This book covers the latest and most to date information on electroporation, electropermeabilisation and pulsed power fields. It provides concise introduction to the topic covering basic knowledge and biomedical applications that anyone in the field needs to understand in order to be successful.

Electroporation-Based Technologies and Treatments

EBTT

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2nd edition

Editors:
Peter Kramar
Damijan Miklavčič

Book of the

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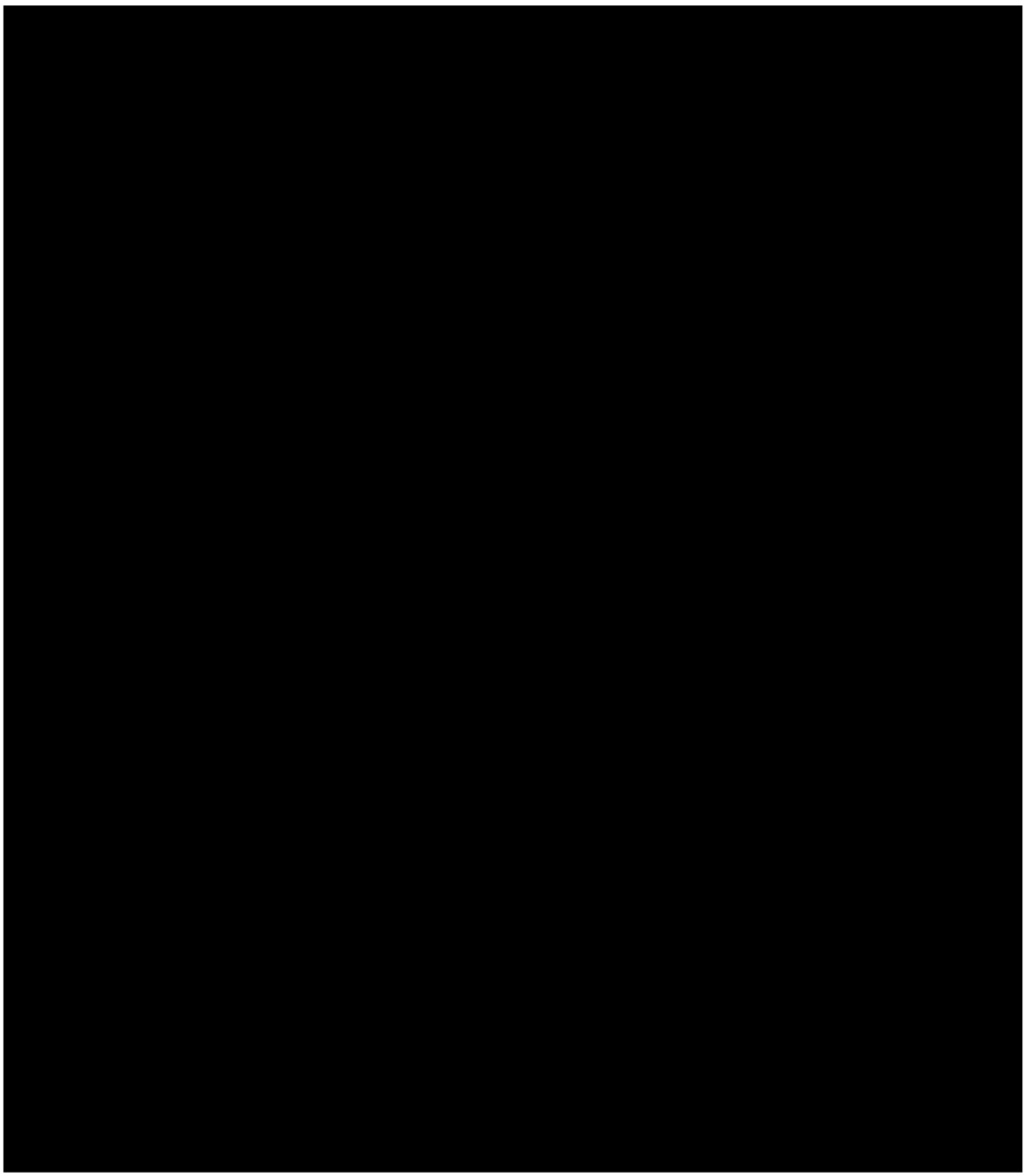

Preface to the 2nd edition of the Book of Electroporation-Based Technologies and Treatments

Originally the Book of EBTT was written by the faculty members together with our "standard" invited lecturers of the Electroporation Based Technologies and Treatments Scientific Workshop and Postgraduate Course in response to unmet needs of students and novices attending the school. For many years the development of the field of electroporation seemed to be much too fluid and rapidly developing to be able to capture the essence and provide course material that would last more than one-to-two years before it becomes outdated.

Rather than trying to capture all the new developments we decided to follow the structure of the school and only provide the "stable" part of the background knowledge and leave the fast-developing area in biomedicine like gene and cell-based therapies, DNA vaccination specifics as well as soft tissue and in particular cardiac ablation by irreversible electroporation to "mature" before we include it as chapters in the book. Electrochemotherapy, however, seemed to be mature enough and was included as a whole: the preclinical and clinical chapters.

The first edition of the Book of EBTT was first published in electronic and printed form in English in 2019, followed by translation into Spanish by Felipe Maglietti and published as printed version in 2020 and finally translated into Portuguese by Jean Carlos dos Santos da Luz published in electronic form in 2022. Electronic book is available free of charge at http://book.ebtt.org and was downloaded English 721, Spanish 498 and Portuguese 173 times, whereas printed versions in English and Spanish were published and distributed at 500 each. With the current numbers and translations, I think the Book of EBTT can be considered as a text book of electroporation.

In the 2nd edition of the Book of EBTT we follow a similar concept. We updated most of the existing chapters and included some new ones which I think you will greatly appreciate. Although you will find some chapters longer than the others – this in no way should be understood or seen as longer chapters being important than the short ones. It is rather the result and image of how different we people are in terms of writing. All chapters nevertheless provide a valuable list of references that should direct you to reading original research and review papers should you require more in-depth knowledge on the specific topic. Also, after attending the school in person, and being in contact with the leading experts in the field, you should feel comfortable in contacting the respective chapter author if you need further information or clarification on the topic.

This book may also serve as an invitation to join us next November in Ljubljana, Slovenia.

See you in Ljubljana next November!

Damijan Miklavčič

Damijan Miklavčič

Chapter 1 7
Cell in Electric Field – Induced Transmembrane Voltage
Tadej Kotnik
Chapter 2 21
The Biophysics of Cell Membrane Electroporation
Lea Rems
Chapter 3 42
Electric Properties of Tissues and their Changes during Electroporation
Damijan Miklavčič and Bor Kos
Chapter 4 65
Insights into Lipid Membranes Electroporation from MD Simulations
Mounir Tarek
Chapter 5 104
Nanoscale and Multiscale Membrane Electrical Stress and Permeabilization
P. Thomas Vernier
Chapter 6 119
Electroporation and electropermeabilisation - pieces of puzzle put together
Lluis M Mir
Chapter 7 124
Nucleic acids electrotransfer *in vitro*
Marie-Pierre Rols
Chapter 8 143
Gene electrotransfer *in vivo*
Maja Čemažar
Chapter 9 158
Prophylactic and Therapeutic Applications of Gene Electrotransfer
Richard Heller
Chapter 10 184
Electrochemotherapy from bench to bedside: principles, mechanisms and applications
Gregor Serša
Chapter 11 201
Electrochemotherapy in clinical practice; Lessons from development and implementation - and future perspectives
Julie Gehl

Chapter 12 **207**
Development of devices and electrodes
Damijan Miklavčič, Matej Reberšek

Chapter 1

Cell in Electric Field – Induced Transmembrane Voltage

Tadej Kotnik

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

The cell and its plasma membrane

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

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

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

From the geometrical point of view, the cell can be characterized as a geometric body (the cytoplasm) surrounded by a shell of uniform thickness (the membrane). For cells in suspension, the simplest model of the cell is a sphere surrounded by a shell. For augmented generality, the sphere can be replaced by a spheroid (or an ellipsoid), but in this case, the requirement of uniform thickness complicates the description of the shell substantially. If its inner surface is a spheroid or an ellipsoid, its outer surface lacks a simple geometrical characterization, and vice versa.[1] Fortunately, this complication does not affect the steady-state voltage induced on the plasma membrane of such cells, which can still be determined analytically.

Spheres, spheroids, and ellipsoids may be reasonable models for cells in suspension, but not for cells in tissues. No simple geometrical body can model a typical cell in a tissue, and furthermore every cell generally differs in its shape from the rest. With irregular geometries and/or with cells close to each other, the induced voltage cannot be determined analytically, and thus cannot be formulated as an explicit function. This deprives us of some of the insight available from explicit expressions,

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

but using modern computers and numerical methods, the voltage induced on each particular irregular cell can still be determined quite accurately.

Resting transmembrane voltage

Under physiological conditions, a voltage in the range of –90 mV up to –40 mV is always present on the cell membrane [2,3]. This voltage is caused by a minute deficit of positive ions in the cytoplasm relative to the negative ones, which is a consequence of the transport of specific ions across the membrane. The most important actors in this transport are: (i) the Na-K pumps, which export Na^+ ions out of the cell and simultaneously import K^+ ions into the cell; and (ii) the K leak channels, through which K^+ ions can flow across the membrane in both directions. The resting transmembrane voltage reflects the electrochemical equilibrium of the action of these two mechanisms, and perhaps the easiest way to explain the occurrence of this voltage is to describe how the equilibrium is reached.

The Na-K pump works in cycles. In a single cycle, it exports three Na^+ ions out of the cell and imports two K^+ ions into it. This generates a small deficit of positive ions in the cytoplasm and a gradient of electric potential, which draws positive ions into the cell, and negative ions out of the cell. But at the same time, the pump also generates concentration gradients of Na^+ and K^+, which draw the Na^+ ions into the cell, and the K^+ ions out of the cell. The K^+ ions are the only ones that possess a significant mechanism of passive transport through the membrane, namely the K leak channels, and through these the K^+ ions are driven towards the equilibration of the electrical and the concentration gradient. When this equilibrium is reached, the electrical gradient across the membrane determines the resting transmembrane voltage, which is continually present on the membrane.

The unbalanced ions responsible for the resting transmembrane voltage represent a very small fraction of all the ions in the cytoplasm, so that the osmotic pressure difference generated by this imbalance is negligible. Also, the membrane acts as a charged capacitor, with the unbalanced ions accumulating close to its surface, so that the cytoplasm can in general be viewed as electrically neutral.

Induced transmembrane voltage

When a biological cell is placed into an electric field, this leads to a local distortion of the field in the cell and its vicinity. As outlined in the introductory section of this paper, due to the low membrane conductivity, in the vicinity of the cell the field is concentrated in the cell membrane, where it is several orders of magnitude larger than in the cytoplasm and outside the cell. This results in a so-called induced transmembrane voltage, which superimposes to the resting component. In the following subsections, we describe in more detail the transmembrane voltage induced on cells of various shapes and under various conditions. In each considered case, the principles of superposition allow to obtain the complete transmembrane voltage by adding the resting component to the induced one.

Spherical cells

For an exposure to a DC homogeneous electric field, the voltage induced on the cell membrane is determined by solving Laplace's equation. Although biological cells are not perfect spheres, in theoretical treatments they are usually considered as such. For the first approximation, the plasma membrane can also be treated as nonconductive. Under these assumptions, the induced transmembrane voltage $\Delta\Phi_m$ is given by a formula often referred to as the (steady-state) Schwan's equation [4],

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

where E is the electric field in the region where the cell is situated, R is the cell radius, and θ is the angle measured from the center of the cell with respect to the direction of the field. Voltage is proportional to the applied electric field and to the cell radius. Furthermore, it has extremal values at the points where the field is perpendicular to the membrane, i.e. at $\theta = 0°$ and $\theta = 180°$ (the "poles" of the cell), and in-between these poles it varies proportionally to the cosine of θ (see Fig. 1, dashed).

The value of $\Delta\Phi_m$ given by Eq. (1) is typically established several μs after the onset of the electric field. With exposures to a DC field lasting hundreds of microseconds or more, this formula can safely be applied to yield the maximal, steady-state value of the induced transmembrane

voltage. To describe the transient behavior during the initial microseconds, one uses the first-order Schwan's equation [5],

$$\Delta\Phi_{\mathrm{m}} = \frac{3}{2} E R \cos\theta \left(1 - \exp(-t/\tau_{\mathrm{m}})\right), \tag{2}$$

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

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

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

In certain experiments *in vitro*, where artificial extracellular media with conductivities substantially lower than physiological are used, the factor 3/2 in Eqns. (1) and (2) decreases in value, as described in detail in [6]. But generally, Eqns. (2) and (3) are applicable to exposures to sine (AC) electric fields with frequencies below 1 MHz, and to rectangular electric pulses longer than 1 μs.

To determine the voltage induced by even higher field frequencies or even shorter pulses, the dielectric permittivities of the electrolytes on both sides of the membrane also have to be accounted for. This leads to a further generalization of Eqns. (2) and (3) to a second-order model [7-9], and the results it yields will be outlined in the last section of this paper.

Spheroidal and ellipsoidal cells

Another direction of generalization is to assume a cell shape more general than that of a sphere. The most straightforward generalization is to a spheroid (a geometrical body obtained by rotating an ellipse around one of its radii, so that one of its orthogonal projections is a sphere, and the other two are the same ellipse) and further to an ellipsoid (a geometrical body in which each of its three orthogonal projections is a different ellipse). To obtain the analogues of Schwan's equation for such cells, one solves Laplace's equation in spheroidal and ellipsoidal coordinates, respectively [10-12]. Besides the fact that this solution is by itself somewhat more intricate than the one in spherical coordinates, the generalization of the shape invokes two additional complications outlined in the next two paragraphs.

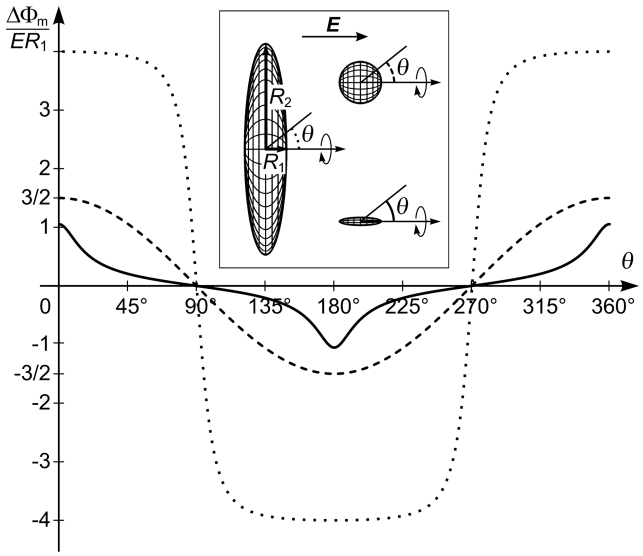

Figure 1: Normalized steady-state $\Delta\Phi_m$ as a function of the polar angle θ for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: an oblate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

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

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

can be found in [10]. Fig. 1 compares the transmembrane voltage induced on two spheroids with the axis of rotational symmetry aligned with the direction of the field, and that induced on a sphere.

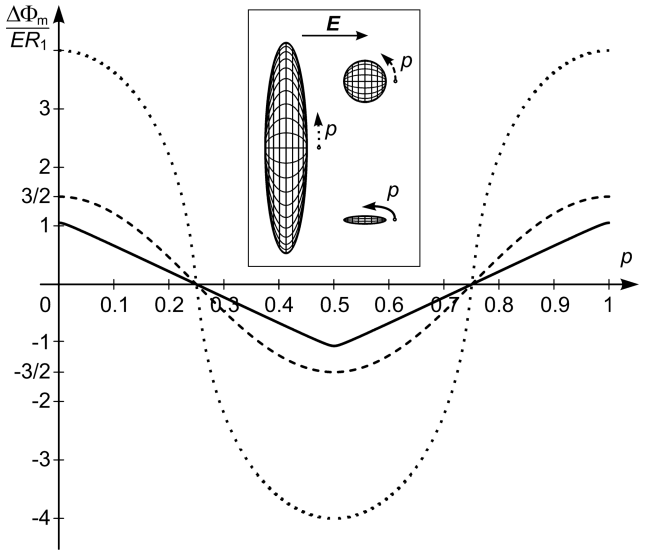

Figure 2: Normalized steady-state $\Delta\Phi_m$ as a function of the normalized arc length p for spheroidal cells with the axis of rotational symmetry aligned with the direction of the field. Solid: a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Dashed: a spherical cell, $R_2 = R_1 = R$. Dotted: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

For nonspherical cells, it is generally more revealing to express $\Delta\Phi_m$ as a function of the arc length than as a function of the angle θ (for a sphere, the two quantities are directly proportional). For uniformity, the normalized version of the arc length is used, denoted by p and increasing from 0 to 1 equidistantly along the arc of the membrane. This is illustrated in Fig. 2 for the cells for which $\Delta\Phi_m(\theta)$ is shown in Fig. 1, and all the plots of $\Delta\Phi_m$ on nonspherical cells will henceforth be presented in this manner.

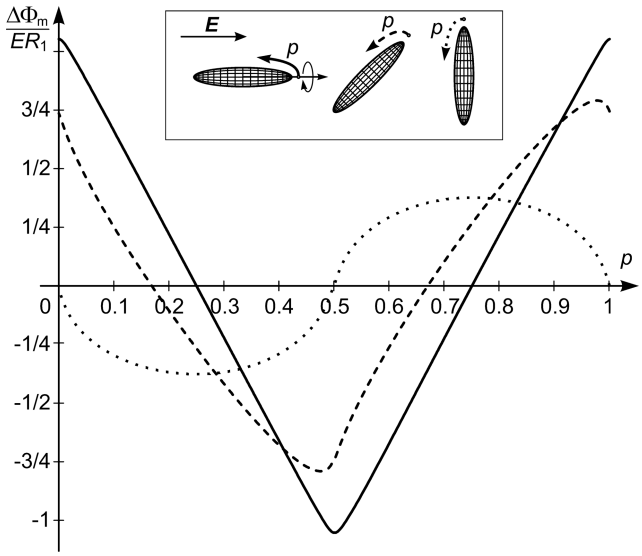

Figure 3: Normalized steady-state $\Delta\Phi_m(p)$ for a prolate spheroidal cell with $R_2 = 0.2 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

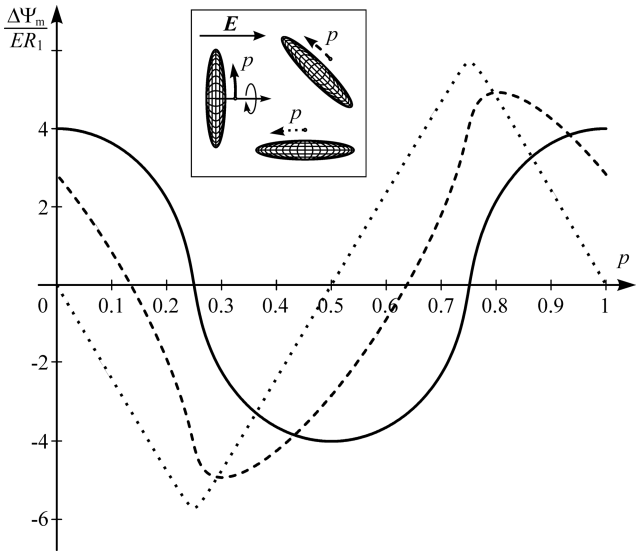

Figure 4: Normalized steady-state $\Delta\Phi_m(p)$ for an oblate spheroidal cell with $R_2 = 5 \times R_1$. Solid: axis of rotational symmetry (ARS) aligned with the field. Dashed: ARS at 45° with respect to the field. Dotted: ARS perpendicular to the field.

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

Irregularly shaped cells

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

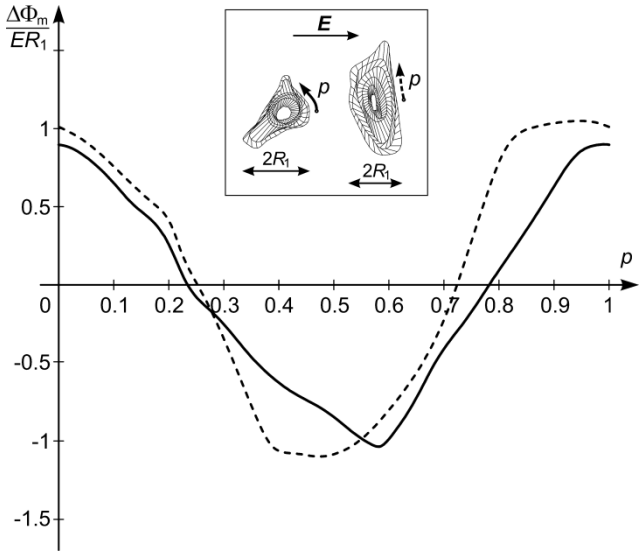

Figure 5: Normalized steady-state $\Delta\Phi_m(p)$ for two irregularly shaped cells growing on the flat surface of a Petri dish.

Cells in dense suspensions

In dilute cell suspensions, the distance between the cells is much larger than the cells themselves, and the local field outside each cell is practically unaffected by the presence of other cells. Thus, for cells representing less than 1 % of the suspension volume (for a spherical cell with a radius of 10 μm, this means up to 2 million cells/ml), the deviation of the actual induced transmembrane voltage from one predicted by Schwan's equation is negligible. However, as the volume fraction occupied by the cells gets larger, the distortion of the local field around each cell by the presence of other cells in the vicinity becomes more pronounced, and the prediction yielded by Schwan's equation less realistic (Fig. 6). For volume fractions over ten percent, as well as for clusters and lattices of cells, one has to use appropriate numerical or approximate analytical solutions for a reliable analysis of the induced transmembrane voltage [15,16]. Regardless of the volume fraction they occupy, as long as the cells are suspended, they are floating freely, and their arrangement is rather uniform. Asymptotically, this would correspond to a face-centered cubic lattice, and this lattice is also the most appropriate for the analysis of the transmembrane voltage induced on cells in suspension.

For even larger volume fractions of the cells, the electrical properties of the suspension start to resemble that of a tissue, but only to a certain extent. The arrangement of cells in tissues does not necessarily resemble a face-centered lattice, since cells can form specific structures (e.g. layers). In addition, cells in tissues can be directly electrically coupled (e.g. through gap junctions). These and other specific features of the interactions between cells in tissues and electric fields will be considered in more detail in the paper that follows this one.

High field frequencies and very short pulses

The time constant of membrane charging (τ_m) given by Eq. (3) implies that there is a delay between the time courses of the external field and the voltage induced by this field. As mentioned above, τ_m (and thus the delay) is somewhat below a microsecond under physiological conditions, but can be larger when cells are suspended in a low-conductivity medium. For alternating (AC) fields with the oscillation period much longer than τ_m, as well as for rectangular pulses much longer than τ_m, the amplitude of the induced voltage remains unaffected. However, for AC

fields with the period comparable or shorter than τ_m, as well as for pulses shorter than τ_m, the amplitude of the induced voltage starts to decrease.

To illustrate how the amplitude of the induced transmembrane voltage gets attenuated as the frequency of the AC field increases, we plot the normalized amplitude of the induced voltage as a function of the field frequency. For a spherical cell, the plot obtained is shown in Fig. 6. The low-frequency plateau and the downward slope that follows are both described by the first-order Schwan's equation, but the high-frequency plateau is only described by the second-order model [7-9], in which all electric conductivities and dielectric permittivities are accounted for.

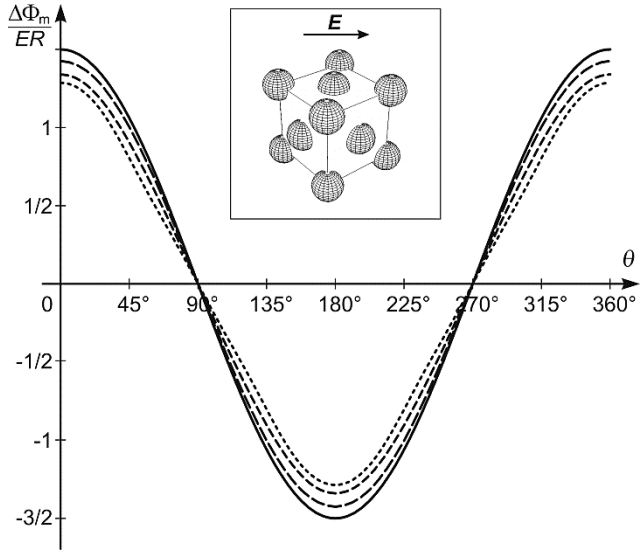

Figure 6: Normalized steady-state $\Delta\Phi_m(\theta)$ for spherical cells in suspensions of various densities (intercellular distances). Solid: The analytical result for a single cell as given by Eq. (1). Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (with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

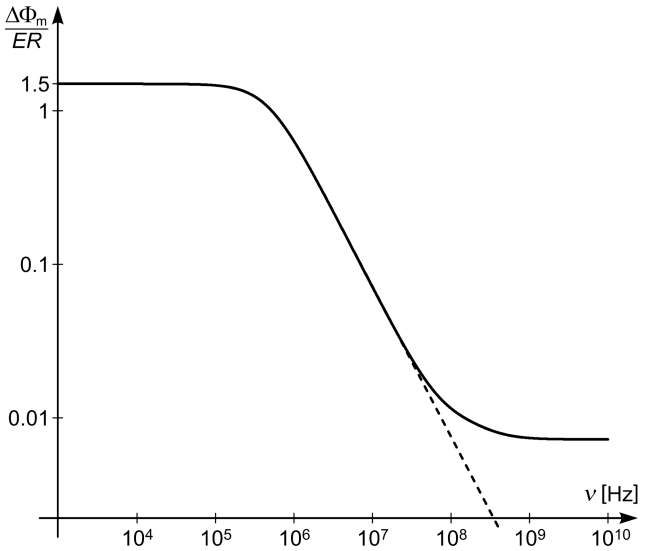

Figure 7: The amplitude of normalized steady-state $\Delta\Phi_m$ as a function of the frequency of the AC field. The dashed curve shows the first-order, and the solid curve the second-order Schwan's equation. Note that both axes are logarithmic.

With field frequencies approaching the GHz range, or with pulse durations in the nanosecond range, the attenuation of the voltage induced on the cell plasma membrane becomes so pronounced that this voltage becomes comparable to the voltage induced on organelle membranes in the cell interior (Fig. 7). In certain circumstances, particularly if the organelle interior is electrically more conductive than the cytosol, or if the organelle membrane has a lower dielectric permittivity than the cell membrane, the voltage induced on the membrane of this organelle can temporarily even exceed the voltage induced on the plasma membrane [17]. In principle, this could provide a theoretical explanation for a number of recent reports that very short and intense electric pulses (tens of ns, millions or tens of millions of V/m) can also induce electroporation of organelle membranes [18-20].

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Chapter 2

The Biophysics of Cell Membrane Electroporation

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Abstract: Exposure of cells to electric field induces a supraphysiological transmembrane voltage, which leads to structural and chemical alteration of the cell membrane that is associated with increased membrane permeability to ions and molecules. This chapter first discusses the known molecular mechanisms of this increased membrane permeability, including lipid pores, lipid peroxidation, and membrane protein denaturation, as well as the associated influence of the cytoskeleton network. Following a historical perspective, both experimental and theoretical support is given for these mechanisms. The chapter then discusses computational simulations of electroporation and the associated transmembrane molecular transport at the single cell level.

Early experiments and models

The preceding Chapter 1 by Kotnik explained how all cells in their physiological state maintain a nonzero resting voltage on their membrane and how exposure of cells to an external electric field leads to an induced transmembrane voltage (TMV) that superimposes onto the resting TMV. If the summed TMV exceeds a few 100 mV in absolute value, the electrical properties of the membrane change considerably with respect to the physiological state. In particular, one can experimentally observe a large increase in membrane permeability to ions (and other molecules) and consequently an increase in membrane electrical conductivity. If the TMV is not elevated for too long, these changes are reversible within

second or minutes (and sometimes longer). While today we term this phenomenon "electroporation", in the earliest studies it was given various names, among them "reversible electrical breakdown".

After the first experimental report on reversible electrical breakdown in a frog nerve fibre in 1958 [1], similar phenomena were observed in membranes of other animal cells, plant cells, bacteria, yeast, and vesicles extracted from cells [2–5]. In parallel, studies in planar lipid bilayers[3] showed that electric pulses resulting in transmembrane voltage exceeding a few 100 mV can lead to stochastic membrane rupture and, under certain conditions, reversible electrical breakdown [6,7].

