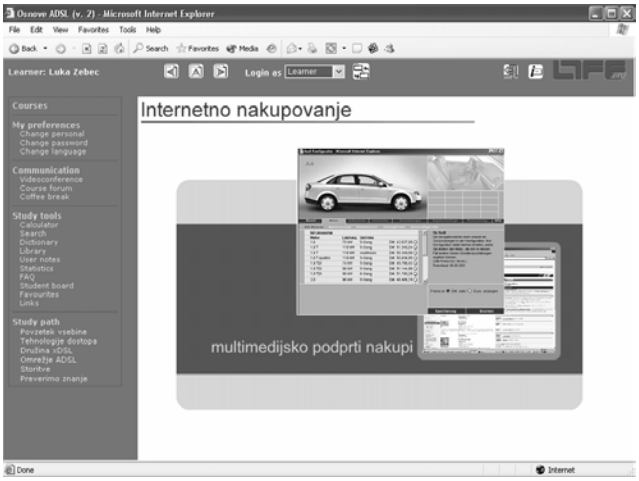

Figure 2: E-CHO user interface [3]

Some of the key features of the system are the access to the hypermedia learning materials, different methods of navigation through the learning material, support of the automatic assessment and monitoring, student's history records, individualized learning environment and simultaneous self examination. The learning process is augmented by different synchrononous and asynchrononous communication mechanisms. The main targeted features are:

- the support of learning service provisioning,
- focus on the management of the courses, users and learning activities,
- flexibility of content development and specially and
- various levels of customisation of the learning activities and personalization of the user interfaces, as well as course catalogue creation,
- enables multilingual user interfaces.

The system supports different fields of knowledge and is independent of the field of education.

User roles

The most frequent role of ECHO user is being a learner. Learner reviews study material by attending courses, communicates with other learners and course tutor using advanced communication channels, such as discussion boards, chats, audio and videoconferencing and participates in tests, exams and surveys.

The pedagogical aspects of learning process are covered by tutor who customizes learning activities of a particular lessons if needed, supports guided discussions among learners and tracks learner's statistical data to have an effective feedback on the learner activity.

Administrative work done by managers of e-learning system consists of managing learners, human resources, learning content, different learning tools and disseminating group statistical data.

Content of e-learning is prepared by content developers. Content delivered by ECHO can include all types of multimedia, supported by the Internet. Developers can develop content any way they are used to and simply upload the materials on the e-learning platform. Once the content is on the system, it can be managed by managers, assigned to courses and reviewed by the learners.

System architecture

The main goal of our approach being put forward while designing ECHO is its simple accessibility with minimum requirements on the users side. Due to large flexibility of information delivery over Internet, ECHO is implemented as a standard Internet application. The client side requires no more than standard Internet browser (such as Internet explorer or Netscape navigator) installed on the local computer, while the main application functionality is assured by the server side.

ECHO is developed using three-tier application design approach. In this approach, we logically divide application architecture into three main layers. The layer that gives a user access to the application is called presentation tier. It presents data to the user and optionally permits data manipulation and data entry. In a three-tier application, the client-side application is skinnier than a client-server application because it does not contain the application logic rules now located in the middle tier - a system in between the user interface and the data storage system. The middle tier ensures that all of the application logic processing is done as required. It serves as an intermediary between the client and the data storage. In this type of application, the client would never access the data storage system directly. Since the parts of the application communicate through interfaces, the internal workings can be changed without affecting the rest of the system, as long as the interface remains the same. The third layer, also termed as a data services layer interacts with persistent data usually stored in a database or in permanent storage. It can be accessed through the application logic layer and on occasion by the user services layer. A three-tier approach allows for any part of the system to be modified without having to change the other two parts.

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NOTES

Lesson on imaging techniques

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Abstract: Tracing and quantifying molecular exchanges and transgene expression mediated by electroporation is required for new progress in basic and practical knowledges of drug transfer and gene therapy. Imaging is particularly well suited for such analysis. Optical imaging techniques are helpful in recording temporal and spatial resolutions of such processes, and can be achieved using isolated cells, tissues and more recently whole animal. Imaging of basic cellular events requires detection systems with high spatial resolution and sensitivity, i.e. capable of producing images with good resolution, and biocompatible imaging probes with high specificity. Fluorescent proteins of the GFP family are easy tools to detect gene expression. New developments of these proteins and the use of cooled CCD camera allow a spatial and temporal detection of gene expression with short time exposition.

INTRODUCTION TO FLUORESCENCE VIDEO IMAGING : From the real to the numerical…

Nowdays, cell proceedings can be resolved from the cellular to the tissue levels using fluorescence imaging. This is performed using fluorescence image acquisition systems from which real images are picked up on amplifyed or not Charge-Coupled-Device (CCD) camera mounted on a fluorescence microscope. Light (photons) is converted in an electrical signal (voltage) through an array of chips (detectors). Quantitative information can be obtained from the analogic image after its mathematic transformation (digitalisation) on a computer using video frame-grabber board. Being now mathematical matrices, numerical images can be corrected from their defects and/or noises before being displayed back on a monitor or recorded for storage. Better understanding of basic cellular events requires new developments in cell imaging which must provide higher resolution in time. Indeed, common acquisition frequencies in video-microscopy imaging are in the range of 33-50 Hz, i.e., 33.37 (NTSC) and 40.0 milliseconds (PAL and SECAM) are required to obtain a complete analogic image. However, whatever the standard, the time need to read a line is the same, about 64 µs. The final image is nominally 525 (NTSC) or 625 (PAL, SECAM) lines per full frame, with two interlaced fields combined to make an entire image. Two half-fields are successively scanned from the CCD chip array, one for the even lines and one for the odd ones. In a first attempt, time-resolution increase can be obtained simply working in non-interlaced condition. Such condition allows to work 2 times quickly (16 or 20 ms per images) but only odd CCD chip lines are read. Higher resolution in time is then achieved to the detriment of the spatial resolution. In the same way, increase in time resolution can be achieved by decreasing the surface of the CCD target which is successively scanned (1/2, 1/4, 1/6 and 1/8 formats, i.e., in european standard, 10, 5, 3.3 and 2.5 ms per image).

In this lecture, examples of video systems and their use in relation to electroporation (calcium ions exchanges, membrane structural changes, trans-membrane potential variations) will be presented. Clear understanding of electroporation's physical and structural bases appears as a necessity owing to the widespread use of exogenous electric field application (electropulsation) in clinical and biotechnological applications. The pulse-induced local perturbation of the membrane structure is in the millisecond time range. Therefore analysis of the electroporation processes requires work at the membrane level of single cells, with high temporal and spatial resolution. We developped a fast and sensitive fluorescence image acquisition system which uses an ultra-low-light intensifying CCD camera able to acquire digitised fluorescence images with a time resolution of 3.33 ms/image [1].

Using our ultra-rapid intensifying video system (3.33 ms/image), we directly observed exchanges of calcium ions between the cytosol of a single cell and the extracellular medium and the interaction of propidium iodide (PI) with the electropermeabilized parts of mammalian cell membrane during and immediately after a millisecond electroporating direct current pulse [2-4].

CELL LABELING.

The cell-permeant Fluo-3 AM (2.2 mM) was loaded (in the presence of 0.2% w/v F-127 pluronic acid) by incubating CHO cells for 15–20 min in culture medium at room temperature and under gentle agitation. Excess external dye was washed off after incubation, and cells were suspended in 1 mM CaCl2-containing PB. When external Ca2+ ions enter the electropermeabilized cell, they immediately bind to pre-loaded Fluo-3 indicators inside the cell and cause

enhancement of fluorescence. For observation of the calcium efflux, we pulsed the cells in 100 nM calcium-containing PB in the presence of 10 mM Fluo-3 free acid indicator. When intracellular calcium ions (220 +/- 20 nM) exit from the cell suspended in 100 nM calcium-containing PB, they immediately bind to Fluo-3 indicator outside the cell and give an enhancement in fluorescence.