In fact, the first theoretical models proposed to explain the observed membrane behaviour were based on experiments in planar lipid bilayers. These models proposed that lipid bilayer rupture is a consequence of severe membrane deformation (thinning) and/or rippling (see [8] for a review). At a critical transmembrane voltage this deformation became unstable, leading to membrane rupture [9–11]. These models treated the membrane as a continuous medium and were based on physical laws developed for bulk materials. Among many shortcomings, these models failed to explain the reversibility of the electrical breakdown. The first model that considered the molecular structure of the lipid bilayer proposed that reversible electrical breakdown results from lipid phase transition due to electric field [12]. The model was later revised [13]; however, this model never became widely accepted.

In 1975, it was independently proposed by Litster and Taupin *et al.* that small aqueous pores can form in a lipid bilayer spontaneously (albeit this is a rare event) [14] and when osmotically stretching the surface area of the bilayer [15]. Two years later, Kinosita and Tsong proposed that formation of nanoscale pores of some type in the membrane of erythrocytes is responsible for their haemolysis following exposure to electric pulses [16]. Another two years later, a group of researchers at the Russian Academy of Sciences in Moscow published 7 back-to-back papers (the first of them being [6]), in which they proposed a theory on how a sufficiently high TMV promotes creation of pores in planar lipid bilayers that afterwards expand leading to stochastic bilayer rupture. In 1982, Neumann *et al.* [17] further proposed that formation of pores in lipid domains of the cell membrane is responsible for intracellular uptake of exogenous DNA following exposure to electric pulses, and accordingly named the phenomenon "electroporation". By mid 1980,

[3] Planar lipid bilayers are model membrane systems, where a lipid bilayer is formed over a micrometer hole in a thin hydrophobic material, separating two electrolyte-filled chambers.

most researchers agreed that formation of aqueous pores in the lipid bilayer (lipid pores for short) is responsible for reversible and irreversible electrical breakdown in planar lipid bilayers and in cell membranes [18–20]. The main hypotheses underlying these early pore models still form the basis for today's models.

Lipid pores

The theory on how lipid pores are formed is the following [6]. To form a pore in a lipid bilayer, an energy barrier needs to be overcome, because it is energetically unfavourable to bring hydrophilic water molecules into the hydrophobic lipid bilayer core. For small pores this energy barrier was estimated as the energy cost to form the edge of the pore. Experimental evidence suggested that pores first form in a hydrophobic configuration, where the lipid tails are directly exposed to water molecules. However, as the pore somewhat expands in size, it becomes energetically favourable for the lipid heads to reorient and move into pore wall to shield the lipid tails from water. Such so-called hydrophilic pore configuration then allows also ions and other molecules to cross the bilayer. The presence of nonzero TMV decreases the energy barrier for pore creation, which is related to the fact that lipids with low dielectric permittivity become replaced by water with high dielectric permittivity [21]. Due to Maxwell stress at the lipid-water interface inside the pore, the TMV also provides a force that drives pore expansion [22,23]. The proposed energy landscape of a pore in the pore radius space, for different values of TMV, is shown in Fig. 1.

The mathematical description of the pore energy landscape was developed mainly based on indirect measurements of current-voltage relationship in planar lipid bilayers [22,24,25]. In 2000s, advances in supercomputing enabled computational simulations of lipid bilayers at the molecular level using molecular dynamics (MD) simulations [26,27], see also Chapter 4.[4] MD simulations confirmed the main molecular picture proposed in earlier models, that is that aqueous pores are indeed formed by sufficiently high TMV in a way that resembles the hydrophobic-hydrophilic pore transition. In addition, an inverse

[4] MD simulation generates a trajectory of atomic positions and velocities in the considered molecular system (e.g., a hydrated lipid bilayer) based on integrating the Newton's equations of motion. The forces acting between the individual atoms are derived from a potential energy function of interatomic interactions, the latter being described through a given force field consisting of the equations chosen to model the potential energy and their associated parameters.

exponential dependence between the average pore creation time and the TMV was observed (Fig. 2), confirming that the TMV decreases the energy barrier for pore creation and exponentially increases the pore creation rate [28].[5]

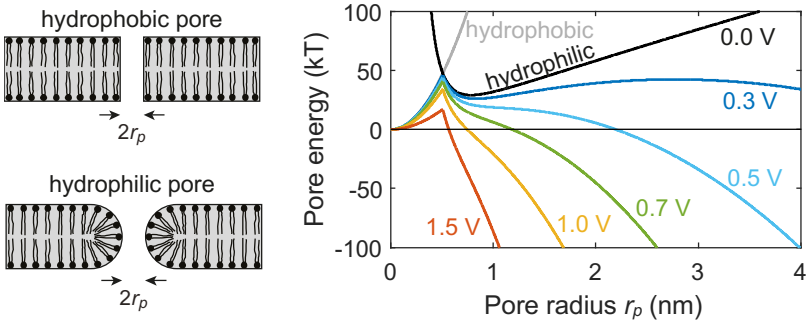

Figure 1: Hydrophobic and hydrophilic pore configuration and the corresponding free energy landscape computed for different values of the induced TMV, according to the model proposed in [24].

MD simulations, paralleled with experiments in model lipid systems, offered further insight into the lipid bilayer electroporation process and demonstrated that certain types of lipids favour and some disfavour poration [30]. Both the architecture of lipid headgroups and tails is important, as well as the presence and concentration of cholesterol, and the lipid phase state (see [31] for a review). A recent MD study investigated electroporation in coarse-grained models of membranes containing >60 different lipid types, mimicking the realistic composition of mammalian cell membranes [32]. Pores in such complex lipid composition do not form homogeneously along the membrane, but colocalize with domains that have specific features. The most important feature that favours poration was found to be the local density of polyunsaturated lipids (Fig. 3). The most important features disfavouring poration were found to be the local density of gangliosides that have large sugar headgroups, lipids with fully saturated tails, and cholesterol. In addition, pores in such asymmetric bilayers form more easily under hyperpolarizing TMV, which could be attributed to greater

[5] Early models of lipid pores proposed that poration time scales with $\exp(-\Delta\Phi_m^2)$, where $\Delta\Phi_m$ is the TMV. In contrast, MD simulations are closer to the scaling $\exp(-\Delta\Phi_m)$, which is also supported by recent experiments in model lipid bilayers [29].

compressibility of the bilayer when the external electric field is directed from the extracellular towards the intracellular side of the membrane.

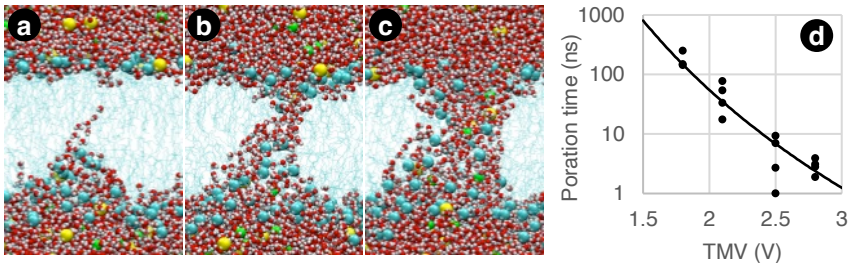

Figure 2: Main steps in pore creation promoted by supraphysiological TMV, as observed in MD simulations. First, water molecules (red and white spheres) start protruding into the lipid bilayer core (a) and eventually bridge with water molecules at the opposite side of the bilayer (b). At this first stage, the lipid tails (cyan lines) are directly exposed to water molecules resembling a hydrophobic pore configuration. As the pore expands further, lipid headgroups (cyan spheres) reorient into the pore wall, resembling the hydrophilic pore configuration, and ions (yellow and grene spheres) start moving through the pore (c). The time it takes to create a pore decreases exponentially with the TMV (d). The results (unpublished) were obtained with MD simulations of a pure palmitoyl-oleoyl phosphatidylcholine (POPC) bilayer.

MD provided detailed information that is to date not accesible with any experimental method. One thing that MD demonstrated very clearly is that lipid pores are not stable in the absence of an external electric field. In simulations, pores were shown to close within a few to a few 100 ns [28]. Cleverly designed experiments in giant unilamellar vesicles (GUVs) confirmed such pore closure time [33]. The short lifetime of lipid pores is in striking contrast to the resealing time of cells membranes following electroporation, which typically takes several seconds or minutes [34]. Therefore, additional mechanisms of increased cell membrane permeability have been proposed – lipid peroxidation, membrane protein damage, and stabilization of pores by the actin cytoskeleton [35]. These mechanisms are further detailed in the following three subsections.

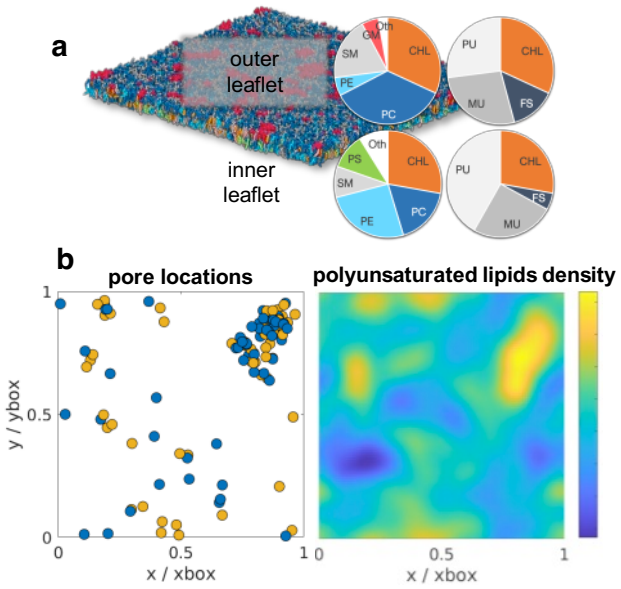

Figure 3: Coarse grained molecular model containing more than 60 different lipid types, mimicking the lipid composition of an average mammalian cell membrane (a). Pore in such membrane do not form homogenouly along the membrane but collocalize with domains enriched in polyunsaurated lipids (b). Results adopted from [32] with permission.

Lipid peroxidation

Lipid peroxidation refers to oxidative degeneration of lipid tails, especially those that are polyunsaturated. This damage is initiated when a free radical, such as HO$^{\bullet}$ or HOO$^{\bullet}$, atacts an allylic or bis-allylic hydrogen next to the double bond in the fatty acid chain, leading to the formation of a lipid radical L$^{\bullet}$ (Fig. 4a). L$^{\bullet}$ reacts with molecular oxygen, which is highly abundant in the membrane's interior, to form a lipid peroxide radical LOO$^{\bullet}$. The latter can further abstract hydrogen from another lipid to form a hydroperoxide LOOH, *i.e.*, the primary peroxidation product. The lipid neighbor with abstracted hydrogen can further propagate the damage to adjacent lipid, initiating a chain reaction, the result of which is creation of highly oxidized lesions (patches) in the lipid membrane (Fig. 4b). Hydroperoxide lipids can then convert directly or indirectly into secondary products of oxidation – lipids with truncated tails ending with either an aldehyde or carboxylic group [36].

The fact that exposure of cells or pure lipid bilayers to electric pulses can lead to lipid peroxidation has been experimentally shown already in the 1990s [37,38]. Lipid peroxidation can be promoted by reactive oxygen species (ROS) already present in the solution before the delivery of electric pulses [39]. Moreover, it has been shown that electric pulses can induce extracellular (electrochemical) as well as intracellular ROS generation [40]. The latter is a consequence of cellular response to electric pulse exposure and can be detected specifically at the permeabilized part of the membrane [41].

The presence of oxidized lipids dramatically alters the membrane and increases its permeability to ions and molecules by up to several orders of magnitude [44,45]. If secondary lipid peroxidation products are present in sufficiently high concentration, this can lead to spontaneous creation of pores within the oxidized membrane lesions [46]. While the presence of primary lipid peroxidation products, *i.e.* lipid hydroperoxides, could explain the lowest values of the measured increase in membrane permeability and conductivity following exposure of cells to electric pulses [42], the presence of secondary lipid peroxidation products could account for the entire range of measurements [43]. Nevertheless, the exact contribution of lipid peroxidation to increased membrane permeability following exposure to electric pulses remains to be elucidated [47].

Membrane protein damage

The cell membrane consist of more than just lipids; in fact, roughly half of the membrane's mass is attributed to membrane proteins [48]. The first report demonstrating that electric pulses can alter membrane proteins dates to 1980 [49], when Teissié and Tsong observed that electroporation of erythrocytes increased also the electric conductivity of transmembrane Na^+/K^+-ATPases, albeit this effect was observed only in a low-conductivity medium. Tsong later proposed that TMV-driven supraphysiological current passing through transmembrane transport proteins and the resulting local heating can lead to denaturation of these proteins [50,51]. He also noted that excision of denatured proteins from the membrane—and thus recovery of its impermeable state—may take many minutes, which agrees with the time scale of membrane resealing observed experimentally.

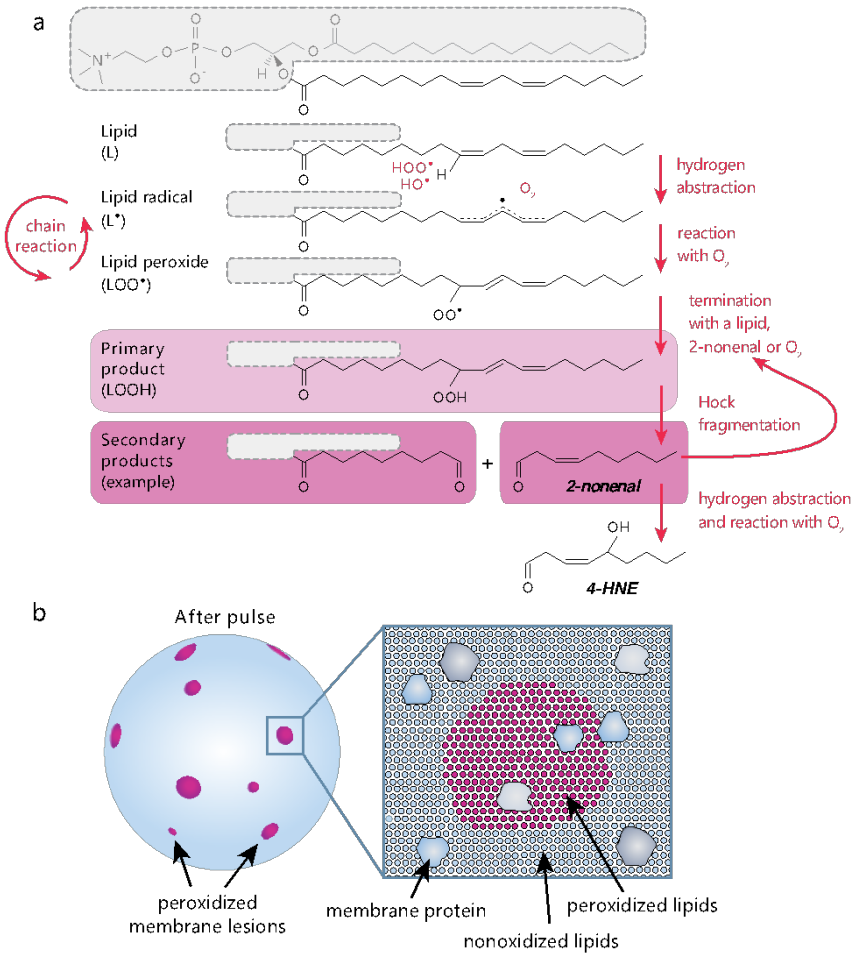

Figure 4: Lipid oxidation. (a) Pathway for primary and secondary oxidation of a phospholipid (L) containing a single bis-allylic site, e.g., linoleic acid. (b) Schematic representation of oxidized membrane lesions, which are expected to form in the cell membrane after exposure electric pulses. The schematic is hypothetical, and the lesions are not drawn to scale. The image on the right depicts the molecular organization in one of the lesions. Figure inspired by [42,43]. See also Fig. 10 in Chapter 4.

Subsequent studies using patch-clamp techniques confirmed that strong electric pulses can affect various types of membrane proteins, particularly voltage-gated ion channels. The first studies in voltage-

clamped frog skeletal muscle cells demonstrated up to ~40% decrease in currents through voltage sodium (Na_V) channels and voltage gated potassium (K_V) channels after exposure to supraphysiological pulses with 4 ms duration. The decrease in ionic currents was more profound when using hyperpolarizing than depolarizing pulses. Several studies also using also demonstrated decreased ionic currents mediated by Na_V and voltage-gated calcium (Ca_V) channels following exposure of cells to pulses of submicrosecond duration [52–55]. However, the molecular mechanisms by which the supraphysiological TMV affects these channels remained unidentified.

MD simulations again offered a method to unravel the molecular events that take place in different voltage-gated ion channels when exposing them to conditions mimicking electroporation (Fig. 5) [56,57]. MD showed that supraphysiological TMV can induce pores in the voltage-sensor domains (VSDs) of these channels and that such pores form more easily in some VSDs than in others. Specifically, poration was found to be more likely in VSDs that are more hydrated and are electrostatically more favourable for the entry of ions [56]. MD further showed that pores in VSDs can expand under electric field, forming so-called complex pores that become stabilized by lipid headgroups. Formation of complex pores is associated with denaturation of the VSD, which makes complex pores considerably more stable than pure lipid pores. Denatured VSDs are expected to become dysfunctional and unable to respond to changes in TMV, which agrees with electrophysiological measurements showing a decrease in the voltage-dependent transmembrane ionic currents after pulse treatment.

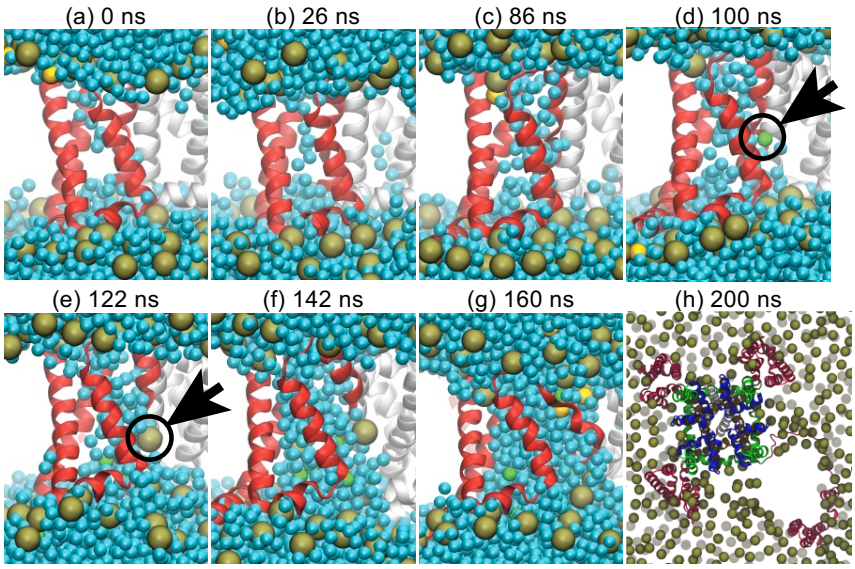

Figure 5: Formation of a complex pore in a VSD of bacterial NavMs channel. (a) VSD was hydrated already in the absence of electric field. (b and c) Upon electric field application, more water molecules entered the VSD. (d) The first Cl^- ion passed through the VSD at ~90 ns after the onset of the electric field. (e and f) More water and ions entered the VSD, and this VSD pore became stabilized by lipid headgroups forming the so-called complex pore. (g) As the complex pore expanded, the VSD began to unfold from the channel. (h) Unfolded VSD viewed from the extracellular side. In (a)–(h) the VSD is colored in red, water in cyan, lipid phosphorus atoms in gold, and sodium and chloride ions in yellow and green, respectively. Black arrows mark the first Cl^- ion within VSD and the first lipid headgroup moving into the pore. Reproduced from [56] with permission.

The influence of the cytoskeleton

All cells contain a cytoskeleton, that gives the cell its shape and provides mechanical support to the cell membrane. The cytoskeleton is a complex, dynamic network of interlinking protein filaments, including the microfilaments (*i.e.*, actin filaments), intermediate filaments, and microtubules. Chemical modification of the cytoskeleton network was shown to affect both the extent and duration of the increased membrane permeability following exposure to electric pulses already in 1992 [58,59]. Similar was later found in genetically engineered plat cells – stabilization of the actin filaments reduced the extent of membrane

permeabilization [60]. A study in mammalian cell line further confirmed that chemical disruption of actin cytoskeleton increases the extent of membrane permeability to small molecules [61]. By examining the temperature-dependent kinetics of intracellular molecular uptake, this study suggested that disruption of the actin cytoskeleton lowers the activation energy barrier for creation of pores in the membrane. Whether this is due to altered mechanical properties of the membrane or altered lipid organization following actin disruption, is not clear.

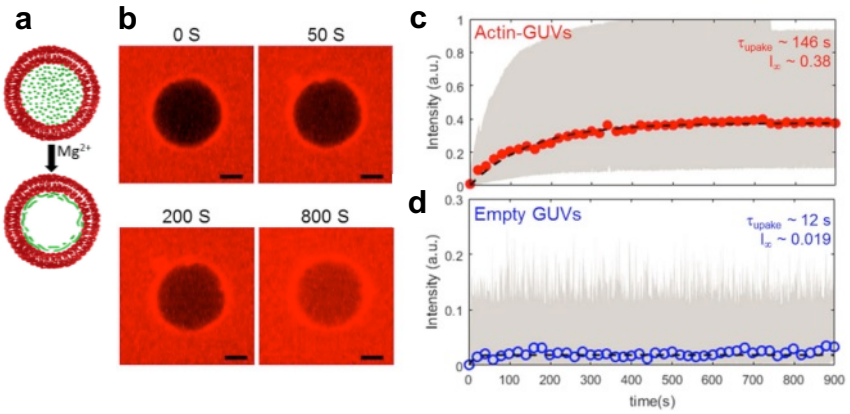

Figure 6: Results from GUVs encapsulating an actin network. The actin network forms spontaneously after initiating actin polymerization with Mg^{2+} ions in GUVs containing actin monomers (a). The kinetics of sulforhodamine B dye uptake during the resealing of actin-encapsulating GUVs shown as four shapshots from a time-lapse captured with a confocal microscope (b), and as a graph of the average increase in dye fluorescence intensity inside the actin-GUV (c, data from 13 different GUVs). The dye uptake in empty GUVs is hardly detectable and exhibits faster kinetics (d, data from 15 different GUVs). Scale bar 5 μm. Reproduced from [62] with permission.

In experiments with biological cells, it is challenging to decouple the influence of the cytoskeleton on membrane permeabilization from other possible biological processes involving actin. Thus, a study was designed where GUVs encapsulating an actin network were used as a model system [62]. Experiments comparing the response of empty and actin-encapsulating GUVs showed that the presence of the actin network stabilizes the membrane against electrodeformation and inhibits the

formation of macropores[6] in the electroporated GUVs. Additionally, experiments on the uptake of dye molecules after electroporation showed that the actin network slows down the spontaneous resealing of the permeabilized membrane (Fig. 6). It should be noted that in cells the resealing process in is mediated by cell membrane repair mechanisms [65,66], and it was shown that chemical disruption of the actin network does not necessarily change the cell membrane resealing time [61].

Several studies further showed that electroporation itself can lead to the disruption of cytoskeletal network, both the actin filaments and microtubules [67–69]. Measurements using atomic force microscopy revealed up to a 40% decrease in membrane stiffness, accompanied by membrane ruffling, cell swelling, and destabilization of actin filaments in the cell cortex underlying the cell membrane followed by cytoskeleton recovery within hours [70,71]. Whether cytoskeleton disruption is a direct effect of the electric field on charged filaments or an indirect effect associated with increased membrane permeability (e.g., cell swelling, ATP leakage, increase in intracellular calcium and its downstream signaling) is not completely clear and might depend on the experimental configuration [72,62]. MD simulations confirmed that sufficiently intense electric field can induce a longitudinal opening of the cylindrical shell of the microtubule lattice, modifying the microtubule structure [73]. Nevertheless, electroporation is typically achieved with much lower electric fields. Interested readers are refered to [74] for further information on cytoskeletal disruption associated with electroporation.

Computational simulations of electroporation and the associated molecular transport at the single cell level

The knowledge of molecular mechanisms of electroporation becomes fully explored in models that allow computational simulations of the electroporation dynamics and that output variables that can be directly compared with experimental measurements. Such models can be used to help interpret experimental results, or as a tool to design, *e.g.*, pulse

[6] Macropores are large holes with diameter of ~1 micrometer that are typically observed in GUVs exposed to strong electric pulses [63]. These pores are large enough to be monitored with conventional optical microscopy. Formation of such macropores has not been reported in cell membranes. In contrast to cells, empty GUVs are highly deformable by electric field; thus it is possible that macropores arise from extensive stretching of the GUV membrane during electrodeformation [64].

parameters that are expected to lead to the desired experimental outcome [75–77].

The first model that enabled simulations of the temporal dynamics of the current-voltage characteristics during electroporation of a planar lipid bilayer was developed in the Weaver's group in the 1990s [25,78]. Their model was based on the theoretical description of lipid pores and their energy landscape (similar as in Fig. 1) coupled to an equivalent electrical circuit representing the bilayer and the surrounding electrolyte solutions. The model was successful in qualitatively reproducing four possible outcomes that were observed in charge-pulse experiments[7] [7] but were not well understood: (i) a slight increase in electrical conductance at low injected charge, (ii) mechanical rupture at intermediate injected charge, (iii) incomplete reversible electrical breakdown resulting in incomplete discharge of the membrane at higher injected charge, and (iv) reversible electrical breakdown resulting in complete discharge of the membrane at the highest injected charge.

Towards the end of 1990s, Krassowska and colleagues adapted the models developed for planar lipid bilayers to enable simulations of the time course of the induced TMV at the single cell level [24]. In their first model, they neglected pore expansion and considered that all pores have a minimum size [79]. This model was successful in qualitatively reproducing the profiles of the induced TMV experimentally measured with a potentiometric dye in sea urchin eggs [80]. They later upgraded their model to also include pore expansion [81]. Computed profiles of the induced TMV are shown in Fig. 7. The model demonstrates how the increase in membrane electrical conductance due to electroporation has a major impact on the temporal evolution of the TMV. Before electroporation, the TMV reaches the highest values at the poles of the cell, following the Schwan equation (see Chapter 1). However, in the regions where the TMV exceeds ~1 V, many pores form through which the membrane discharges and the TMV saturates at ~1 V. The saturation of the TMV then inhibits further pore creation and expansion. Thus, the relationship between the TMV and membrane conductance can be though of in terms of a negative feed-back loop and must be taken into account when modelling electroporation.

[7] In these charge-pulse experiments, a planar lipid bilayer was subject to a short pulse (500 ns to 2.5 μs) of constant electric current that charged the membrane to a certain TMV, depending on the pulse duration and amplitude.

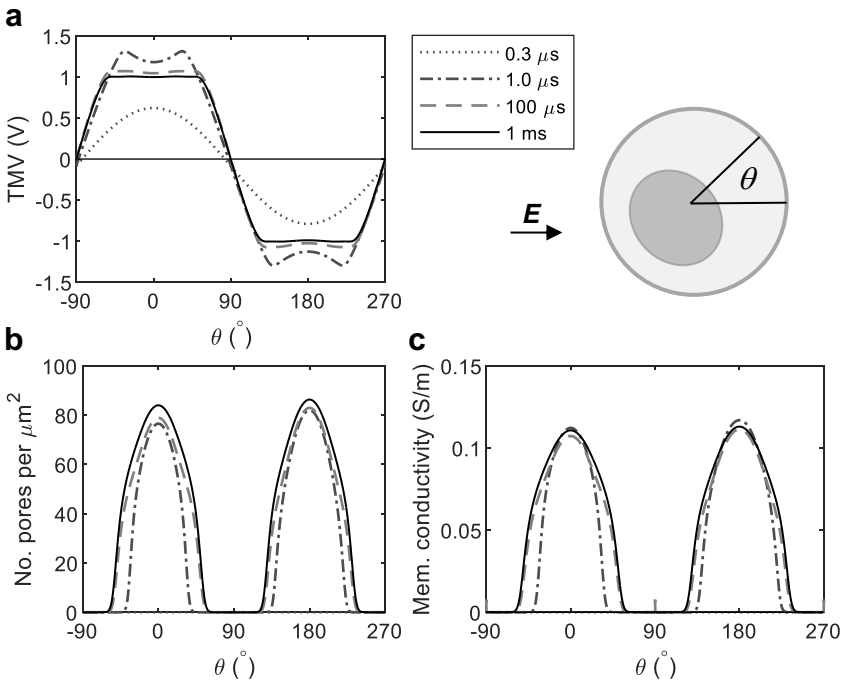

Figure 7: Simulations of the TMV (a), the number of pores per unit membrane area (b), and the membrane conductivity (c), along the membrane perimeter, as predicted by the electroporation model described in [79]. Results are computed for a model of a sea urchin egg, exposed to a 1 ms, 400 V/cm pulse, and plotted at four selected time points during the pulse (see legend).

Models that describe the time course of TMV and increase in membrane conductance (and permeability) can be expanded with Nernst-Planck equations that describe the electrodiffussion of a selected molecule in the extracellular and intracellular space and across the membrane. The description of the transmembrane flux is then coupled to the description of membrane electroporation. For instance, the simplest way to describe the transmembrane flux is to assume that it scales proportionally with the surface fraction of pores in the membrane [82,83], although more complex models accounting for the hindered motion of molecules across pores, depending on the molecule size and charge, have also been developed [84].

Throughout the past two decades, various mechanistic and phenomenological models have been developed to computationally simulate molecular transport across the membrane of an individual cell [85–88]. A recent study [89] critically examined the existing mechanistic

models, which allow spatially and temporally resolved computational simulations of electroporation-induced transmembrane transport of small molecules, by confronting them with different experimental measurements. The study revealed that none of the tested models is reliable enough to be applied universally for all the different pulse parameters and small molecules used in electroporation-based applications. Even more importantly, the study demonstrated that none of the models has been compared to sufficient amount of experimental data to confirm the model validity. These existing models mainly attribute the increase in membrane permeability to lipid pores. Further research into molecular mechanisms of increased membrane permeability, including lipid oxidation and membrane protein denaturation due to supraphysiological TMV, as well as the influence of the cytoskeleton network and its disruption, should help us design better and more reliable electroporation models.

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Chapter 3

Electric Properties of Tissues and their Changes during Electroporation

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

Introduction

The electrical properties of biological tissues and cell suspensions have been of interest for over a century. They determine the pathways of current flow through the body and are thus very important for diagnostics and in the analysis of a wide range of biomedical applications. These applications require the knowledge of tissue response to applied voltages and currents, to be able to successfully develop novel treatments and successfully target them. To analyze the response of a tissue to electric stimulus, data on the

conductivity and relative permittivity of the tissues or organs are needed. A microscopic description of the response is complicated by the variety of cell shapes and their distribution inside the tissue as well as the varying properties of the extracellular media. At low frequency, the electric conductivity is mainly determined by the volume fraction of extracellular medium and the concentration of ions in the medium and their mobility. As the frequency is increased, the tissue complex impedance then also depends on the composition of the cytosol.

Due to this complexity, a macroscopic approach is most often used to characterize field distributions in biological systems. However, even on a macroscopic level the electrical properties are not trivial. They can depend on the tissue orientation relative to the applied field (directional anisotropy), the frequency of the applied field (the tissue is neither a perfect dielectric nor a perfect conductor) or they can be time and space dependent (e.g., changes in tissue conductivity due to electroporation) [1]–[3].

Biological materials in the electric field

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

If a conductive object is placed in an external static electric field, charges will redistribute within the conductive object until the resulting internal field is zero. In the case of an insulator, there are no free charges so net migration of charge can occur, therefore the internal electric field is unperturbed. In polar materials, the positive and negative charge centers in the molecules (e.g. water) do not coincide. An applied field, E_0, tends to orient the dipoles and produces a field inside the dielectric, E_p, which opposes the applied field. This process is called polarization. Most materials contain a combination of dipoles and free charges. Thus the electric field is reduced in any material relative to its free-space value. The resulting internal field inside the material, E, is then

$$E = E_0 - E_p$$

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

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

In practice, most materials, including biological tissue, actually display some characteristics of both, insulators and conductors, because they contain dipoles as well as charges which can move, but in a restricted manner [4], [5].

On a macroscopic level we describe the material as having a permittivity, ε, and a conductivity, σ. The permittivity characterizes the material's ability to trap or store charge or to rotate molecular dipoles whereas the conductivity describes its ability to transport charge. The values for permittivity is typically given as relative permittivity ε_r, with the total permittivity then equal to:

$$\varepsilon = \varepsilon_r \varepsilon_0 \varepsilon = \varepsilon_r \varepsilon_0$$

where ε_0 =8.85 x 10^{-12} F/m is the permittivity of vacuum.

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

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

Consider a sample of material which has a thickness, d, and cross-sectional area, A (Fig. 1).

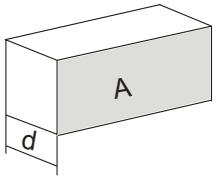

Figure 1: Theoretical small block of a material.

If the material is an insulator, then we treat the sample as a capacitor with capacitance (*C*); if it is a conductor, then we treat it as a conductor with conductance (*G*):

$$C = \varepsilon \cdot \frac{A}{d} C = \varepsilon \cdot \frac{A}{d} \quad G = \sigma \cdot \frac{A}{d} G = \sigma \cdot \frac{A}{d}$$

A simple model for a real material, such as tissue, would be a parallel combination of the capacitor and conductor. If a constant (DC) voltage *V* is applied across this parallel combination, then a conduction current $I_C = GV$

will flow and an amount of charge $Q = CV$ will be stored. However, if an alternating (AC) voltage was applied to this parallel resistor and capacitor:

$$V(t)=\mathrm{V_0}\cos(\omega t)V(t)=\mathrm{V_0}\cos(\omega t)$$

The charge on the capacitor plates is now changing with frequency f. Since the current is the amount of charge that flows in a circuit in a given time period, we can determine the current on the capacitor as a derivative of the charge by time. We call this flow as displacement current:

$$I_d = \frac{\mathrm{dQ}}{\mathrm{dt}} = \text{-}\omega\mathrm{CV_0}\, sin(\,\omega t)I_d = \frac{\mathrm{dQ}}{\mathrm{dt}} = \text{-}\omega\mathrm{CV_0}\, sin(\,\omega t)$$

The total current flowing through the material is the sum of the conduction and displacement currents, which are 90° apart in phase, with the displacement currents leading the applied voltage. The total current is I = I_c + I_d, hence

$$I=GV+C\cdot\frac{\mathrm{d}\,V}{\mathrm{d}\,t} = (\sigma\cos(\omega t) - \omega\varepsilon\sin(\omega t))V_0\cdot\frac{A}{d}\, I=GV+C\cdot\frac{\mathrm{d}\,V}{\mathrm{d}\,t}$$
$$= (\sigma + i\omega\varepsilon)V\cdot\frac{A}{d}$$

For a clearer notation, we introduce the imaginary current, which is a notation for the displacement current using complex numbers. The above equation can then be written as:

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

Where i is the complex number (i^2=-1). The actual material, then, can be characterized as having an admittance, Y*, given by:

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

where * indicates a complex-valued quantity. Here it is important to note, that admittance is a measure of the ability of current to flow in the entire electrical circuit and therefore depends on the geometry of the block of material and the electrodes which enable us to connect the circuit to the material. To be able to generalize this, we need to define material properties, which In terms of material properties we define a corresponding, complex-valued conductivity

$$\sigma^* = \sigma' + i\sigma''$$

Where $\sigma' = \sigma$ is the real part of the conductivity, which determines the conduction currents, and $\sigma'' = \omega\varepsilon$ is the complex part, which determines

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

We can consider the conductivity of a material as a measure of the ability of its charge to be transported throughout its volume in a response to the applied electric field. Similarly, its permittivity is a measure of the ability of its dipoles to rotate or its charge to be stored in response to the applied field. Note that if the permittivity and conductivity of the material are constant, the displacement current will increase with frequency whereas the conduction current does not change. At low frequencies the material will behave like a conductor, but capacitive effects will become more important at higher frequencies. For most materials, however, σ^* and ε^* are frequency-dependent. Such a variation is called dispersion and is due to the dielectric relaxation – the delay in molecular polarization following changing electric field in a material. Biological tissues exhibit several different dispersions over a wide range of frequencies [4,5].

Dispersions can be understood in terms of the orientation of the dipoles and the motion of the charge carriers. At relatively low frequencies it is relatively easy for the dipoles to orient in response to the change in applied field whereas the charge carriers travel larger distances over which there is a greater opportunity for trapping at a defect or interface like cell membrane [2]. As the frequency increases, the dipoles are less able to follow the changes in the applied field and the corresponding polarization disappears. In contrast, the charge carriers travel shorter distances during each half-cycle and are less likely to be trapped. As frequency increases, the permittivity decreases and, because trapping becomes less important, the conductivity increases. In a heterogeneous material, such as biological tissue, several dispersions are observed as illustrated in Fig. 2. In short, alpha dispersion in the kilohertz range is due to cell membrane effects such as gated channels and ionic diffusion and is the first of the dispersions to disappear with tissue death. Beta dispersion can be observed around the megahertz range due to the capacitive charging of cell membranes. Above beta dispersion the impedance of cell membranes drops drastically, allowing the electric current to pass through not only extracellular, but also intracellular space. This dispersion is particularly interesting as it is also apparent in the conductivity of the material. The last, gamma dispersion (above the gigahertz range) is due to dipolar mechanisms of water molecules in the material.

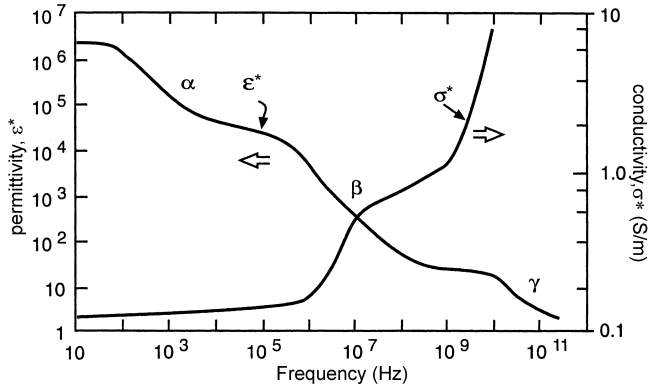

Figure 2: Typical frequency dependence of the complex permittivity ε* and complex conductivity σ* of a heterogeneous material such as biological tissue.

Numerical modeling can show how part of the dispersions emerges from the basic properties of intracellular, extracellular, and membrane conductivity as shown in Fig. 3. The membrane conductivity is several orders of magnitude smaller than the intracellular and extracellular conductivity. When the volume fraction of the cells is increased, the low frequency conductivity decreases, and the beta dispersion around the megahertz range becomes more prominent.

Measurements of dielectric properties of tissues

There is a large discrepancy between various data on electrical properties of biological materials found in the literature. The measurement of tissue dielectric properties is challenging due to several factors, such as tissue inhomogeneity, anisotropy, the physiological state of the tissue, seasonal, age and disease-linked changes and electrode polarization [1].

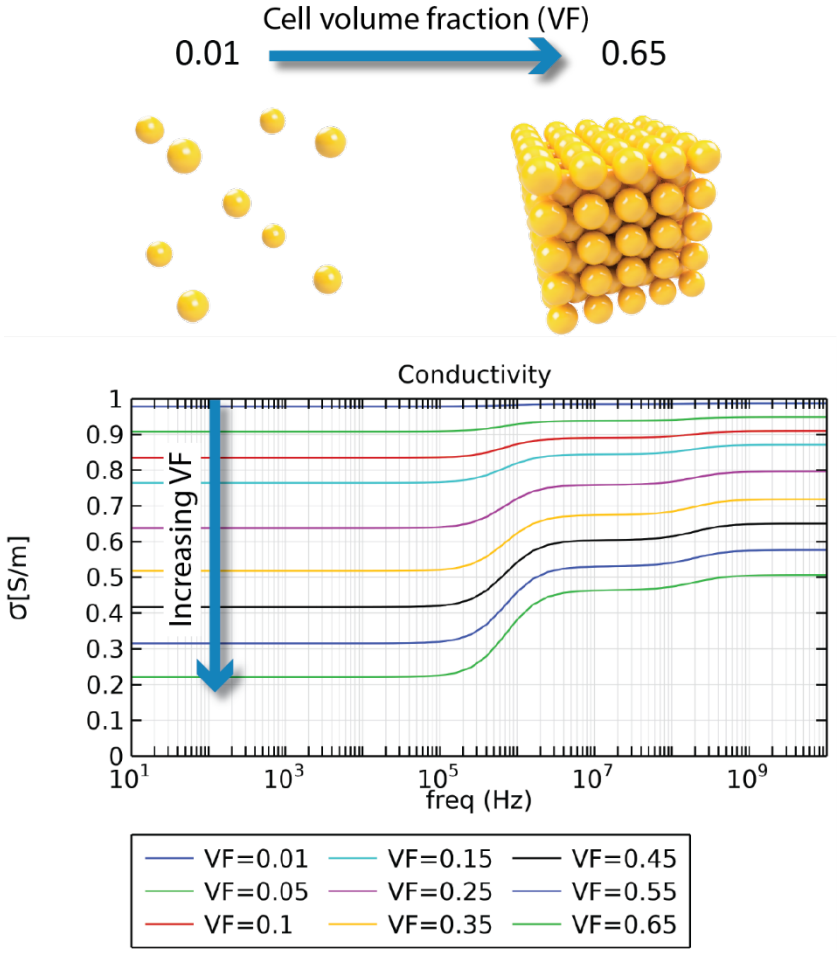

Figure 3: The emergence of beta dispersion with the increase of the volume fraction (VF) of cells in a model of *in vitro* exposure of cells to different frequency sinusoidal voltage.

Inhomogeneity of tissues

Tissue is a highly inhomogeneous material. The cell itself is comprised of an insulating membrane enclosing a conductive cytosol. A suspension of cells can be regarded at low frequencies simply as nonconducting inclusions

in a conducting fluid [6]. The insulation is provided by the cell membrane. At frequencies in the MHz range capacitive coupling across this membrane becomes more important, allowing the electric current to pass not only around the cell, but also through it. In tissue, the cells are surrounded by an extracellular matrix, which can be extensive, as in the case of bone, or minimal, as in the case of epithelial tissue. Tissue does not contain cells of a single size and function. The tissue is surrounded by basal lamela. Furthermore, tissues and organs are enveloped by single or multiple fascia. It is thus difficult (if not impossible) to extrapolate from the dielectric properties of a cell suspension to those of an intact tissue.

Anisotropy of tissues

Some biological materials, such as bone and skeletal muscle, are anisotropic. Therefore, when referring to measured conductivity and permittivity values, one needs to include data on the orientation of the electrodes relative to the major axis of the tissue; e.g., longitudinal, transversal or a combination of both. For example, striated muscles are composed of fibers, very large individual cells aligned in the direction of muscle contraction. Electric current conduction along the length of the fiber is significantly easier than conduction in the direction perpendicular to the fibers. Therefore, muscle tissue manifests typical anisotropic electric properties. The longitudinal conductivity is significantly higher than the transverse conductivity (can be up to 8 times higher) [7].

Heart tissue is an interesting example, since the heart muscle orientation changes from the inside to the outside of the walls of the ventricles [8]. The amount of twisting is species dependent, but ranges from -70 to +80 degrees in humans [9]. This twisting of the fiber orientations is one of the mechanisms which allow the thickening of the ventricular walls during the systole, which enables efficient pumping of the blood without a large change in the outer volume of the heart [10].

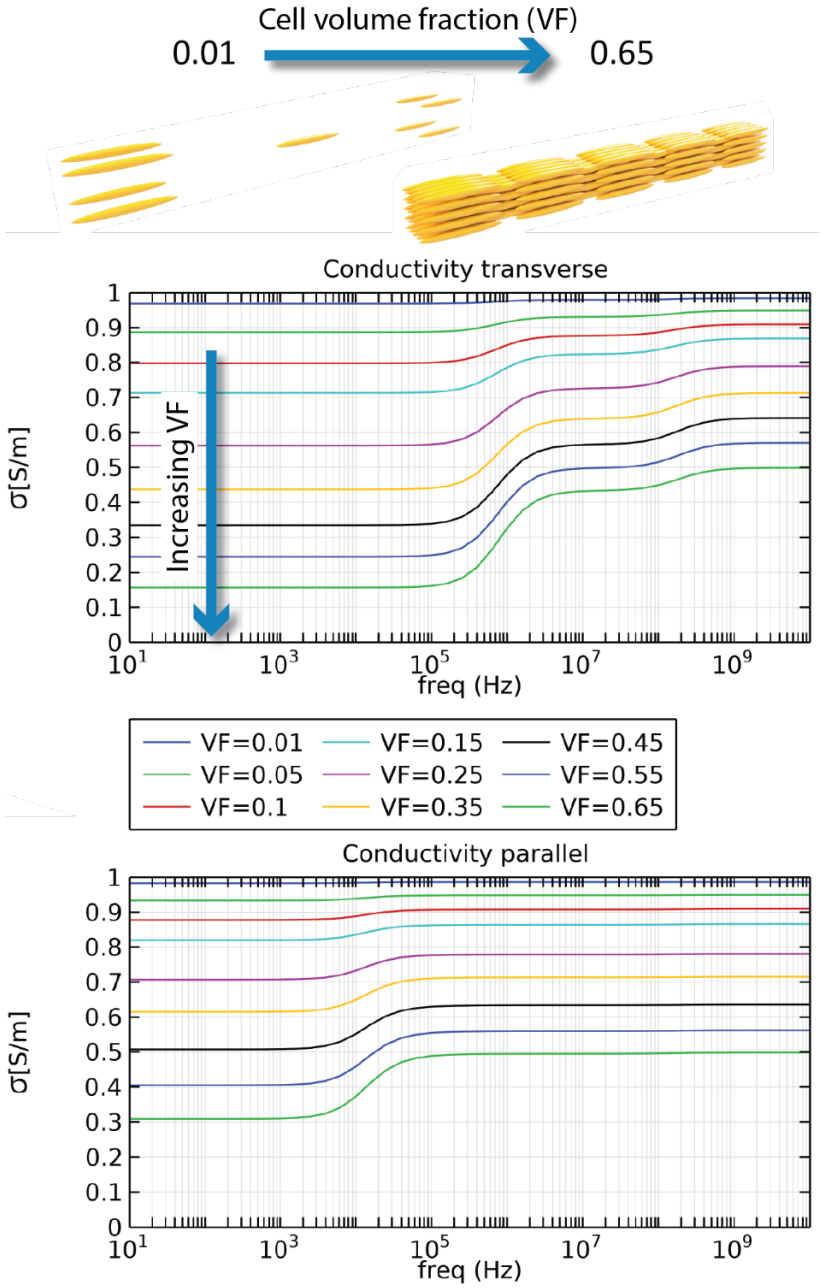

Figure 4: Anisotropy emerges when prolate spheroidal cells are oriented in the same direction. Conductivity in the direction of the long axis of the cells is higher than perpendicular to the cells, when the cell volume fraction (VF) is increased.

Moreover, tissue anisotropy is frequency dependent as can be seen in Figs. 2 and 4. Namely, if the frequency of the current is high enough, the anisotropic properties disappear. Specifically for muscle tissue, that happens in the MHz frequency range, i.e. at beta dispersion.

Physiological factors and changes of tissue

Any changes in tissue physiology can produce changes in the tissue electrical properties. This principle has been used to identify and/or monitor the presence of various illnesses or conditions [11]–[13].

Tumors generally have higher water content than normal cells because of cellular necrosis but also irregular and fenestrated vascularization. Higher conductivity of tumors in the MHz frequency range has been identified as a potential target for imaging applications, such as microwave tomography, which aims to detect or monitor breast cancer noninvasively using microwaves [14]. In addition, there may be differences in the membrane composition. Also, fat is a poorer conductor of electricity than water. Changes in the percentage of body fat or water are reflected in tissue impedance changes [11]. It has also been reported that pathological changes in the liver, such as fatty liver disease or liver cirrhosis can influence the measured properties of tissue at 100 MHz frequencies [12].

Further, tissue excision results in significant changes in electrical properties. Tissue metabolism decreases after the tissue has been excised and often the temperature falls. If the tissue is supported by temperature maintenance and perfusion systems, the tissue may be stabilized for a limited period of time in a living state *in vitro* (*ex vivo*). If the tissue is not supported, however, irreversible, which results in water leakage from cells and other mechanisms which begin to change the electrical properties. For these reasons considerable caution must be taken in the interpretation of electrical measurements which were performed on excised tissues [15], [16].

The electrical properties of tissue also depend on its temperature. The mobility of the ions which transport the current increases with the temperature as the viscosity of the extracellular fluid decreases. The rapid increase of conductivity with temperature was suggested to be used e.g. to monitor the progress of hyperthermia treatment or cryoablation [17]. Also, possible other changes, such as cell swelling and edema, or blood flow occlusion, all affect tissue properties.

Electrode polarization

Electrode polarization is a manifestation of molecular charge organization which occurs at the tissue/electrolyte-electrode interface in the presence of water molecules and hydrated ions. The effect increases with increasing tissue conductivity [18].

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

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

The material of the electrode plays an important role in determining its polarization impedance, the relative importance of which decreases with increasing frequency. It is considered a good practice to measure tissue impedance *in vivo* after waiting a sufficient time for the electrode polarization processes to stabilize. A typical time might be on the order of thirty minutes.

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

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

varying the separation of the electrodes, the contribution of the electrode polarization can be determined and eliminated, however this is a method best applicable to liquid samples, since it requires parallel plate electrodes.

Four-electrode method: Can be used for both DC and AC measurements. Two pairs of electrodes are used: the outer, current electrodes and the inner, voltage electrodes. The current from the source passes through the sample. Voltage electrodes of known separation are placed in the sample between the current electrodes. By measuring the current as the voltage drop across a resistor in series with the sample and the voltage drop across the inner electrodes, one can determine the conductivity of the sample between the inner electrodes. The advantage of this method is that the polarization on the current electrodes has no influence on the voltage difference between the voltage electrodes. Polarization at the voltage electrodes is negligible for both DC and AC due to the high input impedance of the measurement system. The drawback is that measurement results are interpreted based on the assumption of tissue being homogeneous in the entire region where measurement is performed, which is generally not true for most tissues at macroscopic distances.

Electrical response of tissue to electric field

Changes in tissue conductivity have been observed *in vivo* if the tissue is subjected to a high enough electric field. Having said that, we can use the dielectric properties of liver and try to calculate the theoretical electrical response to a short rectangular voltage pulse having the duration of 100 μs and the rise time of 1 μs (typical pulse parameters used for electrochemotherapy). We used the parallel RC circuit to model the electrical response of the tissue (see Fig. 5).

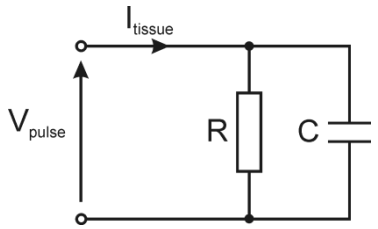

Figure 5: Parallel RC circuit: a theoretical representation of tissue response to electic pulses.

The complications arise from the facts that i) the pulse parameters (the pulse duration, the rise and the fall time) determine the content of its frequency spectrum and ii) the tissue conductivity and permittivity are frequency dependent. The obtained response for the first pulse is presented in Fig. 6. At the onset of voltage pulse, transient capacitive (i.e. displacement) current is observed. As membranes charge, voltage across them rises and the measured displacement current decreases towards zero. Soon steady state is reached and current stabilizes through the conductance of extracellular medium. Since the model describing dielectric dispersions is linear, change of the applied voltage proportionally scales the amplitude of the current.

We can compare this calculated response with the measured response on rat liver *in vivo* for the same pulse as above and different pulse amplitudes spanning up to electroporative field strengths (Fig. 7) [19,20]. For the lowest applied voltage we can see a good agreement with calculated response. As the field intensity is increased, the electrical response of tissue is no longer linear, meaning that the current is increasing faster than the voltage. The current is also increasing *during* the pulse itself, as can be seen from the current rise at the higher voltages in Fig. 7. Measuring the passive electrical properties of electroporated tissues could provide real time feedback on the outcome of the treatment [19,21]. However, care must be taken in the interpretation of the current rise during the application of electroporation treatments, due to the natural variability of the tissue dielectric properties, and because conductivity rise can also be a result of tissue heating [22,23].

A recent study indicates that the changes in total resistance during irreversible electroporation treatments, could be used to differentiate between a lower and higher activation of the immune system after the treatment and consecutive improvement in outcomes [24].

The total increase in current can also be measured directly during the pulse delivery using current density imaging, a special MRI sequence, which uses the perturbation of the magnetic field caused by the current flowing through the medium [25].

Electroporation current can be reproduced in a benchtop test device, which combines passive and nonlinear elements to reproduce various parts of the observed current in tissue [26].

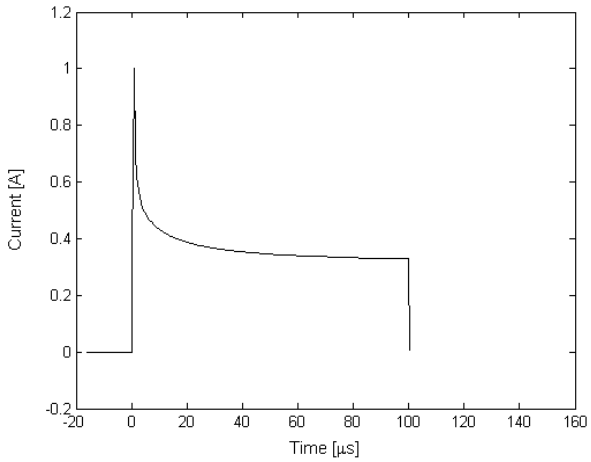

Figure 6: Calculated tissue response during delivery of rectangular voltage pulse with the duration of 100 μs having the rise time of 1 μs and the amplitude of 120 V. Plate electrodes with 4.4 mm interelectrode distance were assumed.

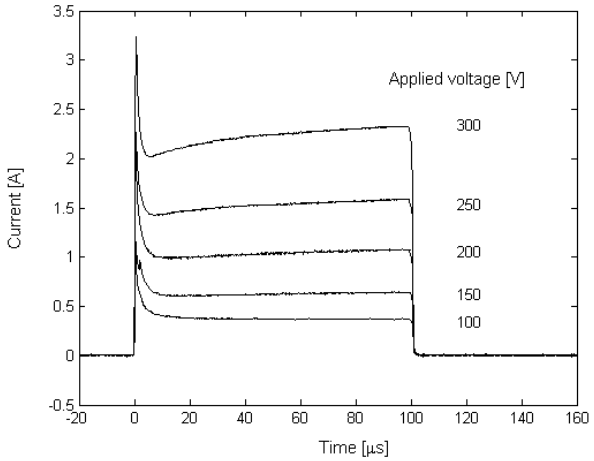

Figure 7: Measured tissue response during delivery of 100 μs rectangular pulses of different amplitudes to rat liver *in vivo*. Adapted from Cukjati *et al.* [19]. Pulses were generated using Jouan GHT1287B; plate electrodes with 4.4 mm interelectrode distance were used.

The measured response is consistent with the hypothesis that the bulk tissue conductivity should also increase measurably (Fig. 8) since on a cellular level electroporation causes the increase of membrane conductance

[6]. In measuring *ex vivo* tissue and phantom tissue made of gel like material [27] and *ex-vivo* liver tissue [28] using MREIT we were able to demonstrate that electric conductivity changes due to membrane electroporation are amplitude dependent and occur in tissue only but not in phantom tissue. It is not clear, however, to which value tissue conductivity increases as a consequence of plasma membrane electroporation. It has been proposed that this could be close to the value in beta dispersion range [29].

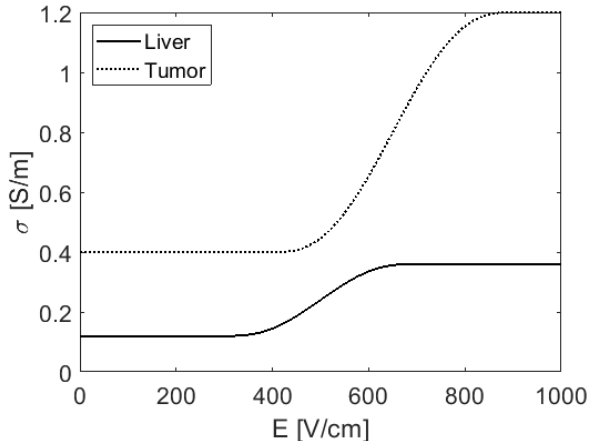

Figure 8: Conductivity of tissues as a function of the local electric field strength.