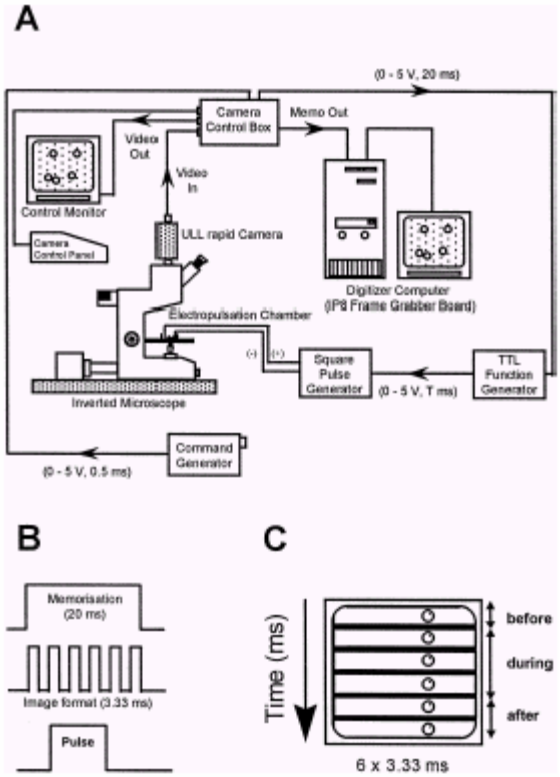

Figure 1: Schematic drawings of the rapid ultra-low-light intensifying imaging system and electroporation system (A), the trigger synchronisation (B), and the typical recorded 3.33-ms image sequence (C). Arrows represent signal directions (TTL and video). The pulse duration was 10 ms.

FLUORESCENCE IMAGE ACQUISITION AND PROCESSING.

The ultra-rapid fluorescence image acquisition system and its synchronization with pulse delivery were previously described [1]. Cells were observed by fluorescence under the 63x 1.4 NA oil immersion objective (Leitz). When PI was used, the wavelengths were selected with a Leitz N2 block filter (530 nm < lexc < 560 nm; 580 nm < lem). For Fluo-3 pre-loaded cells, the Leitz H3 filter block was used (390 nm < lexc < 490 nm; 515 nm < lem). The CCD LH 750-ULL camera (Lhesa) allowed real-time fluorescence image acquisition with a 300-Hz frequency (3.33 ms/image). The size of the image pixel was 0.3 x 0.2 µm. The fluorescence patterns of the dye during and after electropulsation of the cell were recorded in the memory board of the camera control box. The system allowed the acquisition of a single 20-ms video frame that represented six consecutive 3.33-ms images. The first one was acquired immediately before the onset of the pulse (blank image, i.e., the fluorescence image of the selected cell before electropulsation). The recorded images were transferred to a PC computer (AT/486) by using a frame-grabber board (IP8, Matrox). The video signal was converted to a numerical matrix with 8-bit gray scale, i.e., 256 different light levels. The gain of the camera was fixed by manual adjustment. Image acquisition and processing were obtained with Optimas 4.02 software (Bioscan).

RESULTS & CONCLUSIONS.

Exchanges of calcium ions through electroporated membrane of Chinese hamster ovary cells were found to be asymmetrical. Entry of calcium ions during a millisecond pulse occurred on the anode-facing cell hemisphere. Entry through the region facing the cathode was observed only after the pulse. Leakage of intracellular calcium ions from electroporated cell in low-calcium content medium was observed only from the anode-facing side. The exchanges during the pulse were mostly due to diffusion-driven processes, i.e., governed by the concentration gradient. Interaction of propidium iodide, a dye sensitive to the structural alteration of membrane, with cell membrane was asymmetrical during electroporation. Localized enhancement of the dye fluorescence was observed during and after the pulsation on the cell surface. Specific staining of a limited anode-facing part of the membrane was observed as soon as the pulse was applied. The membrane fluorescence level increased during and immediately after the pulse whereas the geometry of the staining was unchanged. The membrane regions stained by propidium iodide were the same as those where calcium exchanges occurred. The fraction of the membrane on which structural alterations occurred was defined by the field strength. The density of defects was governed by the pulse duration. Electroporation is a localized but asymmetrical process. The membrane defects are created unequally on the two cell sides during the pulse, implying a vectorial effect of the electric field on the membrane.

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Bruno Gabriel was born in Lille, France, in 1962. He received a Master in Biochemistry and a Doctorate (D.Sc.) in Cell Biophysics from University Paul Sabatier, Toulouse, in 1987 and 1992 respectively. He is currently Chargé de Recherches at CNRS. His scientific areas are changes in living-cell membrane structure and function in relation with electroporation and associated cell responses (cell death, oxidative stress…). In the last years he has focused on real time imaging (millisecond time range) of electroporation process and cell behaviour. At the present time, he is in charge of the ‘‘Confocal and Dynamic Cell Imaging’’ technical stage in his institute (IPBS-CNRS, Toulouse, France).

NOTES

STUDENTS ABSTRACTS

In vivo Transfer of naked DNA into Skeletal Muscle Using Combinations of Electric Pulses

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INTRODUCTION

To obtain a good gene transfection in skeletal muscles, 8 short squares Electric Pulses (EP) of 200 V/cm and 20ms duration delivered at a repetition frequency of 1Hz [1] are often used. However, in our laboratory, it has been recently showed an increase of gene transfer efficiency for skeletal muscles when using combinations of short, high voltage EP (HV; 800 V/cm, 100 µs) and long, low voltage EP (LV; 80 V/cm, 100 ms) [2].

The efficiency of these combinations is explained by the fact that DNA electrotransfer is based on two different effects of the EP: the electropermeabilization of the target cells (HV) and the electrophoresis of the DNA towards or across the permeabilized plasma membranes (LV). Based upon that principle, a new EP generator has been developed within the CLINIPORATOR EU program (QLK3-1999-00484 of the EU 5th FP.

This generator (Cliniporator™, IGEA Italy), that allows the delivery of these combinations of HV and LV and the recording of various parameters, is used in our laboratory for the optimisation of DNA transfer in skeletal muscle *in vivo* using various high-voltage (HV) and low-voltage (LV) pulse combinations.

MATERIALS AND METHODS

Plasmid DNA. We used a pCMV-Luc+ plasmid containing the cytomegalovirus promoter of pcDNA3 (nucleotides 229-890, Invitrogen) inserted upstream of the coding sequence of the firefly luciferase (photinus pyralis) of the pGL2-Basic Vector plasmid (Promega, pGL2-Basic Vector, Cat. E1641). We prepared the plasmid DNA using the EndoFree Plasmid Giga Kit (QIAGEN).

Animals. Female, 7 to 9-week-old, C57BL/6 mice (maintained under standard conditions) were anesthetized by the i.p. administration of 180 µl of ketamine HCl 10mg/ml (Ketalar, Panpharma) and xylazine 0.1% (Rompun, Bayer). Two days before the experiments, we shave the legs using electric shaver and depilation cream.

DNA injection. We injected 10 µg of plasmid DNA (prepared in 20 µl of PBS (GIBCO, UK)) into tibial cranial muscles using a Hamilton syringe with a 26-gauge needle).

DNA electrotransfer. Muscles were pulsed with various combinations of HV and LV applied with Cliniporator™ (IGEA, Italy) soon (40 ± 15 seconds) after intramuscular DNA injection. We used two opposing stainless-steel plate electrodes, of 1 cm width and 1 mm thickness, and 5,7 mm space (made by IGEA, Italy). The electrodes encompassed the whole leg of the mouse. Contact was ensured by means of ultrasound conductive gel (EKO-GEL).

Luciferase activity measurement. Two days after DNA electrotransfer, mice were killed and the muscles removed and disrupted in 1 ml cell culture lysis reagent solution (Promega Charbonnières, France), supplemented with one tablet of protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). After centrifugation at 12,000 rpm for 10 minutes at 4°C, we assessed the luciferase activity on 20 µl of the supernatant, using a Berthold Lumat LB 9507 luminometer,

RESULTS AND CONCLUSION

We have made some series of experiments where we used different voltages for the HV as well as 1 or 2 LV pulses. We were also testing the combinations of pulses with and without 1 second lag between the HV and LV pulses.

Preliminary results show that a low and variable transfection is obtained by mean of plasmid injection only. A 100X increase is obtain when using only 1LV of 80V/cm and 400ms duration (no HV), and a 1000X increase when using 2 of these LVs, 3000 ms apart. The efficacy of the DNA transfection is improved by a factor 100,000X when delivering 1HV of 100µs at 600V/cm immediately before the 1LV (no lag between HV and LV). The presence of a 1s lag between the HV and LV pulses seems to allow a better transfection than with no lag (about 4X higher luciferase expression).

The best set of Cliniporator electric parameters allows a slightly better transfection compared to the 8 similar square EP of 200 V/cm and 20ms 1Hz, often used.

The influence of other parameters of HV and LV are still under investigation.

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The effect of different $C_{12}E_8$ concentrations on POPC vesicle shape transformation

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INTRODUCTION

Membrane properties of giant 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-0-choline (POPC) unilamellar vesicles, produced by electroformation, have been studied in the past [1]-[3]. The research was focused mainly on the vesicle shape transformations and formation of the nanostructructures-protrusions [2], [3]. Studies with addition of La^{3+}, Gd^{3+}, alcohol and surfactants have shown different transformation patterns.

In this abstract we present the influence of different concentrations of the $C_{12}E_8$ surfactant on the giant POPC vesicle shape transformation.

MATERIALS AND METHODS

Electroformation of giant phospholipid vesicles

Giant phospholipid vesicles were prepared at room temperature by the modified electroformation method [4]. The vesicles were prepared from phospholipid POPC purchased from Avanti Polar Lipids, Inc. In the procedure, 20 ul of POPC, dissolved in 9:1 chloroform/methanol mixture, were deposited to the platinum electrodes. The solvent was allowed to evaporate in low vacuum for at least two hours. The coated electrodes were then placed in the electroformation chamber filled with 0.2 M sucrose solution. An AC electric field with amplitude 1 V/mm and frequency 10 Hz was then applied to the electrodes for 2 h [4a]. The content of the chamber was then poured out in the glass beaker and 0.2 M glucose solution was added. The suspension containing vesicles was gently mixed.

Observation of liposomes after addition of $C_{12}E_8$.

The vesicles were observed at room temperature with ZEISS 200 Axiovert inverted phase contrast microscope at 1000x immersion magnification. The detergent was added to the vesicles either directly or through slow diffusion between two compartments in a special observation chamber. The observation chamber was made on the microscope cover glass by silicon grease in the shape of two rectangular compartments, connected by a very narrow channel so the contents of both compartments could be slowly mixed. One compartment was filled with 30-50 µl of the vesicle solution and the other compartment was filled with 10-15 µl of 0.186-18.6 mM nonionic surfactant octaethylene-glycoldodecylether ($C_{12}E_8$).

RESULTS AND CONCLUSION

Without the addition of the $C_{12}E_8$ surfactant, the observed vesicles remained spherical for several hours and the shape transformation of protrusion was usually slow [2]. Adding the surfactant $C_{12}E_8$ to the solution increases the speed of the vesicle shape transformation. The necks of the bead-like protrusion usually seem to be thicker in the presence of the $C_{12}E_8$.

Addition of $C_{12}E_8$ to the compartment next to the vesicles solution in the final limiting concentrations 0.031-0.31 mM initially caused appearance of long tube-like protrusions in vesicles and their transformation by constant decreasing of the average curvature in one hour. Eventually this lead to stable mother vesicles with daughter inclusions [5].

Addition of $C_{12}E_8$ to the compartment next to the vesicle solution in the final limiting concentration 3.7 mM resulted in transformation of a spherical vesicle to be a bean-like structure, but eventually the membrane disintegrated and the vesicle disappeared.

At even higher limiting concentration of 6.2 mM, slow diffusion of $C_{12}E_8$ caused transformation in the direction of initial increasing and later decreasing of the average membrane curvature, until the vesicle began to shrink gradually and finally disappeared. This probably happened due to using two-compartment observation chamber and slow diffusion of $C_{12}E_8$.

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Electrotransfer of Genes into Injured Mouse Muscle

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INTRODUCTION

Muscle injury is followed by a natural regeneration process which can be helped by conventional treatment but this is many times not sufficient enough to enhance muscle regeneration efficiently. Use of the growth factors involved in the natural regeneration process [1] and the subsequent electrotransfer of the encoding genes could be a way of treating different pathologies involving the altered muscle, from the simple sport injury to myopathic muscles.

METHODS

Injury model: A mouse model of contusion injury (high energy blunt trauma) on the gastrocnemius muscle was used [2]. Autologous conditioned serum produced by mechanical and physical stimulation of blood was injected at the site of injury into the muscle. Control mice were injected with saline at the site of injury.

Assessment of the recovery from injury was done by: 1. Satellite cell activation counts at 30 and 48 hours post injury; 2. Histological evaluation of the regenerating myofibers especially at days 6, 7 and 8 after injury. The autologous conditioned serum was tested for growth factors of interest in the regeneration process.

RESULTS

A preliminary study to determine appropriate muscle regeneration parameters on a contusion model in the mouse gastrocnemius muscle has been conducted. We have tested autologous conditioned serum and we found that compared to the control (saline): 1. the percentage of activated satellite cells was greater at both 30 (84% increase) and 48 (85% increase) hours after injury and 2. on days 6 to 8, the mean diameter of the centronucleated cells was significantly greater (87,42 % ± 0,93 vs 57,08 %± 4,77 had diameters > 25 μm)

Based upon these results, the secondary objectives of this study are to monitor the electrotransfer of a reporter gene (ßgal) after subjecting the gastrocnemius muscle to the same contusion model used. We will monitor whether the efficiency of transfer is affected by the fact that the muscle is in an altered state with oedema and inflammation when the transfection of the gene takes place. Subsequently, the aim is to use one of the known genes of interest in regeneration and transfect this with electric pulses. The previously tested parameters of regeneration will be tested to detect the expected acceleration of muscle regeneration.

CONCLUSIONS

The electroporation of genes of interest into the damaged muscle could be a promising technique to reduce the time to recovery from sports injuries and might also have an application in muscle pathologies such as myopathies.

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Estimation extent of tumor necrosis in Lewis Lung Carcinoma (LLC) after electrochemotherapy

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INTRODUCTION

Electrochemotherapy is an anti-tumour treatment, which combines the use of cytotoxic drug and strong electric fields [1, 2].

The aim of this study was to evaluate necrosis amount in LLC tumors after electrochemotherapy in dependence of pulse strength and cytotoxic drug used.

MATERIALS AND METHODS

C57Bl mice bearing Lewis Lung Carcinoma were used in this study. The animals were divided in to five different groups. Electrochemotherapy was performed using cytotoxic drug bleomycin (dose 5 mg/kg) or cyclophosphamide (dose 100 mg/kg). Three minutes after bleomycin i.v. injection and 3, 30 or 60 min. after cyclophosphamide i.v. injection tumors were subjected to 8 square wave electric pulses with strength of 1100, 1300 or 1500 V/cm and duration of 100 μs. Treated tumors were excised three days after the treatment. Tumor slices were stained with standard hematoxylin and eosin method. Histological preparations were examined under microscope equipped with a digital photo camera. From each slice five photographs were taken from randomly chosen areas. The overall extent of necrosis in each tumor was determined using 100 photographs.

RESULTS

The treatment with bleomycin (5 mg/kg) alone was ineffective and did not result in significant increase of extent of tumor necrosis in compared to control group (Fig. 1).

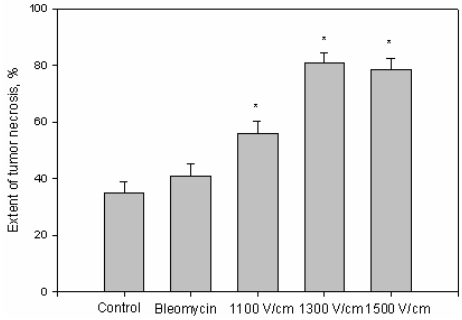

Figure 1: Extent of tumor necrosis in treated LLC tumors using bleomycin

Electrochemotherapy, combining bleomycin (5 mg/kg) with an application of 8 electric pulses (1100, 1300 or 1500 V/cm, 100 μs, 1 Hz) resulted in significant increase ($p<0.05$) in the extent of tumor necrosis. The highest extent of tumor necrosis was obtained when the tumors were treated with bleomycin (5 mg/kg) and 1300 V/cm strength electric pulses. In this group the percent of necrosis was 83.3%.

The injection alone of cyclophosphamide (100 mg/kg) had a significant effect on the tumors necrosis compared to control group (Fig. 2).

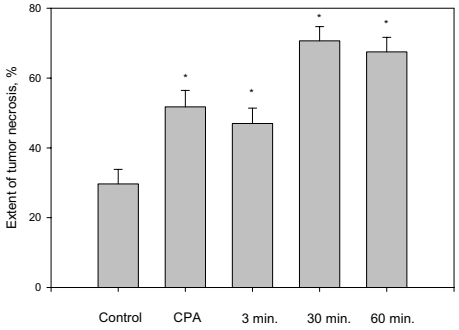

Figure 2: Extent of tumor necrosis in treated LLC tumors using cyclophosphamide.