Further, in applications where electric pulses to skin or tissues underneath (such as subcutaneous tumor) are applied externally, through skin, one might expect very high voltage amplitudes would be required to breach the highly resistive skin tissue and permeabilize tissues underneath. Namely, tissues between the electrodes can be seen as serially connected resistors. Applying voltage on such a circuit (voltage divider) causes the voltage to be distributed between the resistors proportionally to their resistivities [30]. Upon applying electric pulses, almost the entire applied voltage thus rests across the most resistive (least conductive) tissue, in our case the outermost layers of the skin. That would results in a very high electric field strength in skin tissue, while the electric field in other tissues would remain insufficient for successful cell electroporation. If our goal is the electrochemotherapy of the underlying tumor, one might wonder how a successful electrochemotherapy of subcutaneous tumors is possible when external plate electrodes are used. The answer lies in the increase in bulk conductivities of tissues during electroporation, a phenomenon that was also observed *in vivo*. This conductivity increase leads to a change in electric field distribution, which exposes the tumor to an electric field high

enough for successful cell membrane permeabilization [31]. To further support this hypothesis, we described this process with a numerical model, taking into account the changes of tissue bulk electrical properties during electroporation. In Fig. 9 six steps of the electroporation process in the subcutaneous tumor model for the voltage of 1000 V between the electrodes are shown. The electric field distribution shown in V/cm. Step 0 denotes the electric field distribution as it was just before the electroporation process started, thus when all the tissues still had their initial conductivities. When the voltage is applied to the electrodes, the electric field is distributed in the tissue according to conductivity ratio of the tissues in the model and their relative thickness. The field strength is generally the highest in the tissues with the lowest conductivity, where the voltage drop is the largest. In our case, almost the entire voltage drop occurs in the skin layer which has a conductivity of about 10-100 times lower than the tissues lying underneath.

If we look at the last step of the sequential analysis, step 5, at 1000 V (Fig. 9) the tumor is entirely permeabilized, in some areas the electric field is also above the irreversible threshold.

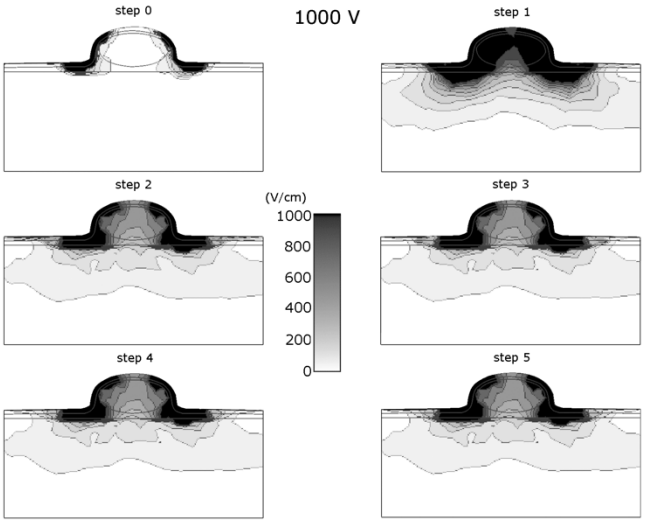

Figure 9: Six steps of the sequential analysis of the electroporation process in the subcutaneous tumor model at 1000 V between two plate electrodes with distance of 8 mm [31]. Time intervals between steps are in general not uniform. Different steps follow a chronological order but do not have an exact time value associated with them. The electric field distribution is shown in V/cm.

A similar situation can be encountered when applying electric pulses on a skin fold with external plate electrodes as a method to enhance *in vivo*

gene transfection in skin [32]. Skin consists of three main layers: *epidermis*, *dermis* and subcutaneous tissue (Fig. 10). The *epidermis* is made up of different layers, but the one that defines its electrical properties the most is the outermost layer, the *stratum corneum*. Although very thin (typically around 20 μm), it contributes disproportionally to the electrical properties of skin. Its conductivity is three to four orders of magnitude lower than the conductivities of deeper skin layers. Again, when electric pulses are applied on skin fold through external plate electrodes, almost the entire applied voltage rests across the *stratum corneum*, which causes a very high electric field in that layer, while the electric field in deeper layers of skin – the layers targeted for gene transfection – stays too low. Similarly as in the case of subcutaneous tumors, the increase in bulk conductivities of skin layers during electroporation exposes the skin layers below *stratum corneum* to an electric field high enough for a successful permeabilization [33]. Research has shown however, that this increase in the conductivity of the *stratum corneum* layer is not homogeneous over the entire volume of the tissue, but rather that the increase happens in localized transport regions, which are cavity-like structures that increase the conductivity and permeability of the *stratum corneum* only in small holes [34,35]. This increase, however is enough to enable the increase transport of molecules across the skin and the increase in total current, which then enables also transcutaneous electroporation to occur.

A recent study shows, how the macroscopic tissue properties, including the anisotropic conductivity, response to electroporation [36]. This study used numerical models of electroporation to determine the change in electrical conductivity of the cell membrane as a function of the applied electric field strength in a unit cell. This data was then used to construct bulk tissue conductivity curves for the conductivity in directions along and across the muscle fibers dependent on electric field strength along and across the muscle fibers. This approach enabled a bottom up construction of a bulk tissue model, which matched the actual *in vivo* experimental results.

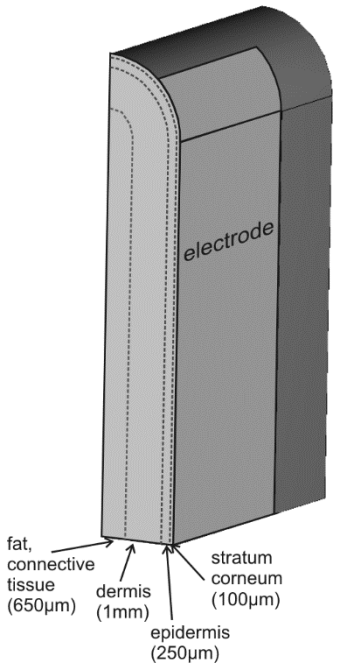

Figure 10: Schematics of a skinfold as described in a numerical model. Only one quarter of the skinfold is presented here.

Tissue properties during high frequency electroporation

High frequency electroporation is recently gaining considerable attention after it had been pointed out in the literature, that electroporation with pulse durations between 1 μs and 100 μs is relatively unexplored [37]. The shorter pulses, combined with some pause between pulses have the potential of reducing nerve and muscle stimulation present in "classical" (8x 100 μs) electroporation pulses [38–40]. Since the pulse protocols of high frequency electroporation typically employ larger number of bipolar pulses of short duration, the frequency content of these pulses is can be markedly different from the clasical 100 μs electroporation pulses [41]. This kind of pulses can also successfully be used for the intracellular delivery of small molecules as in electrochemotherapy [42] and DNA for gene electrotransfer [43]. One possible advantage of this is that there could be a lower conductivity contrast between e.g. tumor and surrounding healthy tissue, however the main frequencies of these pulse trains are still low enough, that induced voltage on the cell membrane can reach high enough values, that

electroporation can occur, and therefore result in a significant rise in the conductivity of tissue.

Conclusions

Theoretical explanation of the process of electroporation offers useful insight into the understanding of the underlying biological processes and allows for predicting the outcome of the treatment [44]. Therefore, due effort needs to be invested into measurements of tissue electrical properties and their changes during electroporation [45].

Further, one of the concerns associated with electroporation could be the amount of resistive heating in the tissue. Excessive heating is unwanted not only to avoid skin burns and assure patient safety, but also to avoid damage to viable cells. Potential excess of the resistive heating during electroporation has been demonstrated [46], therefore thermal aspect in treatment planning and theoretical analysis of specific applications of electroporation-based treatments should be considered [41]. In order to stay within the safety limit while achieving successful treatment, heating needs to be estimated, by means of theoretical models, as a part of treatment planning [47].

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Chapter 4

Insights into Lipid Membranes Electroporation from MD Simulations

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Abstract: The application of short and intense electric pulses enables to transiently alter the properties of cell membranes, making them permeable to a wide range of chemical species. Our knowledge however about all occurring processes at atomistic resolution remains still sketchy. In this contribution we show how we harness the capabilities of computational resources and the predictive power of advanced atomistic level Molecular Dynamics techniques to decipher key steps in several physical and biophysical processes occurring at cell membranes subject to Electroporation Based Technologies and Treatments. The up-to-date results obtained are believed to capture the essence of the several aspects of the electroporation of model membranes and should serve as an additional complementary source of information to the current arsenal of experimental tools.

Introduction

Electroporation disturbs transiently or permanently the integrity of cell membranes [1–4]. These membranes consist of an assembly of lipids, proteins and carbohydrates that self–organize into a thin barrier that separates the interior of cell compartments from the outside environment [5]. The main lipid constituents of natural membranes are phospholipids that arrange themselves into a two-layered sheet (a bilayer). Experimental evidence suggests that the effect of an applied external electric field to cells is to produce aqueous pores specifically in the lipid bilayer [6–10]. Information about the sequence of events describing the electroporation

phenomenon can therefore be gathered from measurements of electrical currents through planar lipid bilayers along with characterization of molecular transport of molecules into (or out of) cells subjected to electric field pulses. It may be summarized as follows: Long and intense electrical pulses induce rearrangements of the membrane components (water and lipids) that ultimately lead to the formation of aqueous hydrophilic pores [6–11] whose presence increases substantially the ionic and molecular transport through the otherwise impermeable membranes [12], see also chapter 2.

In erythrocyte membranes, large pores could be observed using electron microscopy [13], but in general, the direct observation of the formation of nano-sized pores is not possible with conventional techniques. Furthermore, due to the complexity and heterogeneity of cell membranes, it is difficult to describe and characterize their electroporation in terms of atomically resolved processes. Atomistic simulations in general, and molecular dynamics (MD) simulations in particular, have proven to be effective for providing insights into both the structure and the dynamics of model lipid membrane systems in general [14–19]. Several MD simulations have been conducted in order to model the effect of electric field on membranes [20–24], providing the first molecular models of the electroporation process of lipid bilayers.

MD simulations of lipid membranes

Molecular dynamics (MD) refers to a family of computational methods aimed at simulating macroscopic behaviour through the numerical integration of the classical equations of motion of a microscopic many-body system. Macroscopic properties are expressed as functions of particle coordinates and/or momenta, which are computed along a phase space trajectory generated by classical dynamics [25,26]. When performed under conditions corresponding to laboratory scenarios, MD simulations can provide a detailed view of the structure and dynamics of a macromolecular system. They can also be used to perform "computer experiments" that cannot be carried out in the laboratory, either because they do not represent a physical behaviour, or because the necessary controls cannot be achieved.

MD simulations require the choice of a potential energy function, *i.e* terms by which the particles interact, usually referred to as a force field. Those most commonly used in chemistry and biophysics, *e.g*. GROMOS [27] CHARMM [28] and AMBER [29], are based on molecular mechanics and a classical treatment of particle-particle interactions that precludes bond

dissociation and therefore the simulation of chemical reactions. Classical MD force fields consist of a summation of bonded forces associated with chemical bonds, bond angles, and bond dihedrals, and non-bonded forces associated with van der Waals forces and electrostatic interactions. The parameters associated with these terms are optimized to reproduce structural and conformational changes of macromolecular systems.

Conventional force fields only include point charges and pair-additive Coulomb potentials, which prevent them from describing realistic collective electrostatic effects, such as charge transfer, electronic exitations or electronic polarization, which is often considered as a major limitation of the classical force fields. Note that constant efforts are undertaken on the development of potential functions that explicitly treat electronic polarizability in empirical force fields [30–32] but none of these "polarizable" force fields is widely used in large-scale simulations for now, the main reasons for that being the dramatic increase of the computational time of simulation and additional complications with their parameterization. In this perspective, classical force fields provide an adequate description of the properties of membrane systems and allow semi-quantitative investigations of membrane electrostatics.

MD simulations use information (positions, velocities or momenta, and forces) at a given instant in time, t, to predict the positions and momenta at a later time $t + \Delta t$, where Δt is the time step, of the order of a femtosecond, taken to be constant throughout the simulation. Numerical solutions to the equations of motion are thus obtained by iteration of this elementary step. Computer simulations are usually performed on a small number of molecules (few tens to few hundred thousand atoms), the system size being limited of course by the speed of execution of the programs, and the availability of computer power. To eliminate edge effects and to mimic a macroscopic system, simulations of condensed phase systems consider a small patch of molecules confined in a central simulation cell and replicate the latter using periodic boundary conditions (PBCs) in the three directions of Cartesian space. For membranes for instance the simulated system would correspond to a small fragment of either a black film, a liposome or multilamellar oriented lipid stacks deposited on a substrate [33,34].

Traditionally, phospholipids have served as models for investigating *in silico* the structural and dynamical properties of membranes. From both a theoretical and an experimental perspective, zwitterionic phosphatidylcholine (PC) lipid bilayers constitute the best characterized systems [35–38]. Studies have considered a variety of alternative lipids, featuring different, possibly charged, head groups [39–43], and mixed bilayer compositions [44–50]. Despite their simplicity, bilayers built from

PC lipids represent remarkable test systems to probe the computation methodology and to gain additional insight into the physical properties of membranes [15,18,51,52].

Modeling membranes electroporation

The effects of an electric field on a cell may be described considering the latter as a dielectric layer (cell surface membrane) embedded in conductive media (internal: cytoplasm and external: extracellular media). When relatively low-field pulses of microsecond or millisecond duration are applied to this cell (by placing for instance the cell between two electrodes and applying a constant voltage pulse) the resulting current causes accumulation of electrical charges at both sides of the cell membrane. The time required to charge the surface membrane is dependent upon the electrical parameters of the medium in which it is suspended. For a spherical cell it is estimated using equivalent network RC circuits in the 100 ns time scale [20,53–56]. A charging time constant in the range of hundreds of nanoseconds was also obtained from derivations based on the Laplace equation (see e.g. [57] for the first-order analysis on a spherical vesicle; [58] for the second-order analysis; and [59] for the second-order analysis for two concentric spherical vesicles *i.e*. modeling an organelle), see also Chapter 1. If on the other hand, the pulse duration is short enough relative to the charging time constant of the resistive-capacitive network formed by the conductive intracellular and extracellular fluids and the cell membrane dielectric, which is the case for nanosecond pulses, then the response of the system is mainly dielectric and is linked to the polarization of the interfacial water (see below).

Simulations allow ones to perform *in silico* experiments under both conditions, *i.e* submitting the system either to nanosecond, megavolt-per-meter pulsed electric fields or to charge imbalance, mimicking therefore the application of low voltage – long duration pulses. In the following we will describe the results of such simulations.

Electroporation induced by dirrect effect of an electric field

In simulations, it is possible to apply "directly" a constant electric field $\vec{E}$ perpendicular to the membrane (lipid bilayers) plane. In practice, this is done by adding a force $\vec{F} = q_i\vec{E}$ to all the atoms bearing a charge q_i [60]. MD simulations adopting such an approach have been used to study

membrane electroporation, lipid externalization [61], to activate voltage-gated K^+ channels [62] and to determine transport properties of ion channels [63–66].

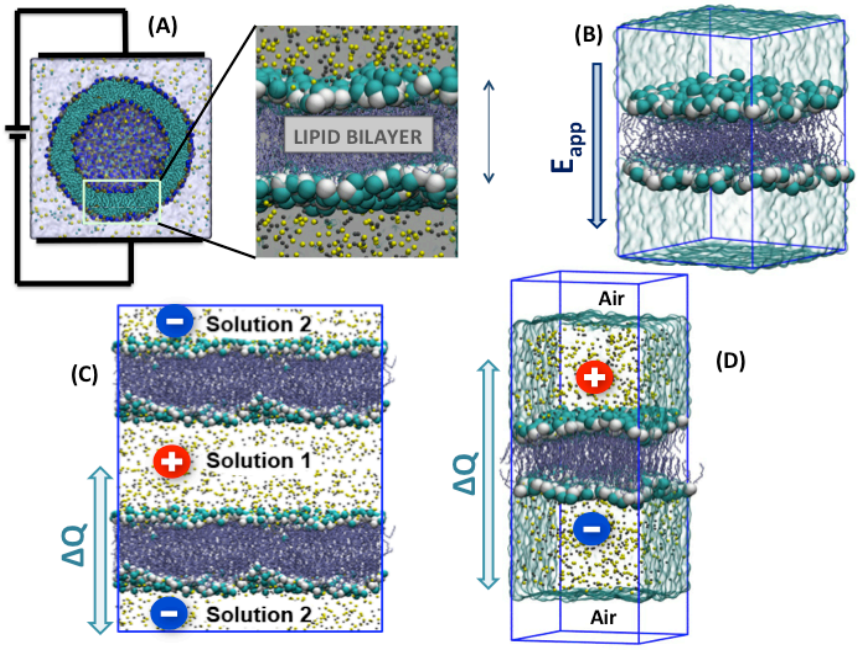

Figure 1. Protocols for atomistic modelling of cell membranes or liposomes lipid bilayers (A) electroporation; (B) nsPEFs (PEF – pulsed electric field) protocol: the system is modeled in absence of salt, and subject to an electric field E_{app} perpendicular to the bilayer (z axis). Note that in some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

The consequence of such perturbation stems from the properties of the membrane and from the simulations set-up conditions: lipid membranes exhibit heterogeneous atomic distributions across the bilayer to which are associated charges and molecular dipoles distributions. Phospholipid head-groups adopt in general a preferential orientation. For hydrated PC bilayers at temperatures above the gel to liquid crystal transition, the phosphatidyl-choline dipoles point on average 30 degrees away from the membrane

normal [67]. The organization of the phosphate (PO_4^-), choline ($N(CH_3)_3^+$) and the carbonyl (C=O) groups of the lipid head group give hence rise to a permanent dipole and the solvent (water) molecules bound to the lipid head group moieties tend to orient their dipoles to compensate the latter [68]. The electrostatic characteristics of the bilayer may be gathered from estimates of the electrostatic profile $\phi(z)$ that stems from the distribution of all the charges in the system. $\phi(z)$ is derived from MD simulations using Poisson's equation and expressed as the double integral of $\rho(z)$, the molecular charge density distributions:

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

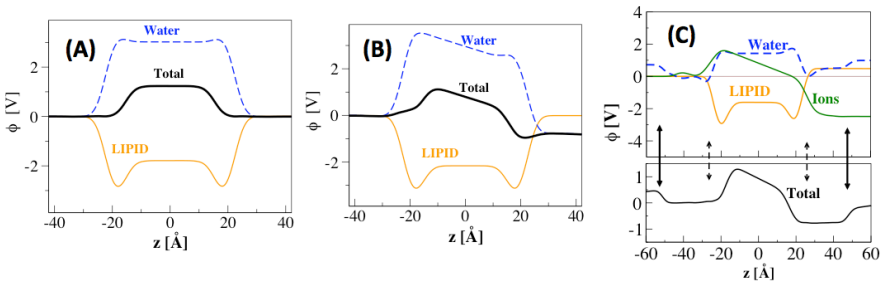

Figure. 2 Electrostatic potential profiles ϕ(z) along the membrane normal (z axis) of a POPC (*palmitoyl-oleoyl phosphatidylcholine*) lipid bilayer. Bilayer (A) at rest, (B) subject to a transverse electric field (nsPEF protocol), and (C) bilayer set with a charge imbalance (μs-msPEF protocol). z=0 represents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

For lipid bilayers, most of which are modelled without consideration of a salt concentration, an applied electric field acts specifically and primarily on the interfacial water dipoles (polarization of water molecules). The reorientation of the lipid head groups appears not to be affected at very short time scales [22,69] and not exceeding few degrees toward the field direction at longer time scales [23]. Hence, within a very short time scale - typically few picoseconds [21] –a transverse field $\vec{E}$ induces an overall TM potential $\boldsymbol{\Delta V}$ (*cf.* Fig. 2). It is very important to note here that, because of the MD

simulation setup (the use of PBCs), $\vec{E}$ induces $\Delta \boldsymbol{V} \approx |\vec{E}|.L_Z$ over the whole system, where L_z is the size of the simulation box in the field direction. In the example shown in Fig. 2, L_z is ~ 10 nm. The electric field (0.1 V/.nm) applied to the POPC bilayer induces $\Delta \boldsymbol{V}$ ~ 1V.

MD simulations of pure lipid bilayers have shown that the application of electric fields of high enough magnitude leads to membrane electroporation, with a rather common poration sequence (*cf.* Fig. 3): The electric field favours quite rapid (within a few hundred picoseconds) formation of water defects and water wires deep into the hydrophobic core [21]. Ultimately water fingers forming at both sides of the membrane join up to form water channels (often termed pre-pores or hydrophobic pores) that span the membrane. Within nanoseconds, few lipid head-groups start to migrate from the membrane-water interface to the interior of the bilayer, stabilizing hydrophilic pores (~1 to 3 nm diameter).

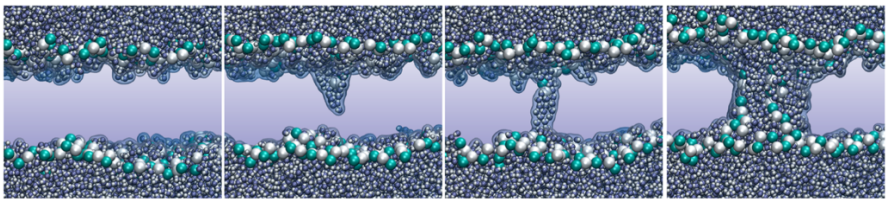

Figure. 3 Pore evolution in a POPC bilayer: The POPC headgroups are shown as cyan and white beads, the lipids tails are not show for clarity. The pore creation, in MD simulations, takes places in the range of nanoseconds.

All MD studies reported pore expansion as the electric field was maintained. In contrast, it was shown in one instance [22] that a hydrophilic pore could reseal within few nanoseconds when the applied field was switched off. Membrane's complete recovery, *i.e* migration of the lipid head group forming the hydrophilic pore toward the lipid/water interface, being a much longer process, was not observed. Systematic studies of pore creation and annihilation life time as a function of field strength have shed more light onto the complex dynamics of pores in simple lipid bilayers [23,70]. Quite interestingly, addition of salt has been shown to modulate these characteristic time scales [71].

For typical MD system sizes (128 lipids; 6 nm x 6 nm membrane cross section), most of the simulations reported a single pore formation at high field strengths. For much larger systems, multiple pore formation with diameters ranging from few to 10 nm could be witnessed [21,22]. Such pores are in principle wide enough to transport ions and small molecules. The first attempt has so far been made to investigate such a molecular

transport under electroporation [22]. In this simulation, partial transport of a 12 base pairs DNA strand across the membrane could be followed. The strand considered diffused toward the interior of the bilayer when a pore was created beneath it and formed a stable complex DNA/lipid in which the lipid head groups encapsulate the strand. The process provided support to the gene delivery model proposed by Golzio *et al.* [72] in which, an ''anchoring step'' connecting the plasmid to permeabilized cells membranes that takes place during DNA transfer assisted by electric pulses, and agrees with the last findings from the same group [73]. Few years later, (see sections below) it was shown that even a single 10 ns electric pulses of high enough magnitude can enhance small siRNA transport through lipid membranes [74] see also chapters 7 and 8.

The eletroporation process takes place much more rapidly under higher fields, without a major change in the pore formation characteristics. The lowest voltages reported to electroporate a PC lipid bilayer are ~ 2 V [23,69] Ziegler and Vernier [24] reported minimum poration external field strengths for 4 different PC lipids with different chain lengths and composition (number of unsaturations). The authors find a direct correlation between the minimum porating fields (ranging from 0.26 V/nm to 0.38 V/nm) and the membrane thickness (ranging from 2.92 nm to 3.92 nm). Note that estimates of electroporation thresholds from simulations should, in general be considered only as indicative since it is related to the time scale the pore formation may take. A field strength threshold is assumed to be reached when no membrane rupture is formed within a100 ns time scale.

Electroporation induced by ionic salt concentration gradients

Regardless of how low intensity millisecond electrical pulses are applied, the ultimate step is the charging of the membrane due to ions flow. The resulting ionic charge imbalance between both sides of the lipid bilayer is locally the main effect that induces the TM potential. In a classical set up of membrane simulations, due to the use of 3D PBCs, the TM voltage cannot be controlled by imposing a charge imbalance Q_s across the bilayer, even when ions are present in the electrolytes. Several MD simulations protocols that can overcome this limitation have been devised (Fig. 1):

The double bilayer setup:

TM potential gradients can be generated by a charge imbalance across lipid bilayers by considering a MD unit cell consisting of three salt-water baths

separated by two bilayers and 3d-PBCs [75] (cf. Fig. 1C). Setting up a net charge imbalance between the two independent water baths at time t=0 induces a TM voltage $\Delta \boldsymbol{V}$ by explicit ion dynamics.

The single bilayer setup:

Delemotte *et al*. [76] introduced a variant of this method where the double layer is not needed, avoiding therefore the over-cost of simulating a large system. The method consists in considering a unique bilayer surrounded by electrolyte baths, each of them terminated by an air/water interface [44]. The system is set-up as indicated in Fig. 1D. First, a hydrated bilayer is equilibrated at a given salt concentration using 3d periodic boundary conditions. Air water interfaces are then created on both sides of the membrane, and further equilibration is undertaken at constant volume, maintaining therefore a separation between the upper and lower electrolytes. A charge imbalance Q_s between the two sides of the bilayer are generated by simply displacing at time t = 0 an adequate number of ions from one side to the other. As far as the water slabs are thicker than 25-30 Å, the presence of air water interfaces has no incidence on the lipid bilayer properties and the membrane "feels" as if it is embedded in infinite baths whose characteristics are those of the modelled finite solutions.

Fig. 2 reports the electrostatic potential profiles along the normal to the membrane generated from MD simulations a POPC bilayer in contact with 1M NaCl water baths at various charge imbalances Q_s, using the single bilayer method. For all simulations, the profiles computed at the initial stage show plateau values in the aqueous regions and, for increasing Q_s, an increasing electrostatic potential difference between the two electrolytes indicative of a TM potential $\Delta \boldsymbol{V}$. Quite interestingly, the profiles show that, in contrast to the electric field case where the TM voltage is mainly due to the water dipole reorientation, most of the voltage drop in the charge imbalance method is due to the contribution from the ions. Indeed the sole collapse of the electrostatic potential due to the charge imbalance separation by the membrane lipid core accounts for the largest part of $\Delta \boldsymbol{V}$.

Using the charge imbalance set-up, it was possible for the first time to directly demonstrate *in silico* that the simulated lipid bilayer behaves as a capacitor [76,77]. Simulations at various charge imbalances Q_s show a linear variation of $\Delta \boldsymbol{V}$ from which the capacitance can be estimated as $C = Q_s . \Delta \boldsymbol{V}^{-1}$. The capacitance values extracted from simulations are expected to depend on the lipid composition (charged or not) and on the force field parameters used and as such constitute a supplementary way of checking the accuracy of lipid force field parameters used in the simulation. Here, in

the case of POPC bilayers embedded in a 1M solution of NaCl, the later amounts to 0.85 μF/cm^2 which is in agreement with the value usually assumed in the literature *e.g*. 1.0 μF/cm^2 [75,78] and with measurements for planar POPC lipid bilayers in a 100 mM KCl solution (0.5 μF/cm^2).

For large enough induced TM voltages, the three protocols lead to electroporation of the lipid bilayer. As in the case of the electric field method, for ΔV above 1.5-2.5 V, the electroporation process starts with the formation of water fingers that protrude inside the hydrophobic core of the membrane. Within nanoseconds, water wires bridging between the two sides of the membrane under voltage stress appear. If the simulations are further expended, lipid head-groups migrate along one wire and form a hydrophilic connected pathway (Fig. 3). Because salt solutions are explicitly considered in these simulations, ion conduction through the hydrophilic pores occurred following the electroporation of the lipid bilayers. Details about the ionic transport through the pores formed within the bilayer core upon electroporation could be gathered.