The best electrochemotherapeutic result using cytotoxic drug cyclophosphamide was obtained when 1300V/cm electric pulses were used 30 min. after injection of the drug. In this case the percent of necrosis was 70.6%.

CONCLUSION

On the basis of the investigations performed, the following conclusions were made.

Electrochemotherapy resulted in significant increase in extent of tumor necrosis when compared to the treatment with bleomycin alone. The highest extent of tumor necrosis was obtained when 1300 V/cm strength electric pulses were used.

A significant increase of the extent of tumor necrosis was observed when 1300 V/cm on combined treatment with cyclophosphamide electric pulses were strength electric pulses were applied 30 min. after the drug injection.

y happened due to using two-compartment observation chamber and slow diffusion of $C_{12}E_8$.

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A model of *in vivo* electric field distribution in muscle and skin determined by finite element method

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INTRODUCTION

In our study finite element numerical models were used in order to describe electric field distribution in biological tissues during electroporation: rat muscle with and without skin and also skin fold. Tissue dielectric properties change during the application of high voltage pulses. We observed relation between tissue conductivity and electric field intensity in the tissue. We modeled electropermeabilization dynamics as a discrete process with sequences of static finite element models, meaning that in each model the tissue conductivity was determined based on electrical field distribution from previous model in the sequence. First we determined threshold values in the model of rat muscle and skin fold, than we used these results in the model of rat muscle with the skin. Our task was to interpret results of *in vivo* experiments on rat muscle and skin. Therefore, we compared computed current and electric field intensity to the results obtained in *in vivo* experiments.

MATHEMATICAL METHODS AND ELECTROPORATION PROCESS MODELING

A condition in homogeneous and isotropic structure describes Laplace's equation:

$$\nabla^2 u = 0 .$$

During the electroporation process, properties of biological tissue are changing, as described by equation in differential form:

$$div(\sigma \cdot \frac{\partial u}{\partial n}) = 0 .$$

where σ is a function of electric field intensity (E).

In the first step of the modeling part of our study we built tissue models based on finite element method (FEM).

Electroporation is a dynamic process, meaning that tissue properties are changing during the constant voltage pulse. In this study we modeled dynamics of electroporation as a discrete process represented with sequence of static models, where each of them describes process at one discrete interval. In each static model in sequence the tissue conductivity was determined based on electric field distribution from previous model in sequence, as described in equation: $\sigma(k) = f(E(k-1))$, where k is the number of static model in the sequence.

Conditions during the high voltage pulse delivery are presented in Figure 1. Model input is applied voltage pulse and model outputs are distribution of electric field intensity and total electrical current in specific sequence. In that way we determined a discrete course of electrical current, which describes an electric current in the tissue during the applied voltage pulse. Electric current is increasing during the pulse, because electric resistance of the model is decreasing. Namely, decreased skin resistance correlates with increased skin permeability.

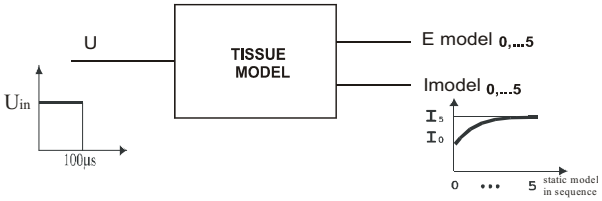

Figure 1: Modeling of tissue response during high voltage pulse application

RESULTS

Based on finite element model and sequential analysis we determined functional dependency of tissue conductivity on electric field intensity and specific threshold values for skin and muscle. We found very good agreement of 51CrEDTA uptake and electric field intensity relationship between *in vivo* experiments on muscle with and without skin and the results obtained with finite element models (Figure 2). In our study we confirmed that electric field distribution in the tissue depends on applied voltage (Uin), model and electrode geometry, and also electrical properties of the tissue. We determined threshold values of electric field intensity for muscle tissue. Threshold values in muscle with skin are obtained at higher values of applied external voltage because of high electric resistance of skin. Obtained results are comparable with data from the literature.[1, 2]

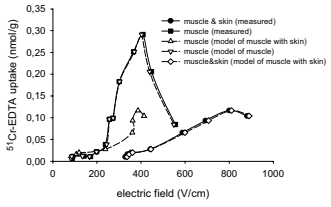

Figure 2: Measured and calculated 51Cr-EDTA uptake in different tissues.

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Electrochemotherapy with Cisplatin or Bleomycin: Clinical Experience in Dogs with Perianal Tumors

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INTRODUCTION

Electrochemotherapy is an antitumour therapy that utilizes locally delivered short intense direct current electric pulse to the tumour nodule and chemotherapy [1]. The aim of the present study was to evaluate the electrochemotherapy treatment of perianal adenomas and adenocarcinomas of different sizes in dogs.

METHODS

Between March 2000 and July 2003, 12 patients with all together 26 measurable cutaneous were included in the study.

Before treatment, the dogs were sedated. Electrochemotherapy consisted of intratumoural administration of cisplatin at doses ranging from 0.5 to 2.5 mg/100 mm³ tumour volume, followed by application of electric pulses to the tumour. If the treatment with cisplatin as the first chemotherapeutic drug was not successful, electrochemotherapy with bleomycin was performed, with bleomycin doses ranging from 2 to 3 mg/100 mm³ tumour volme.

Square wave electric pulses of 100 μsec, 910 V amplitude (amplitude to electrode distance ratio 1300 V/cm), frequency 1 Hz were delivered through two parallel stainless steel electrodes (thickness 1mm; width 7mm, length 8mm, with rounded tips and an inner distance between them 7 mm) with an electroporator Jouan GHT 1287. Each run of electric pulses was delivered in two trains of four pulses, with 1s interval, in two perpendicular directions. The interval between cisplatin or bleomycin administration and the application of electric pulses was 1-2 min. Good contact between the electrodes and the skin was assured by depilation and application of a conductive gel to the treatment area and/or electrodes. The treatment response was evaluated according to WHO classification, in which complete response (CR) is defined as absence of any trace of the lesion, partial response (PR) is defined as more than 50% reduction of the lesion, no change (NC) is defined as less than 50% reduction of the lesion and less than 25% enlargement, and progressive disease (PD) if the lesion enlarged for more than 25%.

RESULTS

Responses to treatment were assessed 4 weeks after therapy; in 91.6% of all tumours treated with electrochemotherapy objective responses (OR) were obtained (CR = 37.5%, PR=54.1%), 8.4% of tumours were in NC and none of the tumours went in the PD. Observation period was between 1 and 42 months. At the end of observation period 87.5% of tumours were in OR (CR=62.5%, PR=25%), 12.5% in NC and none of them in PD.

According to the tumour size, tumours were divided into two subgroups. In the first subgroup (tumours with volume less than 1 cm³) an excellent response to the treatment after 4 weeks was achieved: 100% of tumours were in OR (CR= 53.3%, PR=46.7%). At the end of observation period even more tumours responded completely (86.8%) and the rest of them were in PR (13.2%).

In the second subgroup (tumours with volume bigger than 1 cm³) 77.7% of OR (CR=11%, PR=66,6%) and 24.2% of NC were obtained after 4 weeks. At the end of observation the percentage of CR increased to 22.2%, 55% of tumours were in PR and only 22.2% in NC. These results are indicating that response in bigger tumours to electrochemotherapy is not as pronounced as in smaller tumors.

To achieve above-mentioned results, average number of electrochemotherapy sessions was 1.6 and in range from 1 to maximum 4 sessions per patient. There were no major local or general side effects noted.

CONCLUSIONS

This study shows that electrochemotherapy with cisplatin or bleomycin is an effective treatment of perianal tumours in dogs. The advantages of this therapy are it's simplicity, short duration of treatment sessions, low chemotherapeutic doses, and insignificant side effects, as well as the fact that it can be performed on outpatient basis.

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Capacitance measurement for electroporation of a planar lipid bilayer

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INTRODUCTION

Electroporation is characterised by formation of structural changes within the cell membrane, which are caused by the presence of electrical field. These changes, named "pores", increase the plasma membrane permeability, and enable ions and molecules to enter the cell [1]. This phenomenon is referred to as electropermeabilization and is used to introduce various substances into the cell. Electroporation is used in biotechnology, biology and medicine. For example it is used for water and food conservation, genetherapy, transdermal drug delivery and electrochemotherapy [2].

Lipid bilayer is an artificial model of the cell membrane and can be considered as a small part of total cell membrane. It can be made of only one type of lipid molecules, their mixtures or even lipids and proteins with inserted biosensors [3]. Different compositions of lipid bilayers describe selected properties of real cell membrane. While searching for optimal parameters of electroporation, parameters as breakdown voltage, current, conductance, capacitance and shape of the signal are varied [4]. In this work we describe a method suitable for the measurement of the transmembrane voltage discharge and the estimation of lipid bilayer capacitance.

CAPACITANCE MEASUREMENT

Setup for electroporation of planar lipid bilayer consists of a signal generator, Teflon chamber and device for measurement membrane current and voltage. All sampled signals are analyzed in Matlab™ programme.

One of the most important parts of the signal generator is an analogue switch. It disconnects electrodes from the output of the signal generator and switches them to the 1 MΩ resistor. The switch is fast, it turns off the signal generator in 2 ns.

System (membrane, chamber, Teflon sheet) voltage decay trough the resistor is measured in two steps and from discharge traces the lipid bilayer capacitance is calculated. In the first step we measure capacitance of the system without lipid bilayer (C_{SYS}). The resistance of oscilloscope probes (1 MΩ) and the special resistor (1 MΩ) are connected in parallel (as shown on Figure 1) and are combined into resistance R_{SYS}. Therefore the resistance R_{SYS} is approximately 500 kΩ.

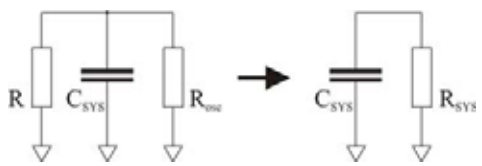

Figure 1: Schematic of electric circuit of the system without lipid bilayer.

In the second step the capacitance of the system with lipid bilayer and salt solution (C_{SBLM}) is measured. Equivalent resistor (R_{SYS}) is still 500 kΩ, because the resistance of the lipid bilayer is greater than 10^8 Ω [3] (Figure 2).

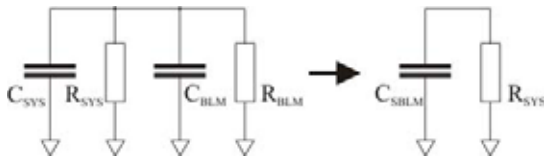

Figure 2: Schematic of electric circuit of the system with planar lipid bilayer and salt solution.

From the discharge trace the time constant $\tau = C \cdot R_{SYS}$ is determined. The capacitance of lipid bilayer is the difference between C_{SYS} and C_{SBLM}:

$$C_{BLM} = C_{SYS} - C_{SBLM} = \frac{\tau_{SYS}}{R_{SYS}} - \frac{\tau_{SBLM}}{R_{SYS}}$$

In the literature the specific capacitance c_{blm} is commonly used for presenting the results due to differences in membrane surface areas.

$$c_{blm} = \frac{C_{BLM}}{A_{BLM}} = 4 \cdot \frac{C_{BLM}}{\pi \cdot d^2}$$

Where A_{BLM} is the surface of lipid bilayer and d is the diameter of the hole in the thin Teflon layer.

CONCLUSION

Setup for electroporation of planar lipid bilayer is suitable for elementary testing of new electroporation protocols. It enables measuring of lipid bilayer properties like the capacitance of lipid bilayer and poration of lipid bilayer with the signals of arbitrary shape.

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The effect of recombinant subunit recPMSB from *Mycoplasma synoviae* hemagglutinin VlhA on MQ-NCSU macrophage cell line

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INTRODUCTION

The drop of productivity caused by *Mycoplasma spp.* in chicken is of significant economic importance. Our main research subject, *Mycoplasma synoviae*, causes avian synovitis, with symptoms ranging from swollen hock joints to pneumonia [1]. Our research is focused on *Mycoplasma* lipoprotein recognition via Toll Like Receptor 2 (TLR-2). *Mycoplasma* lipoproteins, most notably haemagglutinins, are potent imunogens and thus cause a broad immunological response [2]. Our goal is to provide insights into the signal network of cytokines and their effect on immunocompetent cells when chickens become infected with *Mycoplasma sp.*

EFFECTOR MOLECULE recPMSB

In order to provide sufficient amounts of pure *M. synoviae* haemagglutinin VlhA, a 6xHis Tag *E. coli* plasmid construct was previously constructed [3] which contains the N-terminal VlhA subunit termed MSPB. The presence of His tag allows extraction and purification of the recombinant protein (recPMSB) on a Ni-chelated agarose column. *In-vivo,* the N-terminus of the protein undergoes acylation post-translationally. As already shown, the acylated part plays a major role in the recognition of lipoproteins via TLR-2 in some related *Mycoplasma sp.* The structure of the recombinant construct does not allow acylation because of the 6xHis Tag attached to the N-terminus. In this model, any effect caused by recPMSB would indicate a non-acyl, protein sequence based response.

CELL TYPE AND TEST METHODS

MQ-NCSU, a macrophage cell line transformed by the Marek`s disease virus, was used as the target for various concentrations recPMSB. (Table 1) Macrophages play an initial role in the contact of the host organism with a foreign antigen and initialize a system wide response through signal molecule excretion.

Table 1: Concentration of recPMSB used in proliferation assays (BrdU) and signal molecule (NO^-) excretion tests

recPMSB conc. (μg/ml)								
0,0	0,01	0,1	0,25	0,5	1,0	2,0	4,0	10,0

The relative effect of different concentrations of recPMSB on proliferation was measured by the incorporation of BrdU, which is a thymidine analog in DNA synthesis. A subsequent quantitative ELISA reaction with a peroxidase tagged anti-BrdU antibody was performed.

NO^- excretion under different concentrations of recPMSB was used as a general model for signal molecule excretion from macrophages when in contact with reactive antigens.

NO^- was measured with the Griess reaction where the amount of the excreted NO^- is determined colorimetrically.

RESULTS

The recPMSB protein caused no significant change in proliferation at lower concentrations (0,01-1μg/ml). However, at higher concentrations (>2 μg/ml) it induced cell death in comparison to the negative control (0μg/ml). We hypothesised that this cell death might correlate with the signal response. However, the NO- excretion test showed no significant increase of NO- production in comparison to the negative control.

We concluded that the recPMSB protein does not induce any NO^- signal response and that the increased cell death at higher concentrations is not related with NO^-cell signalling.

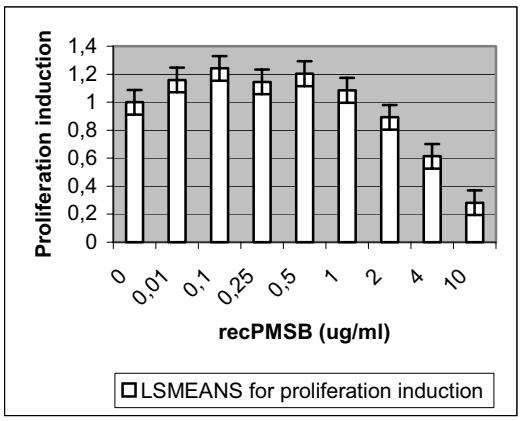

Figure 1: The effect of different concentrations on the proliferation of MQ-CSU

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Modification of the Rate of Endocytosis of Cells in Culture by Low Intensity Pulsed Electric Fields

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INTRODUCTION

Cellular endocytosis is a basic process critical for many aspects of cellular functions including nutrition, signaling, transepithelial transport or control of the membrane surface area. Endocytosis vesicles are constantly formed at the cell surface. They detach in the cytoplasm and fuse with other internal vesicles (endosomes). Membrane surface area is restored by exocytosis, which corresponds to the fusion of internal vesicles to the plasma membrane.

Fluid phase endocytosis is one of the main types of endocytosis and corresponds to the engulfment of liquid media in the endocytic vesicles formed at the cell surface. Any soluble molecule present in the external medium reaches the cell inside, but hydrophilic non permeant molecules remain inside the vesicular compartment.

MATERIALS AND METHODS

We studied the effects of low intensity pulsed electric fields on the fluid phase endocytosis of three different cell lines : B16-F1 cells, a metastatic variant of the B16 murin melanoma, DC-3F cells, a spontaneously transformed Chinese hamster lung fibroblast, A253 cells, a human head-and-neck carcinoma.

The fluid phase endocytosis was monitored by the uptake of Lucifer Yellow which is a fluorescent small molecular weight dye that doesn't enter into the cells by simple diffusion, but that can reach the vesicule compartment of the cell by means of the endocytosis.