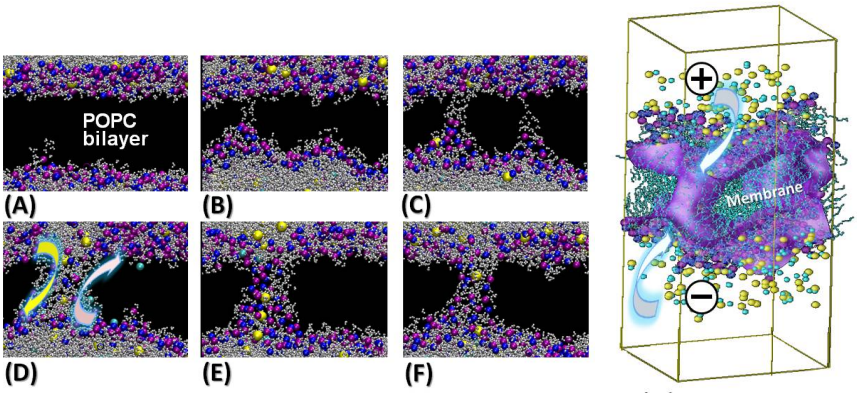

Figure. 4 *Left* Sequence of events following the application of a TM voltage to a POPC lipid bilayer using the charge imbalance method (panels A to F). Note the migration of Na+ (yellow) and Cl- (cyan) ions through the formed hydrophilic pores that are lined with lipid phosphate (magenta) and nitrogen (blue) head group atoms. Panel F represents the state of a non conducting pore reached when the exchange of ions between the two baths lowered Q_s and therefore ΔV to values ≈ 200 mV. *Right* Topology of the nanometer wide hydrophilic pores formed under high ΔV in the planar bilayer. The arrows highlight the ionic flow through the pore.

The MD simulations of the double bilayer system [79,80], and the results presented here for the single bilayer set-up show that both cations and anions exchange through the pores between the two baths, with an overall flux of charges directed toward a decrease of the charge imbalance. Ions

translocation from one bulk region to the other lasts from few tens to few hundreds ps and leads to a decrease of Q_S and therefore to the collapse of $\Delta \boldsymbol{V}$. Hence, for all systems, when Q_S reaches a level where the TM voltage drops down to a couple of hundred mV, the hydrophilic pores "close" in the sense that no more ionic translocation occurs (Fig. 4F). The final topology of the pores remain stable for time spans exceeding the 10 nanoseconds scale, showing as reported in previous simulations [22] that the complete recovery of the original bilayer structure requires a much longer time scale.

Note that to maintain $\Delta \boldsymbol{V}$ constant the we needs to maintain the initial charge imbalance by "injecting" charges (ions) in the electrolytes at a paste equivalent to the rate of ions translocation through the hydrophilic pore. This protocol is, in particular for the single bilayer setup, adequate for performing simulations under constant voltage (low voltage, ms duration) or constant current conditions, which is suitable for comparison to experiments undertaken under similar conditions [81].

Internal electric field distribution and origin of membrane electroporaiton

To determine the detailed mechanism of the pore creation, it is helpful to probe the electric filed distribution across the bilayer, both at rest and under the effect of a TM voltage. Fig. 5A displays the electrostatic potential profiles for a lipid bilayer subject to increasing electric fields that generate TM potentials ranging from 0 V to ~ 3 V. At 0 V, the lipid bilayer is at rest and the profiles reveal, in agreement with experiment [82], the existence of a positive potential difference between the membrane interior and the adjacent aqueous phases.

At rest, the voltage change across the lipid water interfaces gives rise locally to large electric fields (in the present case up to 1.5 V/nm) oriented toward the bulk, while at the center of the bilayer, the local electric field is null (Fig. 5B,5C). When external electric fields of magnitudes respectively of 0.06 and 0.30 V/nm are applied, reorientation of the water molecules gives rise to TM potentials of respectively ~ 0.75 and 3 V. Figs 5B and 5C reveal the incidence of such reorganization on the local electric field both at the interfacial region and within the bilayer core. In particular one notes that the field in the membrane core has risen to ~ 1 V/nm for the highest $\Delta \boldsymbol{V}$ imposed.

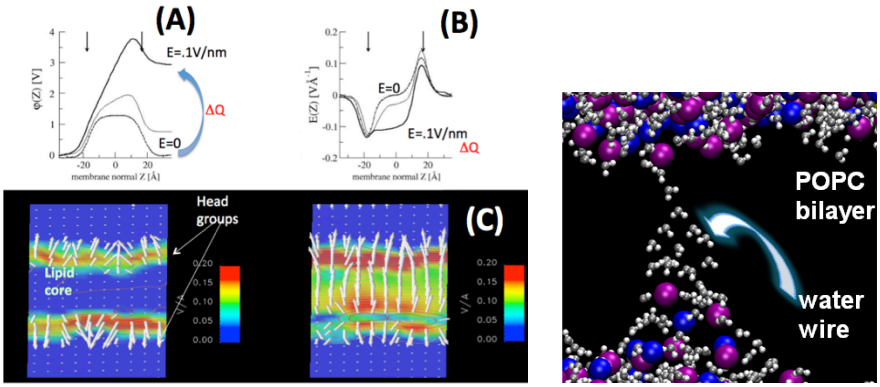

Figure. 5 (A) Electrostatic potential profiles across a lipid bilayer subject to electric fields of 0 V/nm (dotted line) 0.06 V/nm (thin line) and 0.30 V/nm (bold line), or to a charge imbalance ΔQ. (B) Corresponding electric field profiles. (C) 2D (out of plane) maps of the electric field distribution.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

For the charge imbalance method, the overall picture is similar, where again, the TM voltages created give rise within the membrane core to large electric fields oriented perpendicular to the bilayer.

Qualitatively, in both methods, the cascade of events following the application of the TM voltage, and taking place at the membrane, is a direct consequence of such a field distribution. Indeed, water molecules initially restrained to the interfacial region, as they randomly percolate down within the membrane core, are subject to a high electric field, and are therefore inclined to orient their dipole along this local field. These molecules can then easily hydrogen bond among themselves, which results in the creation of single water wires. Such water wires protrude through the hydrophobic core from both sides of the membrane. Finally, these water wires meet up to form water channels (often termed pre-pores or hydrophobic pores) that span the membrane. As the TM voltage is maintained, these water wires appear to be able to overcome the free energy barrier associated to the formation of a single file of water molecules spanning the bilayer (estimated to be ~ 108 kJ/mol in the absence of external electric field [83]. As the electrical stress is maintained, lipid head group migrate along the stable

water wires and participate in the formation of larger "hydophilic pores", able to conduct ions and larger molecules as they expend.

Ziegler *et al.* [24] have shown clearly that the orientation of the lipid headgroups (dipoles) is not a determinant factor in the EP process. The general assumption that the lipid headgroups have a marginal role in the formation of the electropores, is consistent with studies on octane [21] as well as vacuum slabs [84] electroporation: these works have shown that, as in lipid bilayers, water columns can form in any water/low-dielectric/water system subject to high electric fields.

Experimental evidence shows that pores do close when the PEF is turned off. The kinetics of this process determines how long leakage from or delivery to targeted cells can last. MD simulations indicate that this process initiates with a collapse of the pore (closure) due to a rapid leakage of water outwards to the bulk, followed by a much slower reorganization that leads to lipid headgroups re-partitioning toward the external hydrophilic leaflets. Resealing kinetics is independent of the magnitude of the pore initiation electric fields. In general, complete recovery of the original bilayer structure requires a much longer time scale [22,84,85], spanning from nanoseconds to hundreds of nanoseconds, and depends critically on the structure of the bilayer [86]. Note that addition of salt to systems undergoing the nsPEF protocol has been shown to modulate the characteristic time scales of the whole pore life cycle [85,87].

Complex bilayer models: EP thresholds and pore features

Electroporation thresholds

Since the pioneering simulations [22,88], which considered simple bilayers of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and dimyristoyl-phosphatidylcholine (DMPC) lipids, a variety of lipid bilayers have been modeled in order to understand the key elements that might modulate their electroporation thresholds. The increase of the EP threshold upon addition of cholesterol [89–91] was studied using the $\boldsymbol{E}$ field [92] and charge imbalance protocols [90]. For the former, a steady increase of the EP threshold coincides with an increase in cholesterol concentration: a two folds higher electric field was necessary for the electroporation of bilayers with the addition of 50 mol% cholesterol. Under μs-msPEFs conditions, the EP threshold was showed to level-off above 30 mol% cholesterol. Generally, the increase of the EP threshold has been linked to the increase of the stiffness of the bilayer [89,91].

In a series of papers [93,94] Tarek's group investigated the effect on the EP threshold of ester and ether linkages, of branched (phytanoyl) tails, and of bulky (glucosyl-myo-inositol and myo-inositol) lipid head groups. The authors have found that the EP threshold of a lipid bilayer depends not only on the ''electrical'' properties of the membrane, *i.e* its dipole potential or membrane capacitance, but also on the nature of lipids hydrophobic tails. The authors report that there is a correlation between the lateral pressure in the water/lipid interface and the EP threshold. They suggest that an increase of the lateral pressure (in the branched lipid membrane compared with the simple lipid bilayers) hinders the local diffusion of water molecules toward the interior of the hydrophobic core, which lowers the probability of pore formation, increasing therefore the electroporation threshold.

Comparing specifically archeal lipids (glucosyl-myo and myo inositol head groups) to normal PC lipid, the higher electroporation thresholds for the former was attributed [93,94] to the strong hydrogen-bonding network stabilizing the head-group head group interactions. Likewise, Gurtovenko *et al.* [95] reported higher EP threshold for phosphatidylethanolamine (PE) lipid bilayers compared to phosphatidylcholine (PC) lipid bilayers. This effect was linked to inter-lipid hydrogen bonding taking place in the PE bilayer, which leads to a denser packed water/lipid interface and more ordered hydrocarbon lipid chains. Considering an asymmetric bilayer, composed by PC and PE lipid leaflets, the authors observed that the initial electroporation feature, *i.e* the water wires formation is also asymmetric, with initial steps taking place primarily at the PC leaflet. Studying more complex composition membranes, Piggot *et al.* [96] reported that the Gram-positive bacterial S. aureus cell membrane is less resistant to poration than the Gram-negative bacterial E.coli outer membrane (EcOM). The higher EP threshold of the EcOM was linked to the reduced mobility of the Lipopolysacharide molecules that are located in the outer leaflet. Additional factors, such as cholesterol, the presence of impurities, and other compounds, can modify the permeation properties of membrane models by acting on their stability.

Pore features

The MD results support the hypothesis that following the application of a high transmembrane voltage, the cell membrane is permeabilized by the formation of conducting hydrophilic pores stabilized by the lipid headgroups. The properties of the lipids play a determinant role in the electropores life-time and in its structural characteristics (e.g. size, shape, morphology) [84]. Other studies, considering various lipid bilayers,

challenged the standard pore morphology. Tarek and coauthors pointed out that a peculiar EP process may be possible in which large long living ion-conducting water columns are not stabilized by lipid headgroups [90,94,97]. These "hydrophobic" conducting pores originate from constraints of a different nature in the lipid bilayer. The first report [97] focused on a palmitoyl-oleyl-phosphatidylserine (POPS) bilayer characterized by negatively charged headgroups. When this system was subject to a charge imbalance high enough to electroporate the bilayer, the migration of lipids along the water column turn out to be largely hindered (Fig. 6, second panel [97]). Similar conclusions were drawn for PC lipid bilayers containing more than 30 mol % cholesterol [90] or for Archaeal lipids [94] (Fig. 6). This peculiar morphology was ascribed to the repulsion of negatively charged headgroups in the first case [97], to the condensing effect of cholesterol in the second [90], and to the steric hindrance of the bulky headgroups coupled with the branched tails in the latter [94]

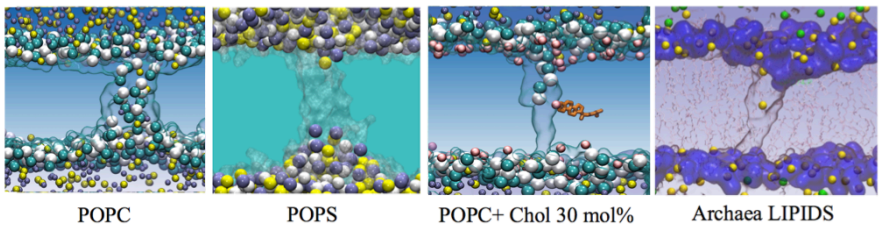

Figure 6. Various morphologies of conducting pores revealed by MD simulations. Note that beside the POPC zwitterionic lipids, pores formed in POPS, a negatively charged lipid, with addition of cholesterol, or in the complex archaea lipids (sugar like head groups), the electropores are not stabilized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

Pore stabilization

When dealing with the characteristics of electropores (e.g. size, conductance, transport of molecules) one would expect the pore to be in an energetically favorable state, *i.e* one that corresponds to a stable configuration. To understand if the pore can be considered in a steady state for a given TM voltage and characterize its size and conductance, the two MD procedures introduced in previous sections need to be improved. Indeed, the main drawback of these two protocols, as usually used, resides in the impossibility of maintaining a stable pore. In the electric field method, the pore tends to expand, leading to the breakdown of the bilayer, when it reaches the dimensions of the simulation cell box. The charge imbalance protocol, on the other hand, suffers from an important shortcoming: the imbalance is not re-set during the simulation. Thus, in the studies carried

out both with the double and single bilayer schemes, the charge imbalance imposed at the beginning decreases significantly within several tens/hundreds ps (depending on the system size) of EP due to an exchange of ions through the pore. The decrease of the charge imbalance results in a TM voltage drop, which ultimately leads to pore collapse and resealing.

When using the nsPEF protocol, the lowering of the electric field intensity after pore creation was shown to result in its stabilization [23]. Using the same strategy, Fernández *et al*. [92] could modulate the size of the pore and showed that it depends only on the strength of the stabilizing electric field. Our group [98] used a scheme to maintain a constant charge imbalance, refining thereby the μs-msPEFs approach to obtain size-controlled steady pores. The protocol used is identical to the procedure proposed by Kutzner *et al*. [81] to study the transport in ion channels using the double layer scheme. In this procedure, named "swapping", the number of ions in the two solution baths is frequently estimated and, if the latter differs from the initial setup, a "swapping" event takes place. An ion of one solution is exchanged by a water molecule of the other solution bath (see the supplementary material for more information). Note that to overcome the limitation of simulating the bilayer in the NVT ensemble (constant volume), the swapping procedure can be coupled with the NPγT ensemble (constant surface tension) to maintain the bilayer surface tension constant (null) and mimic, therefore, experimental conditions [98].

Pore characterization

A first attempt to link experimental evidence of pore conductance and radius estimation was carried out by Kramar *et al*. using a linear rising current technique combined with MD simulations performed under similar conditions [99]. Their findings suggest that the opening and closing of a single pore under conductance in the 100-nS scale would be possible for a pore diameter of ~5 nm.

More systematic investigations, using the nsPEF [92,100] and μs-msPEF [98] modified protocols allowed to better characterize the conductance of electroporesforming in lipid bilayers. For simulations carried out under the two protocols and when applying TM voltages below the EP threshold, the pore formed could be stabilized to different radii for tens of ns. Quite interestingly, the pore radii, and the pore conductance were found to vary almost linearly with the applied voltage. Moreover, the pores were found to be more selective to cations than to anions [98,100,101]. This selectivity arises from the nature of the lipid molecules constituting the pore: the negatively charged phosphate groups that form the walls of the pore attract sodium ions, which hinders their passage across the bilayer, but also makes

the pore interior electrostatically unfavorable for other sodium ions [102]. This, already, suggests that the transport through electropores is sensitive to the type of solutes, showing a different affinity for different charged species.

The pores created in cell membranes are highly dynamic. Their sizes and stability strongly dependent on characteristics of the applied transmembrane voltage [103,104]. Several attempts have been made to measure pore radii by monitoring the selective uptake of molecules of different sizes, *e.g* propidium iodide, YO-PRO-1, bleomycin, sugars, … particularly in cells. However this approach remains questionable, as molecular probes might strongly interact with the lipid bilayer and perturb the pore configuration while diffusing [105–107]. The transport of such molecules across cell membranes needs to be monitored for seconds or minutes after applying pulses. The molecular mechanisms relating to cell membranes permeability in such post-pulse resealing step are yet to be elucidated [108].

As an alternative, conductance measurements can in principle help characterize pores of small sizes. However, in cells, macroscopic currents subject to electric fields generally report conductance through a population of many pores as well as potentially through ion channels [109]. Conductance of single pores can be monitored by imposing of a constant electric current to model membranes. However, accurate characterization of the pore properties from conductance measurements requires the use of a valid and reliable theoretical model, which can quantitatively predict the pore conductance. Typically, more or less simplified expressions derived from the coupled Nernst-Planck and Poisson's (PNP) equations are used to estimate pore sizes. A review of these models [110] and estimates from various experimental studies shows that pore sizes and conductances typically fall into the nm and nS ranges, respectively.

Transport of molecules

Although numerous molecules are implicated in EP and/or concerned by its applications (*e.g* drugs, genetic material, dyes, …), very few have been investigated with MD simulations. Apart from early studies in which electropore-mediated flip-flop of zwitterionic PC lipids [111–113] was reported, most simulations concerned charged species for which transport involved electrophoresis [22,74,105]. In the following, we discuss the results obtained using the two simulations protocols by us.

nsPEFs

nsPEFs can induce externalization of phosphatidylserine (PS), a phospholipid usually confined to the inner leaflet of the plasma membrane that and can trigger recognition, binding and signaling functions. MD studies of PS bilayers [20,114] showed how PS lipids externalization is a pore-mediated event occurring exclusively with an electrophoretic drift.

Two decade ago, Tarek [22] reported the first MD simulation on the transport of a short DNA double strand using high intense electric fields. It was shown that the uptake occurred only in presence of the pore by electrophoretic drift. Since then, to our knowledge, only two MD studies have been reported on the transport of molecules under nsPEFs. In 2012 Breton *et al*. [74] showed that a single 10 ns high-voltage electric pulse can permeabilize giant unilamellar vesicles (GUVs) and allows the delivery of a double-stranded siRNA (-42e charge, 13.89 kDa) through the formed pore, by electrophoresis (Fig. 7 [74]). Comparing experimental evidence with MD simulations we could show in particular that: (i) following the application of an electric field, the siRNA is pushed toward the lipid headgroups forming an siRNA- phospholipids headgroups complex that remains stable even when the pulse is switched off; (ii) no transport is detected for electric fields applied below the EP threshold; (iii) when the applied $\boldsymbol{E}$ is above the EP threshold the siRNA is electrophoretically pulled through the electropore and translocated within a 10 ns time scale; (iv) if the electric pulse is turned off before the complete transition, the pore collapses around the molecule which is, hence, trapped.

Salomone *et al*. [105] used a combination of nsPEFs and the chimeric peptides (CM18-Tat11) as efficient delivery vectors for plasmid DNA using endocytotic vesicles. To provide molecular details about the processes taking place, we modeled therein the peptide and its fragments. MD simulations showed that, when subject to high electric fields, Tat11, a small cationic peptide (residues 47-57 of HIV-1 Tat protein; +8e charge, 1.50 kDa) can translocate through an electroporated bilayer within few nanoseconds without interacting with the phospholipid headgroups. In contrast, the amphipathic peptide CM18, even when located near a preformed pore, remains anchored to the lipid headgroups and does not translocate during a 12 ns high electric field pulse.

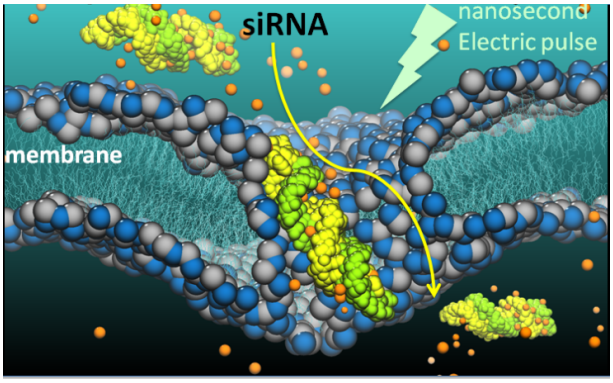

Figure 7: A single 10 ns high-voltage electric pulse can permeabilize lipid vesicles and allow the delivery of siRNA to the cytoplasm. Combining experiments and molecular dynamics simulations has allowed us to provide the detailed molecular mechanisms of such transport and to give practical guidance for the design of protocols aimed at using nanosecond-pulse siRNA electro-delivery in medical and biotechnological applications [74].

µs-msPEFs

We present below the latest results from MD simulations of the uptake of molecules through lipids bilayers subject to µs-msPEFs. We focus our attention on Tat11 and the siRNA double strand to compare their mechanism of transport to the one reported using the nsPEFs [74,105]. These data have been reported in [103].

Transport of siRNA

In 2011 Paganin-Gioanni *et al*. [73] investigated siRNA uptake by murine melanoma cells, when subject to electric pulses (1 Hz of repetition frequency) using time lapse fluorescence confocal microscopy. A direct transfer into the cell cytoplasm of the negatively charged siRNA was observed across the plasma membrane exclusively on the side facing the cathode. Noting that when added after electropulsation, the siRNA was inefficient for gene silencing because it did not penetrate the cell, the authors concluded that the siRNA transport takes place during the electric pulse and is due to electrophoresis through electropores. The same group reported also that 0.17 kV/cm - 5 ms pulses, named EGT, are more effective in terms of silencing than the more intense less lasting HV pulses (1.3 kV/cm - 0.1 ms). They showed on the other hand that a double pulse procedure, consisting of one HV followed by a long below-EP-threshold

pulse does not increase the efficiency of the delivery. Altogether, their evidence suggests that, for msPEFs, the key factors for an efficient delivery are the voltage above the EP threshold and the duration of the pulse.

To investigate the siRNA transfer into cells under conditions similar to the μs-msPEFs experiments, we have performed a set of simulations where the system was subject to several voltages (see Table 1). We first electroporate a bilayer patch by submitting it to a high charge imbalance. Once the pore was large enough (arbitrary value of ~2 nm radius) we lowered Q_S to stabilize it to different radii as in [98]. These configurations were then used to start the simulations with siRNA placed near the pore mouth and were continued at the desired voltage.

For the lowest transmembrane voltages Um run, the siRNA approached the large pore (~4 nm diameter) mouth then started sliding through it while interacting with the lipid headgroups lining it. The complete translocation of the siRNA did not occur however within the first 100 ns of the run. In a completely independent run, we repeated the simulation by maintaining a higher voltage, namely 550 mV. The siRNA approach, pore entry and sliding under these conditions (Fig. 7) were similar to the lower voltage run. However, at 550 mV despite its anchoring to the lipid headgroups, a complete translocation from the upper to the lower water bath occurred in ~30 ns. Two factors contributed probably to this speed up. Compared to the previous conditions, not only the electrophoretic force pulling the siRNA is indeed higher, but the pore size increases too under this higher voltage.

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

System	t_s (ns)	U_m (mV)	R (nm)	t_c (ns)
POPC_1024+siRNA	100	160 ± 160	2.0 ± 0.6	> 100
	35	550 ± 190	3.3 ± 0.2	32.5
POPC_1024+Tat11	40	430 ± 160	1.6 ± 0.2	32.8
	14	700 ± 240	2.0 ± 0.1	11.3

t_s – simulation time; U_m – transmembrane voltage induced by the charge imbalance; R – minimum pore radius maintained by a given U_m; t_c – crossing time of the molecule through the electropore (Adapted from [103]).

All together the simulations mimicking μs-msPEFs experiments, demonstrate that the translocation of siRNA through the pore driven by the application of TM voltages above 500 mV takes place in the nanosecond time scale, as reported for the nsPEFs. Noticeably, in both simulations

carried out under electric field or under the charge imbalance, the siRNA remains anchored to the lower leaflet of the membrane after translocation without diffusing in the bulk solution even if the voltage is maintained.

Experiments performed on mouse melanoma cells applying ms-long pulses evidenced that tuning the duration of the pulse is essential for an efficient siRNA uptake. In fact, the authors found more effective the EGT (0.17 kV/cm, 5 ms) class of pulses than the HV (1.3 kV/cm, 0.1 ms) one. No direct measurement of the TM voltage was carried out during these experiments and the authors assume that it is around 250 mV, since it was observed that the EP threshold value is always about 200 mV for many different cell systems [115]. Corroborated by our findings, one can speculate that the transport of siRNA when subject to longer pulses could be facilitated by the formation of a pore population having larger diameters. This population of larger pores would allow siRNAs to flow through the pore and to access directly the cytoplasm increasing the transport efficiency.

Transport of Tat11

The translocation for Tat11 differs from the highly charged siRNA because no specific interactions between this peptide and the lipid headgroups take place during the process, resulting in a faster uptake. Under a TM voltage U_m 700 mV, the molecule, initially parallel to the membrane and located near the pore opening, first rotates to align its dipole along the local electric field (Fig. 8, $t = 0$ ns), then drifts though the center of the pore with a radius of 2 nm (Fig. 8, $t = 8$ ns), over the same time scale reported by the nsPEFs procedure [105]. The Tat11 reaches the lower bath where it freely diffuses (Fig. 8, $t = 12$ ns). At lower U_m (430 mV) Tat11 translocates in 32.8 ns (see Table 1), presumably as a consequence of a higher hindrance of the pore (the pore radius decreases to 0.4 nm) and of a reduction of the electrophoretic drift.

Considering a patch of 256 lipids, and applying an electric field that generates a 1.6 V across the bilayer, Salomone *et al.* [105] reported that Tat11 translocates through an electropore within 10 ns. This seems inconsistent with our results since one should expect that under our conditions, *i.e* subject to a voltage U_m of 430 mV, the time needed for Tat11 transport would be much longer. Indeed, if one considers only the ratio of electrophoresis, translocation of Tat11 should be three times slower at the lower voltage. In addition, a second inconsistency concerns the sizes of the pores created. Indeed in [105] the pore created has a radius of ~1.7 nm, much smaller than one expected from our results: we generated a pore of radius ~1.6 nm under U_m 430 mV (Table 1). We have identified and reported size effects in simulations of lipid bilayers electroporation and hav

specifically shown [103] that patches of 256 lipids are too small to study electroporation: Pores generated in MD simulations using such patches are much smaller than those generated using larger patches (1024 lipid).

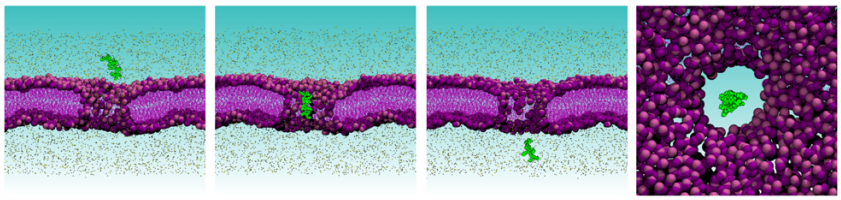

Figure. 8 The process of Tat_{11} transport in three frames corresponding to 0, 8, and 12 ns. In the right panel the top view clearly shows no interactions between the molecule and the pore walls. The POPC headgroups are shown as mauve and violet beads, the tails as purple lines; sodium and chloride ions are colored in yellow and gray; Tat_{11} is green (adapted from [103]).

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

Membranes's oxidation

While there is a consensus that the TMVs induced by an electric field promote the formation of pores in lipid bilayers, the long-standing assumption that the pores created during the pulse are the main transport mean is now highly questioned. The electric-field-induced "electropores" do not seem to be able to explain all aspects of the electroporation phenomenon. Specifically, the mechanisms of sustained permeability of cell membranes, which persists long after pulse application, remain elusive: We do know from experimental investigations that the complete resealing of the cell membrane takes several minutes at room temperature [116–118], which is about 8−9 orders of magnitude longer than the time of electropore closure as reported from MD investigations [85,119].

It has been suggested that pores might retain their permeability as they evolve into more complex pores formed by lipids and other molecules [105–107]. Another possibility is that the long-lived permeability does not involve pores at all, but instead is mediated by leaky peroxidized membrane lesions. Several studies suggest that the long-lived permeability of cell

membranes might be due to lipid peroxidation [120–132] see also Chapter 2.

Electroporation accompanied by lipid peroxidation has been reported for bacteria [120,121], plant cells [122,123], and mammalian cells [123–125], as well as in liposomes made from polyunsaturated lipids [124–127]. Lipid peroxidation can be promoted by reactive oxygen species (ROS) already present in the solution before the delivery of electric pulses [126], although the exact mechanisms are not yet determined. Moreover, electric pulses can induce extracellular (electrochemical) [127,132] as well as intracellular [128–132] ROS generation. The latter is a consequence of the cellular response to the pulse treatment [129]. Both ROS concentration and the extent of lipid peroxidation increase with the electric field intensity, the pulse duration, and with the number of pulses [123–131] and are correlated with the cells' membrane permeability and cells' death [124,125,128–131].