Cells were grown in monolayers on coverslips and were placed into special glass dishes between 2 parallel electrodes. The geometry of the exposure dishes ensures a fair homogeneous electric field over the whole cell monolayer. The glass dishes were placed into a dry thermostatic Block Heater that ensures a constant temperature in the samples of 30±1°C. Both the theoretical considerations about temperature augmentation and the experimental determinations of the temperature during and at the end of the incubation time deny a significant temperature change.

Cells were exposed to bipolar symmetrical square electric pulses to avoid net charge displacements and their consequences (changes in pH around the electrodes, electrophoretic migration of cell surface proteins, etc.). Pulses were delivered for periods of time ranging from 10 minutes to 1 hour. The effects of pulses of various amplitudes (in the range of 1.3 to 8V/cm), various pulse durations (75 to 580 μs) and various repetition frequencies (50 to 400 Hz) were examined.

With the aim of defining if the resting transmembrane voltage of the cells plays a role in the electric field-induced acceleration of the endocytosis, the cells were exposed to pulsed electric fields in mediums with different pH and various ionic compositions. The Lucifer Yellow solution was prepared in various mediums based on the traditional PBS (phosphate buffered saline solution) but differing from this by the relative proportion of potassium ions compared to the sum of the sodium and potassium monovalent ions at constant molarity of monovalent cations

RESULTS

We observed an increase of the fluid phase endocytotic rate when the cells are exposed to bipolar symmetrical square electric pulses of a field intensity (positive part) above a threshold which is cell strain-dependent (between 1.3 and 1.6 V/cm for the B16-F1 cells, 1.3 and 2.6 V/cm for the DC-3F cells and 1.9 and 2.3 V/cm for the A253 cells).

No influence of the pulse duration or the repetition frequency (within the ranges quoted above) was noticed.

In the research of the factors influencing this effect and of the responsible mechanisms, we found that the effect of the electric pulses is not modified by incubation medium pH changes in the range of pH between 6 and 8.

Variations of the extracellular concentration of potassium, which should affect the resting transmembrane cellular difference of potential do not change the fluid phase endocytosis rate. But the confirmation that the variation of the transmembrane voltage is or is not the cause of the cellular response of acceleration of endocytosis under electrical exposure is still under investigation. Further experiments are thus in progress.

ACKNOWLEDGEMENTS

This work was supported by grants of CNRS, IGR, France Télécom and Marie Curie Fellowship Programme.

Electrotransfer in the liver of a doxycycline-inducible system to regulate the secretion of endostatin

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INTRODUCTION

Studies have shown that gene transfer by electroporation to liver is efficient and leads to high expression of the transferred genes [1, 2]. There are several therapeutic implications of this – including the use as protein factory host for patients with deficiency syndromes or production of therapeutic proteins e.g. cytokines.

Martel-Renoir et al. [3] have shown that *in vivo* electroporation of plasmid coding for secreted endostatin (KSA) into muscle results in detectable levels of protein in the blood stream. In addition a regulatory system, which ensures tight regulation of the protein expression by administration of doxycycline, was used.

We will utilise the same system to electrotransfer plasmid coding for the secreted endostatin to the liver. The liver is highly vascularised, plays a critical role in the metabolism and is a major component in the production of proteins. The liver is therefore a good target as a protein factory.

We will use the Tet-on regulatory system, which ensures tight regulation of the transferred genes. The system composes of three elements: a plasmid, pBI, with the endostatin protein under the control of a tTA responsive promoter, a plasmid encoding the transactivator protein, tTA, which in the presence of doxycycline induces transcription, and a plasmid encoding a powerful silencer protein, tTS, which in the absence of doxycycline blocks the expression.

MATERIALS AND METHODS

Preliminary data on feasibility of DNA transfer in the liver using a new device (Cliniporatorä) have been performed using luciferase. The plasmid, pCMV-luc, was prepared using a Qiagen Endofree Giga plasmid kit. Two days after the electrotransfer, the treated liver lobe was collected, disrupted and lysed. Luciferase activity was measured on the lysate supernatant using a Berthold Lumat LB 9507 luminometer.

For electrotransfer, 6 weeks old female C57B/C mice were shaved at the abdomen just prior to treatment. Mice were anaesthetised with ketamine (100 mg/kg body weight and xylazine (40 mg/kg body weight) and 80 µg pCMV-luc in a volume of 100 µl were injected retro-orbitally. An insertion in the abdomen was made and the largest left liver lobe was laid out. A 5 mm plate electrode was fitted around the lobe and conductive gel was applied to ensure contact. 3 min after plasmid injection, the lobe was pulsed using combinations of high voltage and low voltage pulses.

For KSA testing, the mice will be treated as described above. Blood samples will be collected via the retro-orbital sinus in isoflurane-anaesthetised mice and secreted endostatin in the serum will be detected by ELISA.

RESULTS

For optimisation of conditions for DNA electrotransfer in liver, four different combinations of HV and LVs were used. 8 LVs (50 ms, 20V/cm, 1 Hz) were sufficient for DNA electrotransfer in the liver. No significant effect was observed after introducing a HV pulse before the LVs.

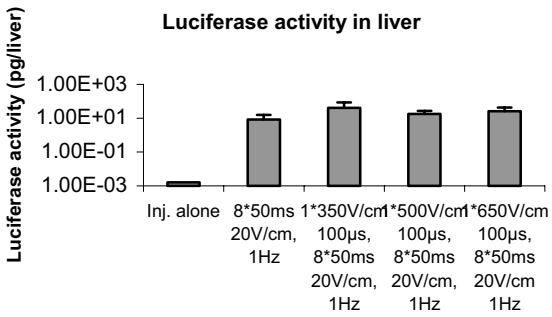

Figure 1: Schematic of electric circuit of the system without lipid bilayer.

For the KSA experiment, mice will be divided into three groups: the control group, which is injected with the plasmids, but not electropulsated, group 1, which is treated and receive doxycycline from day 0 and group 2, which is treated, but first receive doxycycline after 1 week. ELISA test will be performed when all samples are collected.

CONCLUSION

Adequate conditions for electro gene transfer in the liver have been established, even though HV pulse does not seem to ameliorate the efficacy of the LV pulses tested.

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The electroporation pulse delivery algorithm

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INTRODUCTION

In many therapies including treatment of cancer, it is necessary to enable the entrance of the chemotherapeutic drug into the cells. The combined treatment in which delivery of chemotherapeutic drug is followed by application of high voltage electric pulses (electroporation pulses) has been termed electrochemotherapy. This treatment allows increased entry of the drug molecules into the cell and hence greater efficiency of the therapy.

Treatment of tumors located close to the heart muscle presents a threat to life of the patient because the use of electroporation pulses could lead to fibrillation of the heart. Appearance of heart fibrillation is dependent on strength of induced electroporation pulses which must be higher than the threshold for fibrillation [1]. However, fibrillation of the heart can be easily provoked if electroporation pulses are induced in the so called vulnerable period [1]. Moreover, fibrillation of the heart could also elicit electrical stimulation coinciding with heart arrhythmias [1].

METHODS

We built the real-time electroporation pulse delivery algorithm based on the analysis of a single lead electrocardiogram. The algorithm can be split into three major parts (Figure 1): the learning process, the detection process and the decision-making process.

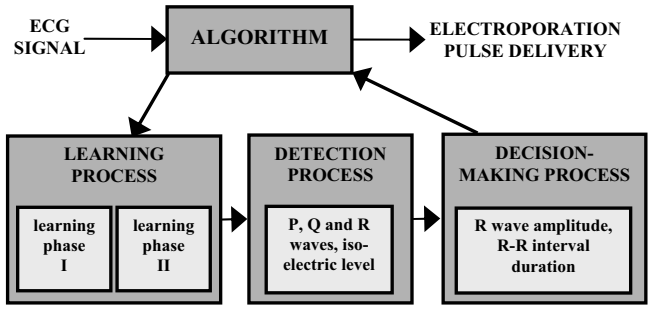

Figure 1: Major structure of the algorithm.

During the learning process certain threshold parameters are automatically set according to characteristics of the particular ECG signal. During the detection process the location of P, Q and R wave, isoelectric level and the amplitude of the R wave are determined. The decision-making process is based on evaluating variations in R wave amplitude and R-R interval. Based on this a decision is made whether the electroporation pulse can be delivered or not. A pulse is delivered if the characteristic points are correctly identified and if the current amplitude of the R wave and heart rate (indicated by the R-R interval) do not deviate significantly from the normal heartbeat.