Lipid peroxidation refers to the oxidative degeneration of lipids, which results from the formation of a hydroperoxide group in the lipid tails. The hydroperoxide group forms in a chain of reactions (Fig. 9), which are initiated when a free radical attacks a weak allylic or bis-allylic C−H bond [133,134]. During this chain of reactions, the intermediate peroxidation products propagate the radical damage to adjacent molecules, which means that a single free radial attack can lead to peroxidation of a patch of lipid molecules.

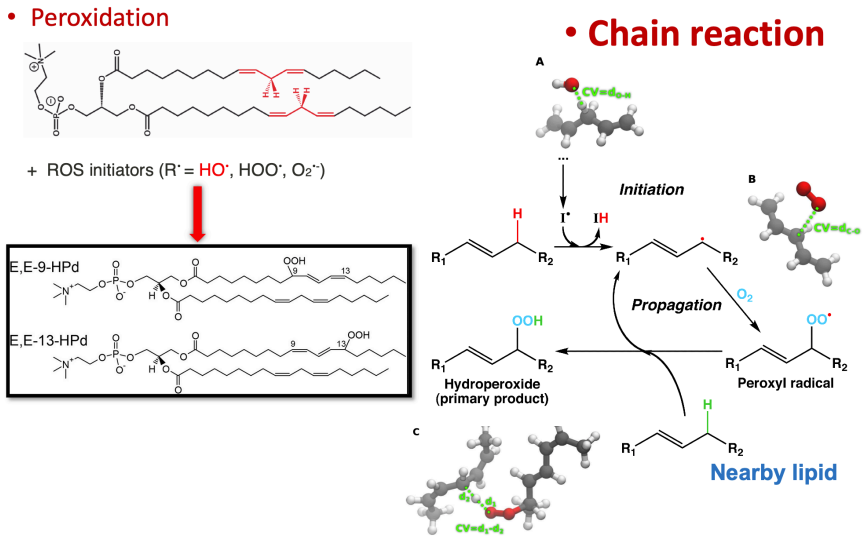

Figure. 9 Schematic pathway for lipid membranes peroxidation (righ panel) and primary products of the DLPC peroxidation products (inset). See text for details

Quantitative assessment of the role of lipid peroxidation in electropermeabilization requires the characterization of the type and amount of lipid peroxidation products in electropermeabilized cell membranes, as well as the quantification of the permeability of the peroxidized parts of the membrane. Recently, we made the first important step in this direction by quantifying the permeability and conductance of peroxidized lipid bilayer patches to sodium and chloride ions using MD simulations and comparing our results with experimental measurements on electropermeabilized cells [135].

We consider herein that exposure of a cell to electric pulses leads to formation of peroxidized lesions in the cell membrane. Such lesions can be formed in membrane domains rich in unsaturated lipids. These peroxidized lesions act as "hotspots" with high permeability and conductance, allowing locally enhanced transmembrane transport.

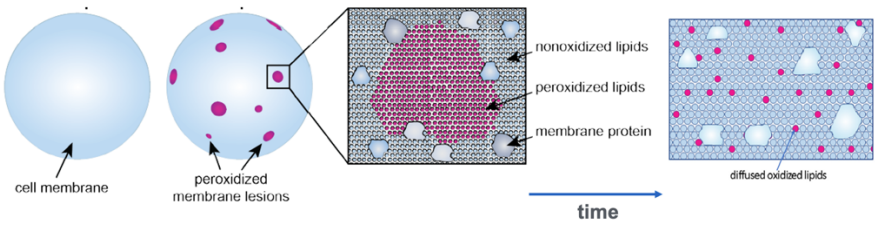

Figure 10. Schematic representation of peroxidized membrane lesions, which are expected to be formed in a cell membrane after exposure to electric pulses. The schematic is hypothetical, and the lesions are not drawn to scale. The squares depicts the molecular organization in one of the lesions (Adapted from [135]).

We studied DLPC (1,2-dilinoleoyl-sn-glycero-3-phosphocholine), a polyunsaturated lipid and its hydroperoxide derivatives (Fig. 9). These can be considered as relevant cell membrane models, since polyunsaturated lipids are the main targets of lipid peroxidation and linoleic acid is one of the most abundant polyunsaturated fatty acids found in mammalian cells.

We calculated the permeability and conductance of bilayer patches containing hydroperoxide lipid derivatives and compared them to experimental measurements on electropermeabilized cells [135]. The calculated values agree rather well for the pure (non peroxidized) lipids. The data analyses show that the permeability and conductance increase dramatically with increasing % of peroxidized lipids: Hence, the

permeability for Na^+ increases by up 8 orders of magnitude, and that of for Cl^-, up to 5 orders of magnitude. The increase in permeability is reflected in the increased membrane conductance (up to 5 orders of magnitude) with respect to the conductance of the non-oxidized DLPC bilayer.

Let's recall that experimental measurements are carried out over the entire cell membrane surface, whereas the bilayer systems investigated in MD are representative of a small part of a peroxidized lesion. If the area of peroxidized lesions in electropermeabilized cell membranes was known, we could calculate the total ionic transport through the lesions and compare it directly to the measurements. This would allow us to estimate the fraction of the ionic transport that can be attributed to lipid peroxidation. However, the area of the lesions is unknown. Therefore, we estimate instead the fraction of the cell membrane that would need to be peroxidized to account for the experimental measurements.

The comparison between the calculated conductance of peroxidized bilayers and experimental measurements suggests that lipid peroxidation could have a measurable effect on the membrane conductance, equal to the lowest measured values in electropermeabilized cells, even if only 1% of the cell membrane was peroxidized. However, the conductance of peroxidized bilayers calculated in our study is too low to account for the entire range of experimental values. The possibility that part of the experimentally measured conductance was mediated by ion channels, which were (de)activated or modified due to electric field exposure, cannot be completely excluded [136,137]. Nevertheless, it is unlikely that ion channels were the dominant conduction pathway, since similar change in conductance was observed in GH3 and CHO cells, whereby CHO cells express very few endogenous ion channels [138]

The hydroperoxide lipid derivatives ultimately reorganize and decompose into secondary products such as the cytotoxic 4-hydroxynonenal [139] and the mutagenic malondialdehyde [140]. The presence of oxidized lipids in a lipid membrane decreases the lipid order, lowers the phase transition temperature, leads to lateral expansion and thinning of the bilayer, alteration of bilayer hydration profiles, increased lipid mobility and augmented flip-flop, influences lateral phase organization and promotes formation of water defects [141–143].

We extended therefore our investigation to study the permeability of membranes that underwent secondary oxidation. Indeed, oxidative lipid damage can result in various products with truncated lipid tails ending with either an aldehyde or carboxylic group [134,144]. Hydroperoxides are converted into secondary products like 2-nonenal and 1-Hexadecanoyl-2-(9-oxo-nonanoyl)-sn-glycero-3-phosphocholine (PoxnoPC) by Hock fragmentation [145]. The 2-nonenal might further be converted to 4-HNE

(4-hydroxynonenal) by reacting with a radical and oxygen, thus potentially contributing to the kinetic's oxidation process (positive feedback loop). Previous MD simulations showed that oxidized lipids with an aldehyde group disturb the bilayer more than the ones with a peroxide one [143]. Bilayers of lipids with aldehyde-truncated tails on the other hand undergo spontaneous pore formation within a few hundred ns and lead in some cases to the bilayer complete disintegration (micellation) [143,146,147] Runas and Malmstadt [148] reported formation of pore defects in GUVs containing only 12.5% aldehyde-truncated PoxnoPC. Spontaneous pore formation in GUVs was reported also by Sankhagowit *et al.* [149] under conditions where aldehyde-truncated lipids were produced.

Our aim in [150] was to thoroughly investigate the properties of lipid membranes that underwent such drastic chemical changes, with regard to their permeability. We specifically considered a model lipid bilayer reflecting properties of a real system (highest fraction of lipids is POPC with a central patch of secondary product lipid oxidation.

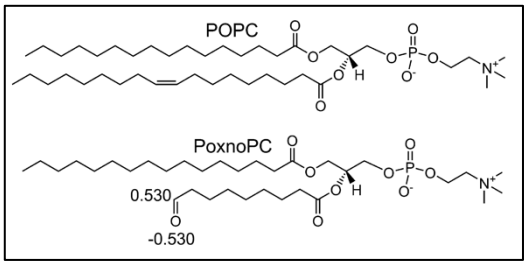

Figure. 11 Schematic representation of POPC and its oxidation product PoxnoPC investigated in [150]. The partial charges for the carbonyl and carboxyl carbon, as well as oxygen atoms are shown.

One system consisted of a large 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer, in which a central patch was replaced by PoxnoPC bilayer of 64 molecules per leaflet (see first up left panel Fig. 12). The whole bilayer model was further solvated in a solution containing sodium, calcium, and chloride ions. The second system was the analogous, but the POPC lipid bilayer contained a 40 mol % of cholesterol to mimic cell membranes' crowding and gel like phases.

The simulations [150] showed that the presence of such a PoxnoPC patch, given the localized high concentration of aldehydes, leads within a few nanoseconds to a pore formation across the bilayer, in the absence of an electric field (i.e. not under PEF conditions). As the simulations are carried out at 0 surface tension, these pores expand quickly to reach a maximum size of about 5-7 nm in diameter (Fig. 12). Such pores are

therefore large enough to enables transport of DNA, and fluorescent dyes such as: Propidium Iodide, or Calcein. Because of the liquid crystalline state of the POPC modeled at 303 K, the PoxnoPC molecules diffuse out of the patch during the ~3 μs MD simulations carried out. This had led to a pore shrinking, basically as the number of central PoxnoPC molecules forming the pore decreased. Within a couple of μs, this pore shrinks hence to a diameter of ~ 0.6 nm, then it closes.

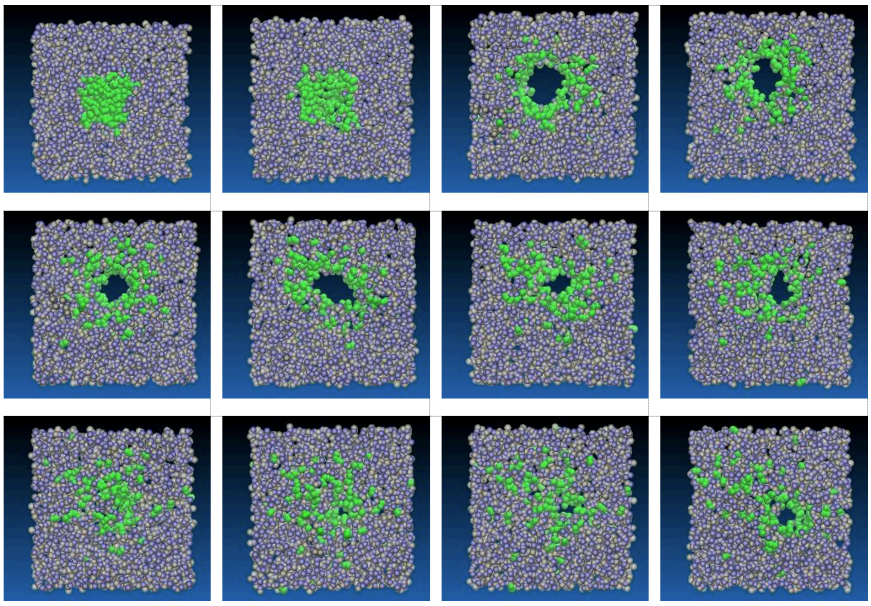

Figure. 12 Time evolution of pore formation in the bilayer systems (top to bottom from left to right). The blue and white spheres represent the choline and phosphate groups of the POPC molecules' zwitterionic head groups. The green spheres represent the head groups of the PoxnoPCs molecules.

For the system with 40% cholesterol, the diffusion of the PoxnoPC out of the initial patch was much less rapid, due to the gel-like dynamics of the remaining lipid bilayer. Consequently, the number of oxidized lipids around the pore remains high enough to maintain an aperture of ~ 1.35 nm diameter for over 5 μs.

We calculated the permeability of such pores and that of bilayer models mimicking the topology of cell membranes well after pore closure. Overall, the modeling results and data analyses indicated that the pore formation due to the presence of secondary oxidation aldehyde lipids (here PoxnoPC) can

quantitatively match the lowest to the highest reported experimental changes in the cells' conductivity and permeation. Along with our previous report this study therefore represents the first molecular level quantitative analyses of the permeation of cell membranes subject to oxidative damage confronted with experimental measurements.

We investigated the influence of membranes' composition on the lifetime of the pores by studying mimics of lipid "gel-like domains" containing cholesterol and have shown that indeed the lipid dynamics in these cases slows down the pores' annihilation. Cell membranes are however crowded, with ~50% of their surface composed of proteins [151]. Furthermore, proteins show strong negative influence on lipid membrane's fluidity [152–154]. We can therefore safely conclude, based on our investigations that the presence of secondary oxidation lipid patches resulting from direct oxidation could form pores with lifetimes far longer than assessed from simulations (or from biophysical experiments on vesicles) where proteins are absent. Since starved cells don't reseal completely [155], it is possible that some pores would remain formed as long as the cell lipid machinery does not take over to repair the damage.

Conclusion

A current goal in improving our understanding of electroporation is the development of a comprehensive molecular description of the phenomenon. This is not an easy task due to the nanoscale dimensions of the lipid electropore and the short time scale (nanoseconds) of their creation, which present challenges to direct experimental observations. For these reasons, MD simulations have become extremely important. In the last two decades, as reported in this chapter, a substantial number of simulations have been conducted to model the effect of PEFs on membranes, providing the most compete molecular model of bilayers electroporation.

Design of MD protocols suitable for the characterization of the transport of uncharged and charged species driven by μs or ms PEFs that can help to shed light on the uptake mechanism of drugs by cell membranes. Systematic studies carried out with these protocols in presence of other relevant drugs (e.g. bleomycin) or dyes (e.g. PI, YO-PRO, …) provide further insights on the molecular uptake mechanism and may lead to improvements in related experimental techniques and therapeutic effectiveness.

Finally, the most recent data now clearly shows that molecular uptake under PEFs experiments may be, at least partially, taking place via diffusion

processes across oxidized/permeabilized lipid bilayers and not uniquely across electropores.

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Chapter 5

Nanoscale and Multiscale Membrane Electrical Stress and Permeabilization

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Abstract: Our understanding of the mechanisms of pulsed electric field interactions with biological cells has been enhanced in recent years by the study of systems in the nanosecond regime, at time scales 1000 times shorter than the pulse widths in conventional electroporation. Experimental and modeling analysis has established that permeabilizing transmembrane voltages cause formation of nanometer-diameter conductive pores in lipid bilayers in less than 2 ns, initiating downstream processes in cells and tissues with macroscale consequences seconds, minutes, hours later. This presents us with a new challenge: accurately representing this multiscale (nanoseconds to hours, nanometers to meters) complex of physiological networks in predictive models for proactive electroporation treatment and process guidelines. Here we discuss the interactions between electric fields and biomolecular structures, focusing on reversible perturbations at nanosecond time scales, and we describe some of the diverse effects of electric fields on biological systems, how short-pulse (ns) studies help to dissect irreversible processes driven by longer (μs, ms) electric field exposures, and some of the engineering challenges involved in generating and accurately monitoring appropriate electrical stimuli for nanosecond bioelectrics experiments..

Introduction

To utilize the diverse *effects* of electric fields on biological systems we must understand the ***causes***. In particular, we want to know the details of the ***interactions*** between electric fields and biomolecular structures. By looking

at very short time scales (nanoseconds) and at single events (non-repetitive stimuli), we reduce the number of larger-scale disturbances and concentrate on reversible perturbations. The analysis is primarily in the time domain, but pulse spectral content may be important for some applications.

Of course, some important *effects* of electropulsation may be a consequence of irreversible processes driven by longer electric field exposures (microseconds, milliseconds). Short-pulse studies can help to dissect these processes.

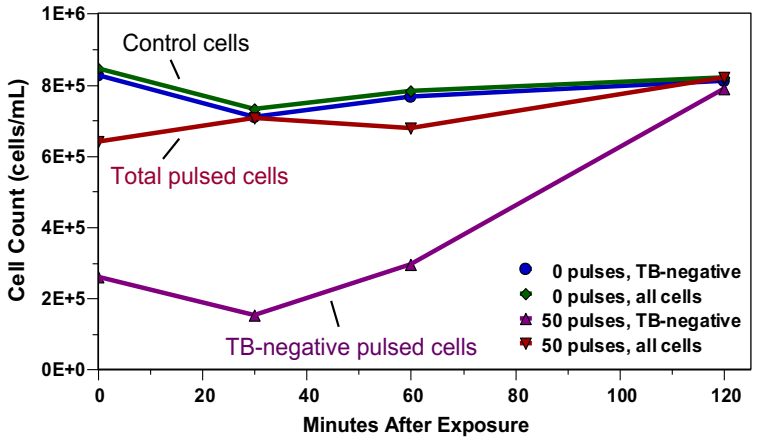

Figure 1. Nanoelectropulsed Jurkat T lymphoblasts recover over 2 hrs from initial Trypan blue permeabilization after exposure to 50, 20 ns, 4 MV/m pulses at 20 Hz.

Although modeling is of necessity a significant component of bioelectrics investigations, experimental observations are fundamental, and to conduct experiments in **nanosecond** bioelectrics, one must be able to generate and accurately monitor the appropriate electrical stimuli, a non-trivial engineering challenge. We will discuss cause and effect here from both **scientific and engineering perspectives**, using data from experiments and simulations. It is commonplace in electrical engineering, and increasingly so in biology, to attack a problem with a combination of modeling and experimental tools. In nanosecond bioelectrics, observations (*in vitro* and *in vivo*) give rise to models (molecular and continuum), which drive experiments, which adjust and calibrate the models, which feed back again to empirical validation. This feedback loop focuses investigations of a very large parameter space on the critical ranges of values for the key variables.

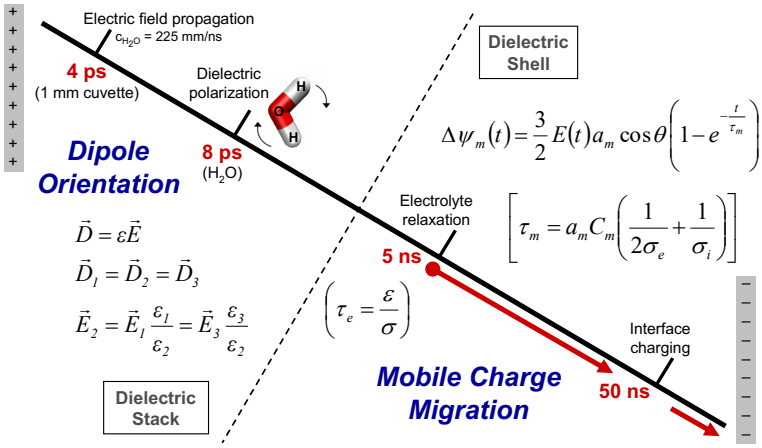

Figure 2. Timeline representing the sequence of events following electrical polarization of a biological tissue or aqueous suspension of cells. The dielectric properties of the system are important in the sub-nanosecond regime. For longer times the distribution of fields and potentials is dominated by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

Nanosecond bioelectrics

From longstanding theory that models the cell as a dielectric shell [1–4] came the notion that sub-microsecond electric pulses could "bypass" the cell membrane, depositing most of their energy inside the cell instead of in the plasma membrane, the primary target of longer pulses. This idea was investigated experimentally beginning in the late 1990s, and apparently confirmed [5–6]. Even though one early report indicated that the electric field-driven conductive breakdown of membranes can occur in as little as 10 ns [7], *and* a theoretical analysis demonstrated that pulses with field amplitudes greater than about 1 MV/m will produce porating transmembrane potentials within about 2 ns [8], *and* a well-grounded model predicted "poration everywhere" in the nanosecond regime [9], procedures used to detect electroporation of the plasma membrane (and the loss of membrane integrity in general) produced negative results for pulses with durations less than the charging time constant of a small cell in typical media (< 100 ns).

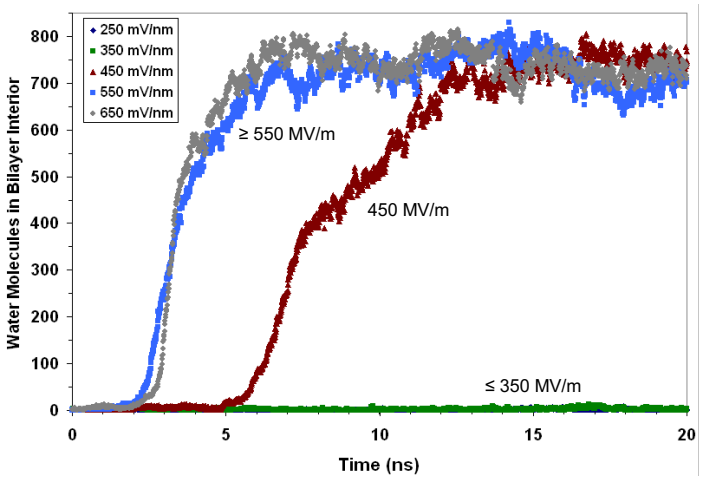

Figure 3. Electric field-driven intrusion of water into a simulated lipid bilayer.

In addition to highlighting the limitations of traditional experimental methods for observing membrane permeabilization, this apparent discrepancy between model and observation points also to inadequacies in the dielectric shell model itself, at time scales below the membrane (cell) charging time. Higher-frequency effects associated with the dielectric properties of high-permittivity aqueous media and low-permittivity biological membranes [10–13] are negligible for the electropermeabilizing conditions that are most commonly studied (μs, kV/m pulses), but for nanosecond pulses they cannot be ignored.

Several lines of experimental evidence indicate that nanosecond electric pulses cause changes in the integrity and organization of the cell membrane.

Trypan blue permeabilization. While remaining propidium-negative, the cell volume of Jurkat T lymphoblasts exposed to a series of 50, 20 ns, 4 MV/m pulses increases, and they become permeable to Trypan blue (TB) (Fig. 1). With increasing time after pulse exposure, these weakly TB-positive cells become again impermeable to TB. Similar observations have been reported for B16 murine melanoma cells exposed to sub-nanosecond (800 ps) pulses at very high fields [14].

Nanosecond porating transmembrane potentials. Fluorescence imaging with a membrane potential-sensitive dye indicates that porating transmembrane potentials are generated during nanoelectropulse exposure [15].

Nanoelectropulse-induced PS externalization. Loss of asymmetry in membrane phospholipid distribution resulting from phosphatidylserine (PS) externalization occurs immediately after nanoelectropulse exposure [16],

consistent with membrane reorganization driven directly by nanosecond-duration electric fields and a mechanism in which nanometer-diameter pores provide a low-energy path for electrophoretically facilitated diffusion of PS from the cytoplasmic leaflet of the plasma membrane to the external face of the cell [8].

Simulations link PS externalization and nanoporation. In molecular dynamics (MD) simulations of electroporation, hydrophilic pores appear within a few nanoseconds [17], and PS migrates electrophoretically along the pore walls to the anode-facing side of the membrane [18–19], an *in silico* replication of experimental observations in living cells [20].

Nanoelectropermeabilization. The first direct evidence for nanoelectropermeabilization was obtained by monitoring influx of YO-PRO-1 (YP1) [21], a more sensitive indicator of membrane permeabilization than propidium (PPD) [22]. Additional direct evidence comes from patch clamp experiments, which reveal long-lasting increases in membrane conductance following exposure to 60 ns pulses [23–25].

Nanosecond activation of electrically excitable cells. Electrically excitable cells provide a highly responsive environment for nanoelectropulse biology. Adrenal chromaffin cells [26] and cardiomyocytes [27] react strongly to a single 4 ns pulse, and muscle fiber has been shown to respond to a 1 ns stimulus [28].

Nanosecond bioelectrics and the dielectric stack model. Fig. 2 depicts a timeline of events in an aqueous suspension of living cells and electrolytes between two electrodes after an electric pulse is applied. Water dipoles re-orient within about 8 ps. The field also alters the electro-diffusive equilibrium among charged species and their hydrating water, with a time constant that ranges from 0.5 to 7 ns, depending on the properties of the media. Pulses shorter than the electrolyte relaxation time do not generate (unless the field is very high) enough interfacial charge to produce porating transmembrane potentials. The dielectric shell model in this regime can be replaced with a simpler, dielectric stack model, in which the local electric field depends only on the external (applied) electric field and the dielectric permittivity of each component of the system.

Nanoelectropermeabilization and continuum models. MD simulations at present provide the only available molecular-scale windows on electropore formation in lipid bilayers. Current models perform reasonably well, but simulations of electroporation still contain many assumptions and simplifications. To validate these models, we look for intersections between all-atom molecular assemblies, continuum representations of cell suspensions and tissues, and experimental observations of cells and whole organisms. For example, a leading continuum model assumes an exponential relation between the transmembrane potential and several

indices of electropore formation [29]. The MD results in Fig. 3, showing water intrusion into the membrane interior as a function of applied electric field, qualitatively demonstrate this same non-linear relation between field and poration. The challenge is to achieve a quantitative congruency of the coefficients.

Nanosecond experiments, models

Experiments and molecular models of membrane permeabilization. Fig. 4 shows a simple and direct response of cells to pulse exposure — swelling [25,30,31]. Electropermeabilization of the cell membrane results in an osmotic imbalance that is countered by water influx into the cell and an increase in cell volume. This phenomenon, initiated by electrophysical interactions with basic cell constituents — ions, water, and phospholipids — on a much shorter time scale (a few nanoseconds) than usually considered by electrophysiologists and cell biologists, provides a simple, direct, and well-defined connection between simulations and experimental systems. By correlating observed kinetics of permeabilization and swelling with rates of pore formation and ion and water transport obtained from molecular simulations and continuum representations, we are improving the accuracy and applicability of the models

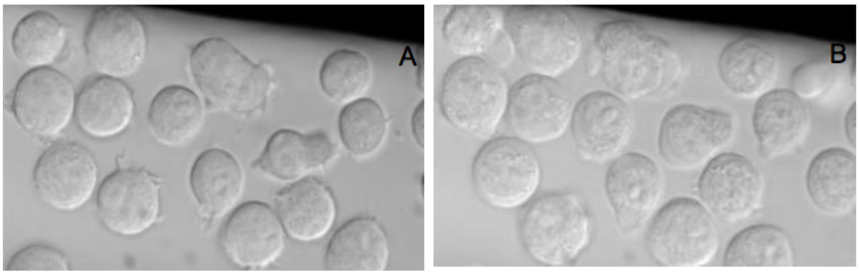

Figure 4. Differential interference contrast (DIC) images of Jurkat T lymphoblasts before (A) and 30 s after (B) exposure to 5 ns, 10 MV/m electric pulses (30 pulses, 1 kHz). Note swelling, blebbing, and intracellular granulation and vesicle expansion, results of the osmotic imbalance caused by electropermeabilization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

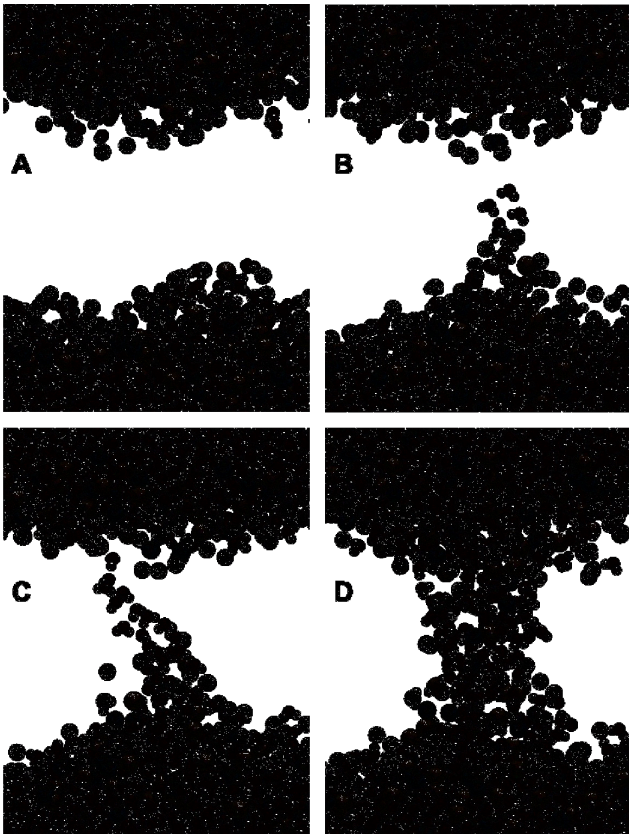

Figure 5. Electropore creation sequence. (A) Molecular dynamics representation of a POPC lipid bilayer. Small red and white spheres at the top and bottom of the panel are water oxygen and hydrogen atoms. Gold and blue spheres are head group phosphorus and nitrogen, respectively, and grey spheres are phospholipid acyl oxygens. For clarity, atoms of the hydrocarbon chains in the interior of the bilayer are not shown. In the presence of a porating electric field, a water intrusion appears (B) and extends across the bilayer (C). Head groups follow the water to form a hydrophilic pore (D). The pore formation sequence, from the initiation of the water bridge to the formation of the head-group-lined pore takes less than 5 ns.

Molecular dynamics and macroscale (continuum) models. Fig. 5 shows the main steps in the electric field-driven formation of a nanopore in a typical MD simulation of a porating phospholipid bilayer, part of a larger scheme for the step-by-step development (and dissolution) of the electrically conductive defects that contribute at least in part to what we call a permeabilized membrane [32]. These molecular simulations permit us to conduct virtual experiments across a wide parameter space currently

inaccessible in practice to direct observation. Although we cannot yet align the detailed energetics and kinetics that can be extracted from MD simulations with laboratory results, it is possible to compare MD data with the predictions of the macroscale models used to describe electroporation.

Fig. 6 shows how pore initiation time (time between application of porating electric field and the appearance of a membrane-spanning water column (Fig. 5C)) varies with the magnitude of the electric field in MD simulations [32]. The value of the electric field in the membrane interior, extracted from simulations by integrating the charge density across the system, is used as a normalizing quantity.

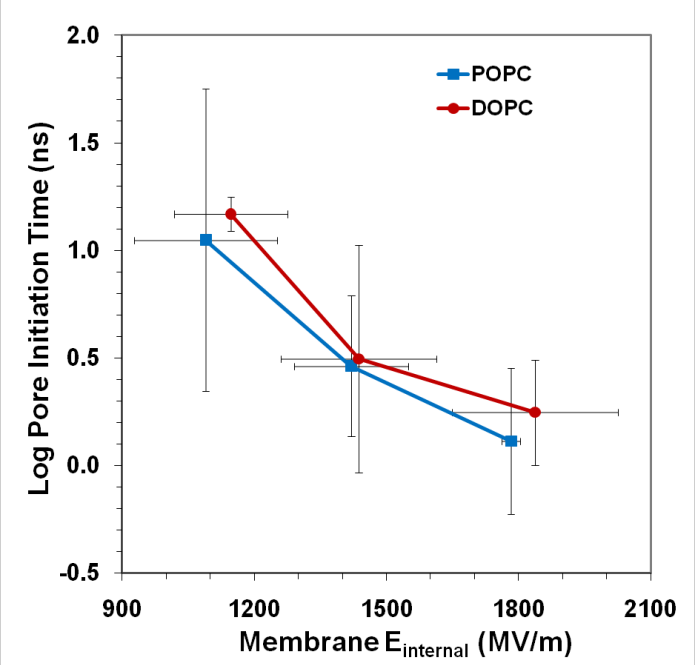

Figure 6. Electropore initiation time is a nonlinear function of the magnitude of the porating electric field. Pore initiation time (time required to form the water bridge shown in Fig. 1C) is exponentially dependent on the applied electric field, expressed here as the electric field observed in the lipid bilayer interior in molecular dynamics simulations. Error bars are standard error of the mean from at least three independent simulations. Data are from Tables 4 and 5 of [32].

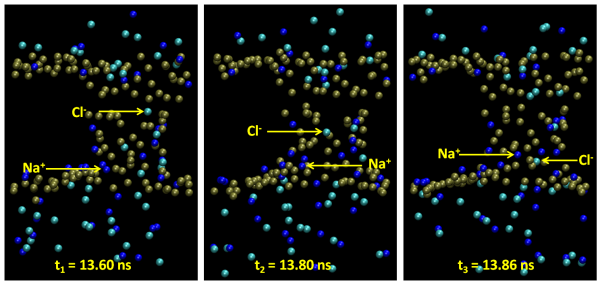

Figure 7. Sodium and chloride ions migrating through a lipid nanopore in the presence of an external electric field.

This membrane internal field results from the interaction of the applied external field with the interface water and head group dipoles, which also create the large dipole potential found in the membrane interior even in the absence of an applied field [33]. The nonlinear decrease in pore initiation time with increased electric field may be interpreted as a lowering of the activation energy for the formation of the pore-initiating structures described above. We can use simulation results like those in Fig. 6 to reconcile molecular dynamics representations with continuum models, and ultimately both of these to experiment. For example, the relation between electric field and pore creation rate is described in the Krassowska-Weaver stochastic pore model in the following expression,

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

where K_{pore} is the pore creation rate, A is a rate constant, $E(r,V_m)$ is the energy of a pore with radius r at transmembrane potential V_m, and k_B, and T are the Boltzmann constant and the absolute temperature [29,34–36]. One of our objectives is to reconcile the pore creation rate in (1) with our simulated pore initiation times, reconciling the two models. We are in the process also of validating the stochastic pore model expression for pore density,

$$\frac{dN}{dt} = \alpha e^{\beta\left(\Delta\psi_m^2\right)}\left(1-\frac{N}{N_{eq}}\right), \qquad (2)$$

where N and N_{eq} are pores per unit area, instantaneous and equilibrium values, α and β are empirical electroporation model parameters, and $\Delta\psi_m$ is the transmembrane potential.

Computing power is needed not only to enable simulations of larger systems. The large variability in pore initiation time indicated by the error bars in Fig. 6 means that independent simulations of each condition must

be repeated many times to ensure valid results. (A surprising number of conclusions in the existing literature have been published on the basis of single simulations.)

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

Better models can contribute also to our understanding of practical problems in bioelectrics. For example, despite years of study, controversy remains regarding the effects, or lack of effects, of exposures to low levels of radio-frequency (RF) electromagnetic fields [37,38]. Part of the reason for failure to establish certainty on this issue arises from the difficulty of conducting experiments with a sufficient number of variables and a sufficient number of samples to generate reliable data sets. With accurate simulation tools, honed by reconciliation with experiment, we can explore the large variable and statistical space in which suspected biophysical effects might occur, narrowing the range of experimental targets and focusing on systems in which effects are most likely and in which mechanisms will be clear.

Experiments and molecular models of ion conductance. The earliest identified and most direct indicators of electric field-driven membrane permeabilization are changes in electrical properties, including an increase in ion conductance [39,40]. Data from careful experimental work can be interpreted as measured values corresponding to the conductance of a single pore [41–44]. By combining continuum models of electroporation with this experimental data and with established values for ion electrophoretic mobilities and affinities between ions and phospholipids, we can draw conclusions about pore geometry and areal density. But the inaccessibility (so far) of membrane electropores to direct observation and manipulation of their physical structure prevents us from definitively bridging the gap between model and experiment.

A recently developed method for stabilizing electropores in molecular dynamics simulations of phospholipid bilayers [45] allows extraction of ion conductance from these model systems and thus provides a new and independent connection between models and experiments, in this case from the atomically detailed models of lipid electropores constructed with molecular dynamics. Fig. 7 shows one of these stabilized pores with electric field-driven ions passing through it.

Although the magnitude of the conductance measured in these simulations is highly dependent on the accuracy of the ion and water models

and their interactions with the phospholipid bilayer interface (and there is much room for improvement in this area), initial results are consistent with expectations from both continuum models and experimental observations.

Nanosecond excitation

Nanoelectrostimulation of neurosecretory and neuromuscular cells. Applications of pulsed electric fields in the clinic, particularly in electrochemotherapy and gene electrotransfer, are well known and described in detail in other chapters of this book. We note here a potential biomedical application specifically of nanosecond electric pulses, the activation and modulation of the activity of neurosecretory and neuromuscular processes, an area which remains relatively unexplored. The sensitivity of electrically excitable cells to nanoelectropulses raises the possibility that very low energy (nanosecond, megavolt-per-meter pulses are high power, but low total energy because of their brief duration) devices for cardiac regulation (implanted pacemakers and defibrillators), remote muscle activation (spinal nerve damage), and neurosecretory modulation (pain management) can be constructed with nanoelectropulse technology. Fig. 8 demonstrates functional activation of an adrenal chromaffin cell after a single 5 ns, 5 MV/m pulse [46,47].

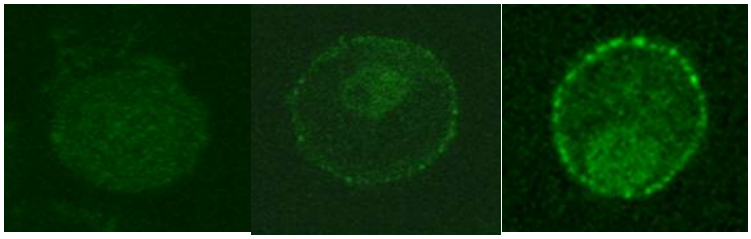

Figure 8. Immunocytochemical labeling of dopamine-β-hydroxylase (DβH) using an anti-DβH antibody coupled with a fluorescently-tagged 2º antibody. DβH is externalized by exocytotic fusion of vesicles with plasma membrane. Left panel, control. Center panel, 2 min after treatment with the pharmacological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

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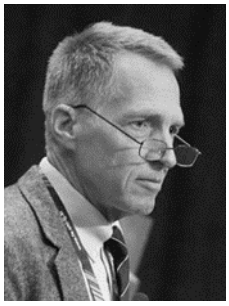

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Chapter 6

Electroporation and electropermeabilisation - pieces of puzzle put together

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European Associated Laboratory (LEA) on the pulsed Electric fields in Biology And Medicine (LEA EBAM).

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

The models built to describe the phenomena occurring at the cell membrane (even at artificial membranes, whether these artificial membranes were planar membranes or membranes of vesicles of different sizes and compositions) have been mainly based on the physical principles that could explain the transport of molecules across the membrane. The input of the bioimpedance measurements, while very useful in practical

terms, has brought a limited contribution to the understanding of these phenomena. However, in the transport phenomena there are parameters not related to the structural features of the membrane before, during and after the pulses. Indeed, as already mentioned, there is an impact of the size of the molecules, their charge, the gradient of concentration between the inside and the outside, the sensitivity of their detection inside the cells, etc. There are a number of examples, whatever the duration of the pulses, nanosecond pulses or microseconds pulses, that can be reported. In this context, it is important to highlight that penetration of Calcium ions can be detected at electric field amplitudes for which many other electropermeabilization markers do not yet reveal the electropermeabilization of the cells. This allows manipulating cytosolic calcium content in conditions where cell survival is fairly well protected [2,3].

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

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

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

Because biological objects are immersed in water-based media, Confocal Raman microscopes must be used to eliminate the non-resonant Raman contribution of the water. Coherent Raman microspectroscopy, like the Coherent AntiStockes Raman Scattering microspectroscopy, seems more attractive because of the enhancement of the signal caused by the "coherence" provided by the use of two lasers accordingly tuned. Enhancement of the signal with respect to spontaneous Raman signal can reach 10^8 times. Coherent AntiStockes Raman Scattering microspectroscopy has recently provided us with information on changes in the interfacial water (the few layers of water molecules organized at the

surface of the membranes) and even of the interstitial water. After the pulses delivery, an important loss of the interfacial water signal has been recorded, which means that the alterations of the membrane structure consecutive to the pulses application also affects the water surrounding the membrane (to be submitted). We are thus acquiring information on the changes occurring in the membranes independently of any transport phenomenon. This information has now to be introduced into the models that tentatively describe the phenomena occurring at the membranes, to continue improving the knowledge of the electroporation/electropermeabilization of cells as well as of even much smaller biological objects [7].

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

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

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

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Lluis M. Mir was one of the pioneers of the research of electropermea-bilization (electroporation) and the applications of this technique for antitumor electrochemotherapy and DNA electrotransfer. He is the author of 193 articles in peer-reviewed journals, 21 chapters in books, and over 500 presentations at national and international meetings, invited lectures at international meetings and seminars. He received the Award for the medical applications of electricity of the Institut Electricité Santé in 1994, the Annual Award of Cancerology of the Ligue contre le Cancer (committee Val-de-Marne) in 1996, the Award

of the Research of Rhône-Poulenc-Rorer in 1998, the medal of the CNFRS under the auspices of the French Sciences Academy in 2012, the Frank Reidy Award in Bioelectrics in 2015 and the Balthazar van der Pol Gold Medal of the International Union of Radio Sciences in 2017. He is an Honorary Senator of the University of Ljubljana (2004). He is also fellow of the American Institute of Biological and Medical Engineering. He has been visiting professor of the Universities of Berkeley (USA), Bielefeld (Germany) and Jerusalem (Israel). He is the director of the laboratory of Vectorology (UMR 8203 of CNRS, Universiy Paris-Sud and Institut Gustave-Roussy), and he is also the founder and co-director of the European Associated Laboratory on Electroporation in Biology and Medicine of the CNRS, the Universities of Ljubljana, Primorska, Toulouse and Limoges, the Institute of Oncology Ljubljana and the Institut Gustave-Roussy.

Chapter 7

Nucleic acids electrotransfer *in vitro*

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Abstract: Cell membranes can be transiently permeabilized by application of electric pulses. This process, called electropermeabilization or electroporation, allows hydrophilic molecules, such as anticancer drugs and nucleic acids, to enter into targeted cells and tissues. The knowledge of the processes involved in membrane permeabilization and in gene transfer is mandatory for this promising method to be efficiently and safely used. The behavior of the membranes and the cells both while the electric field is on and after its application has therefore to be addressed. The description of the full mechanisms takes benefit from studies performed on different biological models (lipid vesicles, cells in 2D and 3D culture) and from different microscopy tools that allow to visualize the processes. Single cell imaging experiments revealed that the uptake of molecules (antitumor drugs, nucleic acids) takes place in well-defined membrane regions and depends on their chemical and physical properties (size, charge). Small molecules can freely cross the electropermeabilised membrane and have a free access to the cytoplasm. Heavier molecules, such as plasmid DNA, face physical barriers (plasma membrane, cytoplasm crowding, nuclear envelope) which engender a complex mechanism of transfer. Gene electrotransfer indeed involves different steps, occurring over relatively large time scales. As will be presented, these steps include the initial interaction with the electropermeabilised membrane, the crossing of the membrane, the transport within the cell towards the nuclei and finally gene expression.

Introduction

Gene therapy is a treatment option for a number of diseases as inherited disorders and cancer. Despite the fact that a lot of methods of vectorization

have been developed during the last decades, the technique has still to be improved to be both efficient and safe [1,2]. Among the different approaches, electroporation appears as the most promising one. This physical method can be efficiently used for the targeted deliver of molecules in a wide range of cells and tissues [3]. Electroporation is nowadays a well-known technique of cell transfection used in the laboratories. Vaccination and oncology gene therapy are major fields of application of DNA electrotransfer in clinics [4,5]. Translation of preclinical studies into clinical trials started 10 years ago. The first clinical trial of plasmid electroporation carried out in patients with metastatic melanoma has shown hopeful results [6]. The method has also been successfully used for the treatment of companion animals. However, despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated more than 30 years ago [7], many of the mechanisms underlying membrane electropermeabilization and DNA electrotransfer remain to be elucidated. Even if *in vitro* electrotransfer is efficient in almost all cell lines, *in vivo* gene delivery and expression in tumors can be not as efficient as in viral vectorization. It is therefore mandatory, for increasing gene transfer and expression while preserving safety, to increase knowledge about the mechanisms. This chapter aims to describe the basics aspects of membrane electropermeabilization and gene delivery in cells and by doing so to give some tips to perform experiments and optimize protocols.

Membrane electropermeabization

The basics

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

The way to conduct an experiment

Electropermeabilization can be performed in different manners depending on the way cells are grown. For cells grown on Petri dish, culture medium can be removed and replaced by a low ionic, iso-osmotic buffer. This pulsation buffer allows to limit the Joule effect and therefore help to preserve the cell viability (See also Chapter 11). The composition of this medium is generally a 10 mM phosphate buffer, 250 mM sucrose and 1 mM $MgCl_2$. On a practical point of view, the bottom of the Petri dish can serve as an electropulsation chamber. For cells in suspension, cells resuspended in the pulsing buffer are placed in purchased cuvettes or in "home-made" chambers that can be easily obtained by placing the electrodes on the bottom of the Petri dish (see Fig. 1). The electric pulses are delivered through a set of electrodes connected to the pulse generator. In most experiments, square-wave electric pulses generators are used. Contrary to exponential decay generators, they allow the independent control of the amplitude of the electric field pulses E and their duration T. This is important for mammalian cells which have no cell wall and therefore are more affected by electric pulses than bacteria and yeast. The electric pulse parameters have to be selected considering the characteristics of the cells in particular their size. One key step to further ensure DNA electrotransfer and expression is to determine the best electric conditions allowing both the permeabilization of the plasma membrane and the preservation of the cell viability.

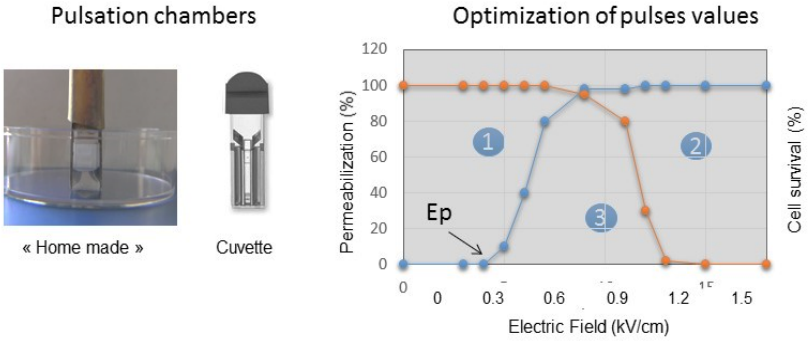

Figure 1. Tips for your experiments. Cells are pulsed on Petri dish or in cuvettes. Permeabilized (blue) and viable (orange) cells are plotted to define the optimum conditions ((1) E < Ep or just above, poor permeabilization; (2) E >> Ep, viability loss; (3) best values).

The use of video microscopy allows visualization of the permeabilization phenomenon at the single cell level. Fluorescent indicators of membrane

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

Kinetics of membrane permeabilization

Electropermeabilization of cells is a fast process that can be detected immediately after the application of electric pulses. Usually, transport across the membrane is not homogeneous on the whole cell membrane. It occurs at the sides of the cells facing the electrodes in an asymmetrical way where it is more pronounced at the anode-facing side of the cells than at the cathode (Fig. 2), i.e. in the hyperpolarized area than in the depolarized area, which is in agreement with both theoretical and experimental considerations as explained in other chapters. Electropermeabilization can be described as a 3-step process in respect with electric field: (i) before electropulsation, the plasma membrane acts as a physical barrier that prevents the free exchange of hydrophilic molecules between the cell cytoplasm and external medium; (ii) during electropulsation, when pulses parameters have been correctly defined, E > Ep, the formation of transient permeable structures facing the electrodes allows the exchange of molecules; Propidium iodide is observed to rapidly access the cell interior in the region of the cells facing the electrodes, mainly at the anode facing site; (iii) after electropulsation, membrane can stay permeable before resealing occurs [8]. Lifetime of permeabilization can be assayed by adding the fluorescent dyes at various times following the pulses. If the cell membrane is still permeable, then the cell will be fluorescent. Resealing varies from a few seconds (when cells

are put at 37°C just after pulsation) to several hours (when cells are maintained on ice) according to the experimental conditions (temperature and pulse parameters). However, one has to take into account that viability can be affected since ATP release will occur. It is therefore better to avoid to maintain the cells at low temperature after pulse delivery.

Whatever the molecules used to detect permeabilization (if they are small enough and charged), a direct transfer into the cell cytoplasm is observed. When added after electropulsation, molecules can still penetrate into the cells but less efficiently because electric field acts on both the permeabilization of the membrane and on the electrophoretic drag of the charged molecules from the bulk into the cytoplasm. The electrotransfer mechanism involved is indeed specific for the physico-chemical properties of the molecule [9].

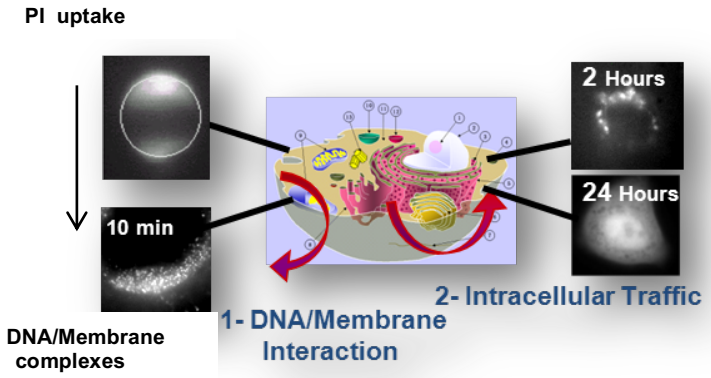

Figure 2: Molecule electrotransfer mechanisms. Left: During electric pulses application: Plasma membrane is electropermeabilized facing the 2 electrodes (PI uptake). DNA aggregates are formed at the plasma membrane within 10 min. This interaction takes place only on the membrane facing the cathode. Right: About 2 h after electric pulses application, DNA molecules are present around the nucleus. Finally, eGFP expression is detected for hours. The arrow indicates the direction of the electric field.

Progress in the knowledge of the involved mechanisms, in particular in the elucidation of membranes structures that are responsible for molecules transfer, is still a biophysical challenge. Hydrophilic pores have been proposed to be created and their formation confirmed by molecular dynamics modelling. But their existence in permeabilized cells is difficult to be visualized and has still to be proven. Phospholipid scrambling and changes on lateral mobility of proteins have been observed suggesting that part of the membrane surface is occupied by defects or pores and that these

structures propagate rapidly over the cell surface [10]. One can also took advantage of atomic force microscopy to directly visualize the consequences of electropermeabilization and to locally measure the membrane elasticity. Results obtained both on fixed and living CHO cells give evidence of an inner effect affecting the entire cell surface that may be related to cytoskeleton destabilization. Thus, AFM appears as a useful tool to investigate basic process of electroporation on living cells in absence of any staining [11,12].

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

Mechanisms of electrotransfer of DNA molecules into cells

What is known about the process

The first electroporation-mediated gene transfer experiment was published in 1982 [7]. Translation to the clinic benefited from increased knowledge of the mechanisms involved in the electrotransfer of nucleic acids during the last 4 decades. As for electropermeabilization, single-cell studies aided in describing the process of DNA electrotransfer.

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

around the membrane in the direction of the anode. Beyond the critical field value, above which cell permeabilization occurs (E > Ep), the DNA interacts with the plasma membrane.

DNA/membrane interaction

Interaction only occurs at the pole of the cell opposite the cathode and this demonstrates the importance of electrophoretic forces in the initial phase of the DNA/membrane interaction. When the DNA-membrane interaction occurs, the formation of "microdomains" whose dimensions lie between 0.1 and 0.5 μm is observed (Fig. 2). Also seen are clusters or aggregates of DNA which grow during the application of the field. However, once the field is cut the growth of these clusters stops. DNA electrotransfer can be described as a multi-step process: the negatively charged DNA migrates electrophoretically towards the plasma membrane on the cathode side where it accumulates. This interaction, which is observed for several minutes, lasts much longer than the duration of the electric field pulse. Translocation of the plasmid from the plasma membrane to the cytoplasm and its subsequent passage towards the nuclear envelope take place with a kinetics ranging from minutes to hours.

Dynamic of the process

DNA/membrane interaction and as a direct consequence gene expression depend on electric pulse polarity, repetition frequency and duration. Both are affected by reversing the polarity and by increasing the repetition frequency or the duration of pulses. These observations revealed the existence of 2 classes of DNA/membrane interaction: (i) a metastable DNA/membrane complex from which DNA can leave and return to external medium and (ii) a stable DNA/membrane complex, where DNA cannot be removed, even by applying electric pulses of reversed polarity. Only DNA belonging to the second class leads to effective gene expression [13]. Dynamics of membrane/complexes formation has been poorly understood because direct observations have been limited to time scales that exceed several seconds. However, experimental measurement of the transport of plasmid DNA and propidium iodide with a temporal resolution of 2 ms has been performed thanks to high speed and sensitive camera and allowed the visualization of the DNA/membrane interaction process during pulse application [14]. Plasmid complexes, or aggregates, start to form at distinct sites on the cell membrane during the first pulse. Increasing the number of pulses do not lead to the creation of new sites, but to the increase in the

amount of DNA. The formation of plasmid complexes at fixed sites suggested that membrane domains may be responsible for DNA uptake and their lack of mobility (as directly observed under the microscope or quantify by Fluorescence Return After Photobleaching (FRAP) measurements) could be due to their interaction with the actin cytoskeleton. As will be described later in this chapter, several publications reported evidences for the involvement of cytoskeleton [15,16]. The dynamics of the entire process is reported in Table 1. If pulse delivery occurs in a relative short time scale (μs to ms), the subsequent traffic of plasmid DNA occurs during the minutes and hours following pulse delivery.

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

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

DNA transfer through the cytoplasm

The process of plasmid transfer through the cellular cytoplasm to the nuclear envelope is a complex process [17]. In principle micro sized aggregates of DNA or vesicles filled with DNA could be too large to pass through the pores formed by electroporation. However individual DNA molecules, while they can pass through electropores, have a limited mobility within the cell and may well be totally degraded before reaching the nucleus. It is possible and worth investigating the possibility that the actin cytoskeleton reacts to the presence of DNA aggregates and plays an important role in the subsequent intracellular transport. It seems reasonable

that only aggregates beyond a certain size (a few hundred nanometers) can induce a biological cellular response and can be transported by the cell. In addition, the fact that the DNA is in aggregate form means that the DNA in the center of the aggregate is relatively protected from degradation. Therefore, for gene therapy purposes, it is optimal for DNA to enter the cell as single molecules, but the subsequent transport toward the nucleus is, for biological (possibly by inducing a response of the actin cytoskeleton) and physical (diminishing enzymatic degradation) reasons, optimized if the DNA is in a micro-sized aggregate form.

Even if the first stage of gene electrotransfection, i.e. migration of plasmid DNA towards the electropermeabilised plasma membrane and its interaction with it, becomes understood, guidelines to improve gene electrotransfer can not only result from the way pulses parameters have been selected. Expression of the pDNA is controlled by the viability of the pulsed population and successful expression of the plasmid depends on its subsequent migration into the cell. Therefore, the intracellular diffusional properties of plasmid DNA, as well as its metabolic instability and nuclear translocation, represent cell limiting factors that must be taken into account. The cytoplasm is composed of a network of microfilament and microtubule systems, along with a variety of subcellular organelles present in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration means that there is substantial molecular crowding in the cytoplasm which hinders the diffusion of plasmid DNA. These apparently contradictory results might be reconciled by the possibility of a disassembly of the cytoskeleton network that may occur during electropermeabilisation and is compatible with the idea that the cytoplasm constitutes an important diffusional barrier to gene transfer. In the conditions induced during electropermeabilisation, the time a plasmid DNA takes to reach the nuclei is significantly longer than the time needed for a small molecule (hours compared to minutes). Therefore, plasmid DNA present in the cytosol after being electrotransferred can be lost before reaching the nucleus, for example because of cell division. Finally, after the cytoskeleton, the nuclear envelope will represent the last, but by no means the least important, obstacle for the expression of the plasmid DNA. See also Chapter 2.

Passage through the nuclear envelope and gene expression

A high transport does not always result in a high level in expression. The relatively large size of plasmid DNA makes it unlikely that the nuclear entry occurs by passive diffusion. Single particle tracking experiments of DNA aggregates in living cells showed how electrotransferred DNA is

transported in the cytoplasm towards the nucleus. The modes of DNA aggregates motion in CHO cells have been analyzed. Fast active transport of the DNA aggregates occurs over long distances. Tracking experiments in cells treated with different drugs affecting both the actin and the tubulin network clearly demonstrate that transport is related to the cellular microtubule network (Fig. 3, [18]).