We evaluated the performance of the algorithm using ECG records of the standard Long-term ST database (LTST DB) [2] and some experimentally acquired ECG signals with and without disturbances. Measures of reliability of the algorithm were calculated based on a beat-by-beat comparison of the results of the algorithm with true events.

RESULTS

The algorithm is an effective tool for QRS detection and electroporation pulse delivery. A large proportion of correctly detected (98.8% in average) and an insignificant proportion of incorrectly detected QRS complexes (0.0008% in average) were achieved with ECG records from the LTST DB. Moreover, a large proportion of correctly delivered (91.8% in average) and on the other hand only small proportion of incorrectly delivered electroporation pulses (0.0023% in average) were achieved. Practically ideal results were obtained on experimentally acquired ECG signals without disturbances. Good results were also obtained on experimentally acquired ECG signals with disturbances. The ability of the correct QRS detection and electroporation pulse delivery of the algorithm is degraded only in presence of disturbances due to body movements which are similar to QRS complex. The performance of the algorithm is improved with regard to the preliminary algorithm [3].

CONCLUSIONS

The algorithm for online synchronization of electroporation pulse delivery with ECG presents a significant improvement over the existing practice of electroporation pulse delivery with respect to the safety of the patient. The algorithm allows the electroporation pulses to be delivered only outside the vulnerable period of the heart beat and prevents the pulses from being delivered in case of the appearance of different heart arrhythmias.

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Induction of apoptosis by electrotransfer of positively charged proteins (Cytochrome C, Histone H1) in cancer cells

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INTRODUCTION

Programmed cell dead, called apoptosis is an important process for the maintenance of homeostasis in multicellular organisms. Virtually all cells in such organisms have the capacity to undergo this form of dead. The release of Cytochrome C from mitochondria is certainly involved in the processes of apoptosis [1]. On the other hand it was established that the basic protein Histone H1 detach the cytochrome C from the surface of liposomes containing acidic phospholipids.[2]. Both Cytochrome C and Histone H1 have unusually well-conserved primary structure. This fact suggests that these proteins could be involved in many biological functions.

MATERIAL AND METHODS

Leukemia cell lines as K562, SKW3 and HL60 and human lymphocytes were used.

Compounds and chemicals: Cytochrome C (horst heart), Sigma, HistoneH1 isolated from mouse liver with MW 21 kD, Methylated Albumin (mBSA) was prepared by method given in [3].

Electroporation protocol: 100 ml with 106cells/ml were electroporated in Bio Rad chamber, 600 V/cm, 1 rectangular pulse with duration 5 ms.

Viability test – by MTT assay [4]

DNA fragmentations were checked by Flow cytometry and gel electrophoresis.

RESULTS

On the Fig. 1 is given the representative histograms from Flow cytometry analysis after the treatment with different positively charged proteins.

CONCLUSION

It was shown that cancer cells, electroloaded with Cytochrome C, Histone H1 or mBSA undergo the processes of apoptosis.

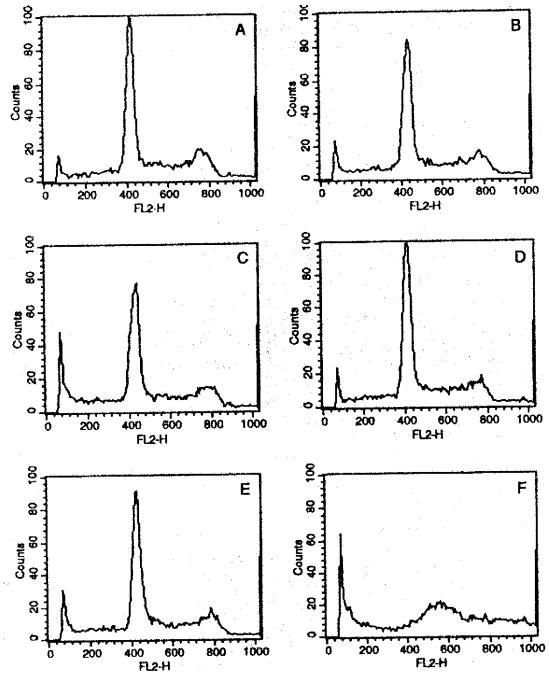

Figure 1: Flow cytometry of K562: A) control minus pulse B) control + pulse C) Histone H1 + pulse, D) FCS + pulse, E)cytochrome C + pulse, F) mBSA + pulse

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DNA transfer in skin by means of electroporation.

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INTRODUCTION

Skin is an interesting target for DNA transfer because of its accessibility and the large surface offered for such gene transfer. Besides viral methods, chemical and physical methods have been developed to enhance gene expression in the skin [1]. Electroporation seems particularly effective to improve transfection after intradermal and topical delivery without any significant alteration of skin structure.

However, the effect of electrical parameters and electrode design on the efficacy of transfection and mechanism of enhancement has not been studied systematically so far. But it has been shown for muscle tissue that efficient cell electrotransfection can be achieved using combinations of high and low-voltage pulses [2].

MATERIALS AND METHODS

Two reporter genes were used. Plasmid coding for green fluorescent protein (GFP) was used to localise its expression in skin. Plasmid coding for protein luciferase was used to quantitatively measure the expression in the skin. We also performed a kinetic study of the expression. We were measuring the protein's activity from 1 up to 25 days after the electroporation.

The side effects of electric pulses delivered by Cliniporator on the skin were also studied by standard mehods used to assess the effect of skin treatment (transepidermal water loss, chromametry and histology [3]).

All the above experiments were performed on rat skin. We used Wistar rats, 8-10 weeks old, whose hair was removed before the experiment. Plasmid was administered intradermally, each injection contained 50ug of DNA.

RESULTS

The control epidermis showed some autofluorescence of the hair follicles but no fluorescence in the dermis. When only one high voltage (HV) pulse or only one low voltage (LV) pulse was applied, the expression of GFP remained very low both in the epidermis and to a lesser extent in the dermis. Using a combination of a HV pulse and a LV pulse, the expression of GFP was enhanced.

In luciferase experiments we tried a number of different electroporation protocols, all consisted of one HV followed by one LV pulse. In Figure 1 we can see two of our best protocols compared to old electroporation protocols found in literature and control groups.

Kinetic study showed the highest expression with the lowest results in the control group the second day after the electroporation. After that the luciferase concentration drops rapidly.

Tolerance study showed higher transepidermal water loss shortly after electroporation, then after 30 minutes TEWL values went back to normal. Histology shows no alteration of skin, neither does chromametry, although we could see some redness of the skin with the naked eye.

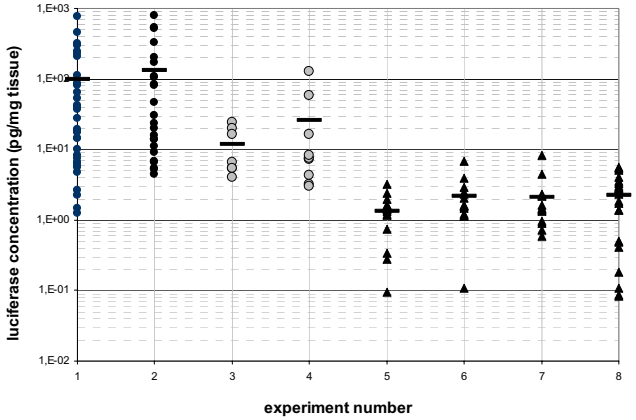

Figure 1: Luciferase expression after different electroporation protocols (exp.1: HV:1000V/cm (100us), LV: 140V/cm (400ms), exp.2: HV:700V/cm (100us), LV: 200V/cm (400ms), exp.3: 6x 1750V/cm (100 us), 8Hz, exp.4: 6x 250V/cm (20 ms), pause 980ms, exp.5: only intradermal injection, no electroporation, exp.6: only 1 HV, exp.7: only 1 LV, exp.8: skin sample.

CONCLUSIONS

The combination of one high followed by one low voltage pulse enhances cell electrotransfection in skin. Compared to the protocols found in literature, our protocols seem to be better. Tolerance study showed that this kind of pulsing, with the voltage amplitudes we used, has no major side effects on skin.

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In vitro Transfer of DNA into Cells Using Combinations of Electric Pulses

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INTRODUCTION

DNA transfer into the cells using electric pulses is a widely accepted and popular method. It is generally agreed that the main action of the electric pulses in DNA electrotransfer is cell permeabilization *in vitro* [1] as well as *in vivo* [2]. It has also been suggested that the negative charge of DNA might lead to its electrophoretic movement by electric forces thereby bringing the DNA in contact with the cell membrane and facilitating its transfer [1,2]. Working on the project "*In vitro* and *in vivo* transfer of DNA into cells using combinations of electric pulses" with a new machine (Cliniporator™, IGEA Italy) we are trying to optimise DNA transfer on cultured cells in suspension using different high-voltage (HV) and low-voltage (LV) pulse combinations.

MATERIALS AND METHODS

In our experiments we are using DC-3F, B16F1 and B16F10 cells which were grown in Minimum Essential Medium (MEM, GIBCO, UK) with added Fetal bovine serum (8%) and antibiotics (Penicillin and Streptomycin).

After trypsination with trypsin-EDTA (GIBCO, UK), cells were centrifuged for 10 min at 1000 rpm and resuspended in Spinner Minimum Essential Medium (S-MEM, GIBCO BRL, UK). A 50µl droplet of cells suspension (106 cells) with DNA (8µg of pCMV Luc coding for the firefly luciferase under the control of the CMV promoter) was placed between two parallel plate stainless steel electrodes 2 mm apart and different combinations of high-voltages and low-voltages pulses were applied with Cliniporator™ (IGEA, Italy). After pulse delivery cells were incubated for 5 min at room temperature and then cultured for two days in a 100 mm diameter Petri dish. Two days after DNA electrotransfer cells were collected and lysed, and Luciferase activity was measured on the lysate supernatant using a Berthold Lumat LB 9507 luminometer.

RESULTS

We have made some series of experiments where we were using different voltage number and duration of HV as well as different voltage, number and duration of LV pulses. Also we were using the combinations of pulses with and without lag between the HV and LV pulses.

Preliminary results show that efficient transfection is already obtained using the minimal voltage settings for the LV pulses (12V). In fact, oscilloscope measurements showed that the actual voltage delivered during LV pulses is still lower (4V). Then the ratio of applied voltage to electrodes distance is 20 V/cm. Thus good tranfection levels are obtained using 8 LV pulses of 12.5 ms at 20 V/cm. No difference between the absence or the presence of a 1 s lag between the HV and the LV pulses has been noticed in the experiments performed until now.

The influence of the number, duration and field strength of HV are still under investigation.

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RESULTS

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Real time QRS detection

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INTRODUCTION

QRS detectors are a part of most microprocessor based ECG systems used for monitoring of vital functions of patient in intensive care, they are part of ECG holters, defibrilators and other medical equipment. There is no ideal QRS detector that could detect 100% of QRS complexes, even when tested on referent signal from standard ECG signal data bases. During the recording often the morphology of the ECG signal is essentially changing due to physiological changes in heart activity or because of different external influences like different types of interference and/or artefacts.

DETECTOR STRUCTURE

Common structure of the QRS detector consists of a preprocessing stage (linear filtering and nonlinear transformation) and a processor stage. The preprocessing stage is used for noise reduction and for emphasizing of the spectral content of the QRS complex frequencies in range from 5-30Hz. For linear filtering, usually FIR type band pass filter, is implemented. For further preproccesing nonlinear transformation (nonlinear operations) on the signal like squaring, limiting, averaging, rectifying etc. are implemented. The processor is making classification of potential QRS complexes based on defined conditions which the signal must suffice. Conditions include a fixed or an adaptive detection threshold(s), a predicted RR interval, defined refractory period in which the detector is making no detection and which is used for avoiding of manifold QRS detections, etc.

ALGORITHMS

We analyzed and realized five algorithms for QRS complex detection: Ahlstrom – Tompkins, Balda's, Okada's, Pan – Tompkins, and Engelse – Zeelenberg's. We found the Engelse – Zeelenberg's algorithm most appropriate for this application because of minimal phase delay between moment of the appearance of QRS complex and time of detection of the QRS complex, approx. 180ms (at 8051 platform).

The detector has high value of positive predictability (PP=99,50%) compared to a little bit smaller sensitivity (S=98,42%). Engelse – Zeelenberg algorithm is simple, it does not need a lot of processing power and is suitable for realization in 8051 microcontroller family.

The algorithm is realized at MatLab first, at higher level of abstraction, than is translated into C language and adapted for specifics of the microcontroller. Digital filter id FIR type, band pass filter with central frequency of 20 Hz. Filter has a notch at 50Hz.

RESULTS

We have developed a system for real time QRS detection. This device is intended for use in medical equipment that needs high positive predictability of QRS detector for example the electroporator. Further improvement of algorithm will enable the decrease of the phase delay and increasing of the detector sensitivity.

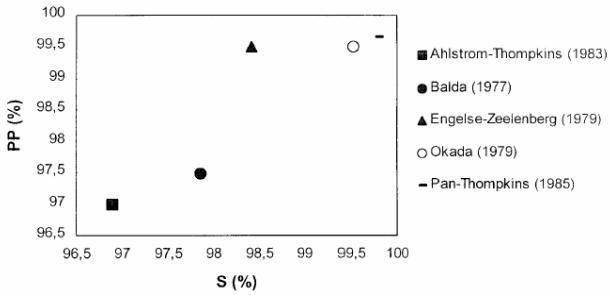

Figure 1: Comparison of characteristics of different QRS detectors.

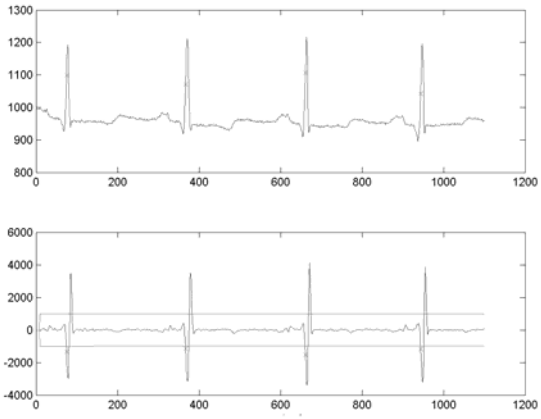

Figure 2: ECG (MITDB record 100) with QRS anotations and filter output signal with two thresholds.

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Electrofusion in Hypoosmolar Conditions of Monocytes and of B16F1 Cells

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INTRODUCTION

Monocyte-subset CD14 $CD11c^+$ is the precursor population of dendritic cells (DC). Dendritic cells are antigen–presenting cells, important for eliciting immunity against cancer. So, DC loaded with tumor antigen can induce protective immune response, and one way to do it is electrofusion of DC with tumor cells in hypoosmolar low-conductivity fusion-buffer (120-180 μS/cm) [1,2] The aim of the present study was to optimize the electrofusion procedure and, consequently, to prove the formation of hybridoma cells. We are reporting the results of electrofusion of B16F1 cells or monocytes alone under specific conditions.

METHODS

Monocytes cultered for three days in RPMI and supplemented with 10% FCS and 800 U/ml hGCSF or B16F1 cells, cultured for two days in EMEM with 10% FCS were used. Before the fusion, the cells were stained with lipid soluble dyes DiI and DiO. The concentration of cells in fusion chamber was $2x10^7$ cells/ml for monocytes and $3x10^6$ cells/ml for B16F1. The volume of electrofusion chamber was 250 μl with a gap 0,2 mm between electrodes. The electrofusion was performed with Multiporator Eppendorf (Germany) in 25%, 50% or 75% hipoosmolar buffer with a low-conductivity (120-180 μS/cm). The square wave pulses of 40 V (2000 V/cm) with the duration of 30 μs for monocytes or 50 μs for B16F1 repeated twice were used. The duration period of pre- and post- alignment was 30 s, while the applied voltage was 10 V (500 V/cm) and frequency 2 MHz. The fusion rate as well as the viability were determined by flow-cytometry. To determine the viability of the cells propidium iodide was used.

RESULTS

The viability of monocytes before fusion was 85%. The viability after fusion in 25% hypoosmolar fusion buffer was 80% and the fusion rate under these conditions was 1%.
The viability of monocytes after fusion in 50% hypoosmolar fusion buffer was 53% and the fusion rate under the same conditions was 24%. The viability of monocytes in 75% hypoosmolar buffer was not determined, but the fusion rate was 15%.
The B16F1 were fused in 50% hypoosmolar buffer resulting in a fusion rate of 25%, while the viability was 40%.

CONCLUSIONS

These results show that electrofusion in hypoosmolar conditions is successful. It resulted in up to 24 % fusion rate for monocytes and 25% fusion rate for B16F1 cells. The procedure has to be further optimized to be useful for preparation of tumor vaccines.

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