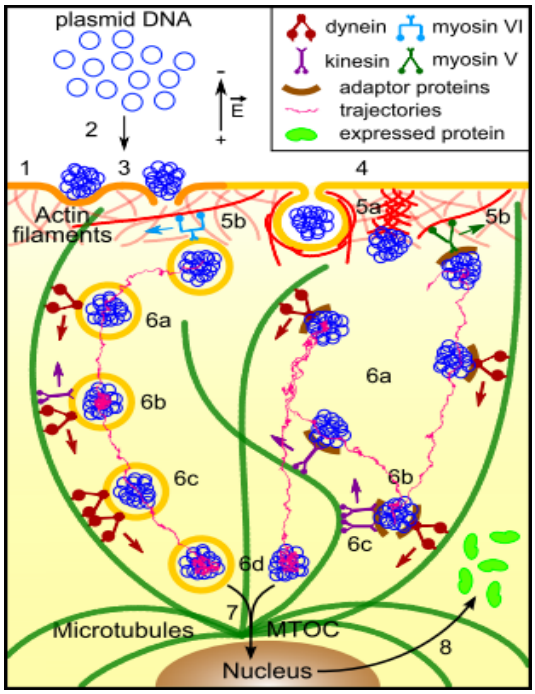

Figure 3: Schematic representation of the mechanism of DNA electrotransfer. During the electric pulses, (1) the plasma membrane is permeabilized, (2) DNA is electrophoretically pushed onto the cell membrane, which results in (3) DNA-membrane interactions. After resealing of the membrane, (4) DNA is internalized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

Active transport of DNA aggregates

Several studies point towards the contribution of endocytosis in the electrotransfer of DNA, but further research is needed in order to understand what type(s) of endocytosis would be involved. It is necessary to understand as well how electric fields could stimulate such processes. Also notably, any endocytosis model would only explain the internalization of large molecules as it does not support the free membrane crossing of small molecules. It has therefore to be considered to occur in parallel to another model valid for small molecule transmembrane exchange. One model that could reconcile all the DNA internalization models would be that DNA accumulates where pores are formed and that its electrophoretically driven insertion in the membrane pulls the pore and the plasma membrane around. This would generate membrane curvature that could be recognized as an emerging endocytic vesicle and induce a similar response from the cell as for an endocytic process, with the recruitment of actin, clathrin, caveolin, dynamin and other endocytic regulators [19,20].

Electrotransferred DNA trajectories possess portions of active transport interrupted by phases of nearly immobility [16]. During the phases of active transport, DNA aggregates featured a motion on average having a velocity of 250 nm/s, persisting for 6 s and leading to a displacement of 1.3 μm. However, the distributions were rather broad with velocities from 50 nm/s to 3400 nm/s, displacements from 0.1 μm to 12 μm and active transport durations from 2 s to 30 s. These ranges are in agreements with other types of intracellular particle dynamics as observed for viruses, polyplexes, lipoplexes, receptors, endosomes and mitochondria. Lower velocities were shown to correspond to actin-associated transport. Indeed, after disruption of the microtubules using the nocodazole drug, active transport of the DNA still occurred and the measured velocities were in the range expected for myosin motors operating on actin – between 50 nm/s and 300 nm/s for myosin VI and between 250 nm/s and 500 nm/s for myosin V. In addition to motor driven transport, actin-related movement could be also due to bursts of actin polymerization which was reported to drive viruses, bacteria or endosomes from the plasma membrane to the cytosol with mean velocities ranging from 50 to 600 nm/s.

New challenges to increase gene expression

As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA in the intracellular medium. Electrotransferred plasmid DNA, containing specific sequences could then use the microtubule network and its associated motor proteins to move through the cytoplasm

to nucleus [21]. Clear limits of efficient gene expression using electric pulses are therefore due to, in addition to the passage of DNA molecules through the plasma membrane, to the cytoplasmic crowding and transfer through the nuclear envelope. One of the key challenge for electromediated gene therapy is to pinpoint the rate limiting steps in this complex process and to find strategies to overcome these obstacles. One of the possible strategies to enhance DNA uptake into cells is to use short (10-300 ns) but high pulse (up to 300 kV/cm) induce effects that primarily affect intracellular structures and functions. As the pulse duration is decreased, below the plasma membrane charging time constant, plasma membrane effects decrease and intracellular effects predominate. An idea, to improve transfection success, is thus to perform classical membrane permeabilization allowing plasmid DNA electrotransfer to the cell cytoplasm, and then after, when DNA has reached the nuclear envelope, to specifically permeabilize the nuclei using these short strong nanopulses. Thus, when used in conjunction with classical electropermeabilisation, nanopulses gave hope to increase gene expression [22]. However, this work was not yet replicated. Another idea is to combine electric pulses and ultrasound assisted with gas microbubbles. Although electroporation induced the formation of DNA aggregates into the cell membrane, sonoporation induced its direct propulsion into the cytoplasm. Twenty-four hours later, cells that received electrosonoporation demonstrated a four-fold increase in transfection level and a six-fold increase in transfection efficiency compared with cells having undergone electroporation alone [23]. Sonoporation can therefore improve the transfer of electro-induced DNA aggregates by allowing its free and rapid entrance into the cells [24].

Lipid vesicles and 3D-cell cultures as other models to study gene electrotransfer

Coming back to a mechanistic point of view and due to the complexity of the composition of the plasma membrane, other experimental tools can be useful to characterize the membranes domains observed during gene electrotransfer. For that purpose, giant unilamellar vesicles (GUV) represent a convenient way to study membrane properties such as lipid bilayer composition and membrane tension [25]. They offer the possibility to study and visualize membrane processes due to their cell like size in absence of any constraint due to cell cytoskeleton. They can be obtained by simple methods such as electroformation and their composition can be very simple (one type of phospholipids) or more complex (several lipids

including cholesterol). Experiments showed a decrease in vesicle radius which was observed as being due to lipid loss during the permeabilization process. Three mechanisms responsible for lipid loss were directly observed: pore formation, vesicle formation and tubule formation, which may be involved in molecules uptake. However, no interaction between plasmid DNA and the GUV membrane could be observed; a direct transfer of DNA into the GUVs took place during application of the electric pulses [26]. That gives clear evidence that "lipid bubble" is not always relevant as a cell and a tissue is not a simple assembly of single cells. Therefore, it is necessary to develop and use different models, from simple lipid vesicles to tumor multicellular tumor spheroids more closed to the *in vivo* situation, for the understanding of the membrane permeabilization and DNA electrotransfer process in tissues. Each of this model has advantage and limits. Together combined they can help in the study of the full processes (Table 2).

Even if the high majority of studies underlying molecule transfer by electric fields have been performed on 2D cell culture in Petri dish or in cells cultured in suspension, 3D multicellular spheroids and engineered reconstructed human skin represent a nice, relevant, cheap, easy-to-handle *in vitro* model. Upon growth, spheroids display a gradient of proliferating cells. These proliferating cells are located in the outer cell-layers and the quiescent cells are located more centrally. This cell heterogeneity is similar to that found in avascular micro regions of tumors [27]. Confocal microscopy allowed to visualize the repartition of permeabilized cells in spheroids submitted to electric pulses. Results revealed that cells were efficiently permeabilized, whatever their localization in the spheroid, even those in the core, mimicking previously observed *in vivo* situations. Propidium iodide uptake was observed to be present but spatially heterogeneous within the 3D multicellular spheroid after electroporation, with a progressive decrease from peripheral to interior cells. In the case of large molecules as plasmid DNA, spheroids allowed showing that electrophoresis, and not tissue deformation or electroosmosis, is the driving force for interstitial transport. In addition, and at the opposite of cells in 2D cultures, only cells on one side of the outer leaflet expressed the reporter gene [28]. For siRNA uptake, we also showed that it was not uniform across 3D multicellular spheroids. The electrophoretic migration of nucleic acids upon delivery of unipolar electric field pulses could explain the asymmetry of siRNA uptake. Moreover, a gradient was observed from external layers toward the center, leading to siRNA silencing of GFP positive cells located in the outer rim. While siRNA delivery experiments on spheroids may differ from intratumoral injections, the levels of transfection in spheroids are comparable to levels observed in published studies *in vivo*. Taken together,

our results provide fundamental information about siRNA 3D distribution during electrotransfer, indicating that multicellular spheroids remain a relevant alternative to animal experimentation [29].

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

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

The clarification of spheroids carried out confirmed that all the cells of the spheroids have been efficiently permeabilized and that no cells were transfected in the internal layers. This in-depth method for microscopy observation is however not the most relevant for the detection of cells expressing plasmid DNA since the labelling does not seem to be stable. Yet, while efficient *in vitro*, gene-electrotherapy remains challenging in tumors. To assess the differences of gene electrotransfer in respect to applied pulses in multi-dimensional (2D, 3D) cellular organizations, we herein compared pulsed electric field protocols applicable to electrochemotherapy and gene electrotherapy and different "High Voltage-Low Voltage" pulses. Our results show that all protocols can result in efficient permeabilization of 2D- and 3D-grown cells. However, their efficiency for gene delivery varies. The gene-electrotherapy protocol is the most efficient in cell suspensions, with a transfection rate of about 50%. Conversely, despite homogenous permeabilization of the entire 3D structure, none of the tested protocols allowed gene delivery beyond the rims of multicellular spheroids. Taken together, our findings highlight the importance of electric field intensity and the occurrence of cell

permeabilization, and underline the significance of pulses' duration, impacting plasmids' electrophoretic drag. The latter is sterically hindered in 3D structures and prevents the delivery of genes into spheroids' core [30].

This low expression is in fair agreement with *in vivo* experiments on tumors. Close contacts between cells and extracellular matrix may act as physical barrier that limit/prevent (uniform) DNA distribution and explain the absence of gene expression in the inner region of spheroid. The limited access of plasmid DNA to central region of spheroid remains a significant barrier to efficient gene delivery in tissues. Taken together, these results, in agreement with the ones obtained by the group of R. Heller [31], indicate that the spheroid model is more relevant to an *in vivo* situation than cells cultured as monolayers and therefore can be useful to address the mechanisms of DNA electrotransfer. In order to assess the effects of the extracellular matrix composition and organization, as well as intercellular junctions and communication, other 3D reconstructed human connective tissue model can be used. Cell sheets, reconstructed *in vitro* by a tissue engineering approach, presents multiple layers of primary dermal fibroblasts embedded in a native, collagen-rich extracellular Matrix (ECM) and can be a useful tool to study skin DNA electrotransfer mechanisms. Cells within this standardized 3D tissue can be efficiently electropermeabilized by milliseconds electric pulses [32,33]. Moreover such a tissue-engineered dermal model recapitulates the mechanical properties of human native dermal tissue unlike the classically used monolayer and spheroid models [34]. A better comprehension of gene electrotransfer in such a model tissue would help improve electrogene therapy approaches such as the systemic delivery of therapeutic proteins and DNA vaccination.

Conclusions

“Intracellular delivery of materials has become a critical component of genome-editing approaches, *ex vivo* cell-based therapies and a diversity of fundamental research applications. Limitations of current technologies motivate development of next-generation systems that can deliver a broad variety of cargo to diverse cell types. Every day in research institutes and clinical centres around the world, scientists use kits and protocols based on viral vectors, lipid transfection agents, and electroporation, among other options. The complex mechanisms of established methods and their often-unpredictable impact on cell behaviour have dramatically limited the scope of biological experiments and reduced efficacy of potentially promising cell

therapy concepts. The biomedical research community would benefit greatly from a more mechanistic and transparent understanding of intracellular delivery, both to further the development of more robust techniques and to realize key medical and industrial applications" [32]. In this context, the so- called electroporation technology is probably the most promising one.

Classical theories of electropermeabilization present some limits to give a full description of the transport of molecules through membranes. Certain effects of the electric field parameters on membrane permeabilization, and the associated transport of molecules, are well established but a great deal of what happens at the molecular level remains speculative. Molecular models of lipid bilayers and electropore formation are giving interesting new insight into the process. Electroinduced destabilization of the membrane includes both lateral and transverse redistribution of lipids and proteins, leading to mechanical and electrical modifications which are not yet fully understood. One may suggest that such modifications, that may vary according to the microenvironment, can be involved in the subsequent transport of molecules interacting with them such as the DNA molecules. Experimental verification of the basic mechanisms leading to the electropermeabilization and other changes in the membrane, cells and tissues remain a priority given the importance of these phenomena for processes in cell biology and in medical applications. *In vivo* gene electrotransfer will face other challenges such as the necessity to control electric field distribution and gene expression both in space (targeted DNA delivery to the cells) and in time. Guidelines for successful DNA delivery are still required but we can be optimistic that further working to improve gene electrotransfer mechanisms will yield effective treatments, as it becomes the case for cancer eradication by reversible or irreversible electroporation [35]. Electric fields are among physical stimuli that have revolutionized therapy. Occurring endogenously or exogenously, the electric field can be used as a trigger for controlled drug release from electroresponsive drug delivery systems, can stimulate wound healing and cell proliferation, may enhance endocytosis or guide stem cell differentiation. Understanding the multiple effects of this powerful tool will further help harnessing its full therapeutic potential in an efficient and safe way [36].

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Chapter 8

Gene electrotransfer *in vivo*

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Abstract: Gene electrotransfer consists of the administration of nucleic acids (DNA, RNA oligonucleotides…) and the subsequent application of electric pulses to the tissue of interest to allow the introduction of the nucleic acids into the cells and thus the therapeutic effect of the delivered genetic material. Due to the size of the nucleic acids, the electrical parameters of gene electrotransfer vary widely depending on the tissue to be transfected and the desired level and duration of expression, as well as the associated tissue damage. In addition to optimizing the electrical parameters for a particular application, the design of the therapeutic plasmid DNA or RNA molecules can also influence the therapeutic outcome. The initial gene electrotransfer studies mainly focused on evaluating the electrical parameters for efficient gene delivery into various tissues such as skin, muscle, liver, and tumors using different reporter genes encoding fluorescent proteins, luciferase, and β galactosidase. The therapeutic field of gene electrotransfer is mainly divided into two areas: DNA vaccination and cancer gene therapy. DNA vaccination against infectious diseases and cancer on the one hand, and anti-angiogenic and immunomodulatory gene therapies against cancer on the other hand are the predominant areas of research. In addition, more and more clinical trials are being registered, mainly in the USA, using electroporation to deliver therapeutic plasmid DNA. The prospects of therapeutic gene electrotransfer for cancer therapy lie mainly in its combination with standard local ablative therapies, such as radiotherapy or electrochemotherapy, with the aim of converting local treatments into systemic ones. In addition, much preclinical work is devoted to the optimization of therapeutic plasmid DNAs, the development of new electrodes, and the evaluation of electrical parameters, which will lead to better planning and design of clinical trials.

Introduction

The *in vitro* application of electroporation for the introduction of DNA into the cells was evaluated and tested in 1982 by Neumann et al [1], 6 years before the use of electroporation for delivery of antitumor chemotherapeutic drugs (electrochemotherapy) into the tumor cells [2]. However, *in vivo* studies only slowly followed, and the first *in vivo* study was performed in 1991 by Titomirov et al [3], who investigated the usefulness of exponentially decaying pulses for delivery of genes to the mouse skin. Later on, the transfection of brain, liver, tumor and muscle with various reporter genes were successfully demonstrated using different types of electric pulses[4–8]. Due to the physicochemical properties and size of nucleic acids compared to small chemotherapeutic agents, the entry mechanism of nucleic acids is different from that of small molecules. In tissues, other tissue- and cell-related parameters also influence transfection efficiency, such as cell size, shape, and organization in the tissue, the presence of the extracellular matrix, and the heterogeneity of the tissue (presence of similar cells in a given tissue). In addition, the construction of the plasmid and its administration may also affect the degree of transfection as well as its duration. Therefore, a large number of studies have been conducted in the field of *in vivo* gene electrotransfer to evaluate the different parameters of electrical pulses for different tissue types and applications (Fig. 1). Currently, the therapeutic use of gene electrotransfer is mainly focused on two areas: DNA vaccination and cancer gene therapy [9,10], but it is also becomingincreasingly important in other fields, such as ophthalmology [11].

Preclinical gene electrotransfer of reporter genes

The reporter genes used in preclinical gene electrotransfer studies mainly encode either various fluorescent proteins or luciferase. Both allow visualization of tissue transfection (gene expression in cells in tissues) *in vivo* with various types of *in vivo* imaging, either whole body or at the cellular level [12,13]. Most studies have been performed in muscle and skin because these tissues are easily accessible and therefore represent an obvious target tissue for DNA vaccination. Apart from being easily accessible for gene electrotransfer, muscle cells are long-lived and can produce relatively large amounts of therapeutic proteins, which are also released into the bloodstream and thus have a systemic effect. On the other hand, the skin is also an excellent target tissue, not only because of its easy

accessibility, but mainly because of the numerous immune cells in the skin that can trigger an effective immune response of the organisms needed for DNA vaccination [14–16](Fig. 2).

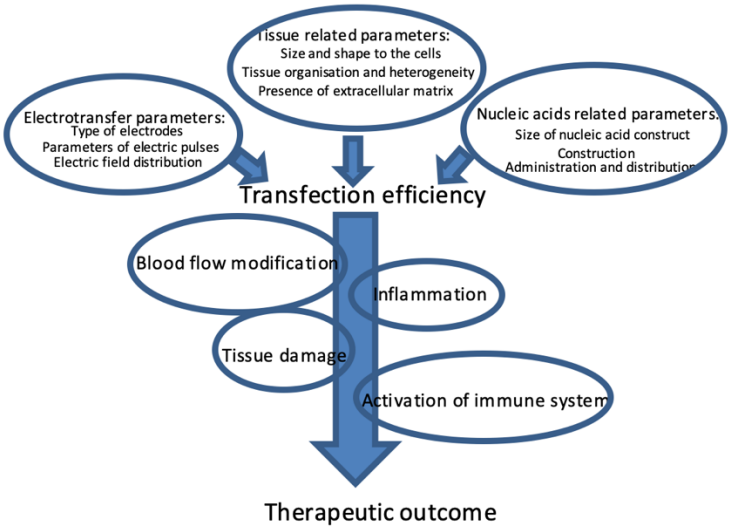

Figure 1: Different parameters can influence the transfection efficiency and therapeutic outcome of gene electrotransfer.

As mentioned in the introduction, many different parameters of electric pulses were used, either short (~100μs) high voltage pulses (in the range of ~1000 V) or long (up to 100 ms) low voltage (up to few 100 V) pulses. Moreover, different combinations of high voltage and low voltage pulses with different frequencies were tested and showed improved transfection in skin and muscles compared to single type of pulses used for transfection [15–18]. In tumors, the combination of pulses did not result in improved transfection [19]. In addition, the influence of orientation and polarity of the applied electric pulses were also evaluated in tumors, demonstrating that increased transfection efficiency is obtained only by changing the electrode orientation, but not pulse polarity [20].

The main type of electrodes used in the studies was either plate or needle and more recently also non-invasive multielectrode arrays [21–23]. Other types of electrodes that were tested for gene electrotransfer were spatula electrodes for gene delivery to muscle [24] and other types of noninvasive electrodes, such as needle free, meander and contact electrodes for skin delivery. Selection of electrode is very important for appropriate electric field distribution in the tissue which is a prerequisite for effective gene electrotransfer [25–28].

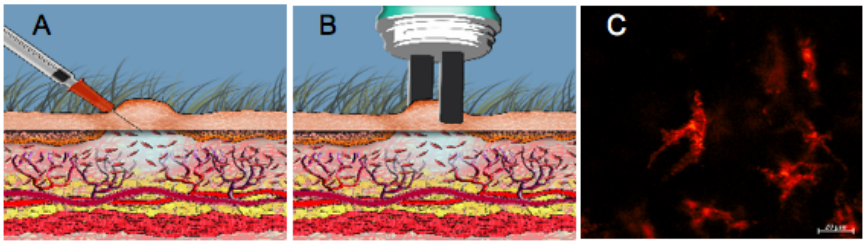

Figure 2: Gene electrotransfer to skin. **A** injection of plasmid DNA subcutaneously. A bubble on the skin will be formed. **B** If using plate electrodes, they are positioned in a way that the bubble is encompassed between the two plates. **C** Intravital confocal microscopy of cells in mouse skin expressing DsRED fluorescent protein at the depth of 30 μm. See also Fig. 9, Chapter 3.

Besides electrical parameters, the type of nucleic acid used for electrotransfer can also affect the transfection efficiency. Namely, it was shown that smaller siRNA can more easily crossed the plasma membrane compared to larger plasmid DNA molecules, however the duration of the expression (or effect) is shorter [29–31]. Therefore, plasmid DNA is still most commonly used in gene electrotransfer studies. To improve the safety and targeting of plasmid DNA transfer and minimize unwanted tissue damage, plasmids with tissue-specific promoters, no antibiotic resistance gene, and minimal or no bacterial backbone were constructed and evaluated in combination with electroporation [32–36].

Because of the size of plasmid DNA and the presence of nucleases in blood and tissues, the most appropriate way to administer plasmid DNA is by local injection. The distribution of plasmid DNA in different tissues occurs in different time periods, so the timing between the injection of plasmid DNA and the application of electrical pulses is also very important. For muscles, it has been shown that this should be as soon as possible, while for tumors, depending on the histological type, it can be up to 30 minutes after the injection of the plasmid [37–39]. Improved distribution and consequently better transfection efficiency can be achieved also by pretreatment of muscles and tumors with extracellular matrix degrading enzymes, such as hyaluronidase and collagenase [40,41]. Recently, it has also been shown that moderate tissue preheating at 43 °C results in good gene expression and allows the applied voltage to be lowered and the number of pulses delivered to be reduced [42–44].

In vitro, it was shown that size, orientation and shape of the cells influence the permeabilisation of the cell membranes and thus also transfection efficiency. The same is also valid *in vivo*. Tissues with more organized structure, such as muscle are easier to transfect than highly

heterogenic tissue, such as tumors [45]. In addition, in tumors with large cells higher transfection efficiency was obtained compared to tumors with smaller cells [46,47].

The importance of careful selection of plasmid DNA and electrical parameters for specific application, was recently reinforced by experiments showing that gene electrotransfer of plasmids devoid of therapeutic gene can induced complete regression of tumors and that cytosolic DNA sensors activating innate immune response were upregulated following gene electrotransfer [48]. The inflammation and induction of immune response was demonstrated also for muscle and skin transfection. In addition, it was demosntratedf that also non-immune cellspresent in the skin contribute to the observed effects [49–51].

Preclinical and clinical gene electrotransfer of therapeutic genes

The preclinical studies in which therapeutic genes have been used have been mainly devoted to the evaluation of gene electrotransfer for DNA vaccination or treatment of various diseases such as cancer, where the therapies are designed either to target tumor cells directly or to enhance the immune response of the organism against cancer cells.

In general, gene therapy can be performed using two different approaches. The first is *ex vivo* gene therapy, in which cells, including stem cells, are removed from the patient, transfected in vitro with the plasmid or viral vector, selected, amplified, and then re-injected into the patient. The other approach is *in vivo* gene therapy, in which exogenous DNA is delivered directly into the host target tissue, e.g., locally into the tumor or peritumorally and for systemic release of the therapeutic molecule into skeletal muscle, depending on the type of therapeutic molecules and the treatment goal [52].

Gene electrotransfer was first used for DNA vaccination in 1996 [53]. Currently, numerous studies, using gene electrotransfer mainly to muscle and skin for DNA vaccination against infectious diseases, arthritis, multiple sclerosis, inflammation are undergoing. In addition, several clinical trials, against infectious diseases, such as HIV, hepatitis are going on. Gene electrotransfer of plasmid DNA resulted in stimulation of both arms of adaptive immune system, humoral and cellular [9,10].

In cancer gene therapy, gene electrotransfer of therapeutic genes directly into tumors facilitates local intratumoral production of therapeutic proteins, enabling sufficient therapeutic concentration and thus therapeutic outcome.

This is especially important in the case of cytokines, where high systemic concentrations are associated with severe toxicity.

The first evaluation of intratumoral gene therapy using electrotransfer for cancer treatment was performed 3 years after the first DNA vaccination study in 1999 in murine melanoma tumor model [54]. Since then, a variety of therapeutic genes, mostly encoding cytokines but also tumor suppressor proteins, and siRNA molecules have been tested against various targets, such as oncogenes, in numerous animal tumor models. Overall, the results of preclinical studies suggest that intratumoral therapeutic gene electrotransfer allows efficient transgene expression with sufficient production of therapeutic proteins that can even lead to complete tumor regression and, in some cases, induction of long-term antitumor immunity in treated animals [55–58].

Some of the most significant antitumor effects to date in cancer gene therapy have been achieved with employment of active nonspecific immunotherapy, i.e. use of cytokines. Gene electrotransfer of genes, encoding different cytokines, has already shown promising results in preclinical trials on different animal tumor models. Cytokine genes, which showed the most potential for cancer therapy, are interleukin (IL)-2, IL-12, IL-18, interferon (IFN) α, and GM-CSF [10,59–61]. Currently, the most advanced therapy is using IL-12, which plays important role in the induction of cellular immune response through stimulation of T-lymphocyte differentiation and production of IFN-γ and activation of natural killer cells [62]. Antitumor effect of IL-12 gene electrotransfer, has already been established in various tumor models, e.g. melanoma, lymphoma, squamous cell carcinoma, urinary bladder carcinoma, mammary adenocarcinoma and hepatocellular carcinoma [63]. Results of preclinical studies show that beside regression of tumor at primary and distant sites, electrogene therapy with IL-12 also promotes induction of long-term antitumor memory and therapeutic immunity, suppresses metastatic spread and increases survival time of experimental animals [63]. On preclinical level, gene electrotransfer to tumors was also employed in suicide gene therapy of cancer, replacement of oncogenes therapies, introduction of wild type tumor suppressor genes, production of checkpoint-inhibiting antibodies within the tumors by DNA base delivery etc. Another approach in cancer gene therapy, which is currently being widely investigated, is based on inhibition of angiogenesis of tumors. The basic concept of antiangiogenic gene therapy is transfection of cells with genes, encoding inhibitors of tumor angiogenesis. Electrotransfer of plasmids encoding antiangiogenic factors (angiostatin and endostanin) was demonstrated to be effective in inhibition of tumor growth and metastatic spread of different tumors. Recently, RNA interference approach was evaluated, using siRNA molecule against

endoglin, which is a co-receptor of transforming growth factor β and is overproduced in activated endothelial and also certain tumor cells. Gene electrotransfer of either siRNA or shRNA molecules against endoglin resulted in vascular targeted effect in mammary tumors as well as antitumor and antivascular effect in melanoma tumors that are expressing high level of endoglin [64–70] .

Muscle tissue is used as a target tissue in addition to DNA vaccination because it allows high production and secretion of therapeutic proteins. Gene electrotransfer into muscle has been studied with the aim of treating various muscle diseases, local secretion of angiogenic or neurotrophic factors, or systemic secretion of various therapeutic proteins such as erythropoietin, coagulation factors, cytokines, monoclonal antibodies, and so on. In gene therapy of cancer, gene transfer of plasmid DNA encoding cytokines IL -12, IL -24 and antiangiogenic factors has been studied with encouraging results.

Clinical studies on gene electrotransfer with plasmid DNA encoding cytokine IL-12 in patients with melanoma, as well as in veterinary patients show great promise for further development of this therapy [71,72]. In a human clinical trial, 24 patients with subcutaneous metastases from malignant melanoma were treated 3 times. Response to therapy was observed in both treated and distant, untreated tumor nodules. A systemic response was observed in 53% of patients, resulting in either stable disease or objective response. The main adverse event was transient pain after the application of electrical pulses. Tumor necrosis and infiltration of immune cells were observed in biopsies after treatment. This first human clinical trial of IL -12 electrogene therapy for metastatic melanoma has demonstrated that this therapy is safe and effective [71]. In veterinary oncology, 8 dogs with mastocytoma were treated with IL-12 gene electrotransfer. A good local antitumor effect was obtained with significant reduction of treated tumors from 15% to 83% (mean 52%) of the original tumor volume. In addition, a change in the histological structure of the treated nodules was observed, as evidenced by a reduction in the number of malignant mast cells and infiltration of inflammatory cells in the treated tumors. In addition, a systemic release of IL-12 and IFN-γ was observed in the treated dogs without any noticeable local or systemic side effects [73]. Again, data suggest that intratumoral IL-12 gene therapy via electrotransfer could be used to control both local and systemic disease.

For example, results of intramuscular IL-12 gene electrotransfer in canine patients indicate that it is a safe procedure, which can result in systemic shedding of hIL-12 and possibly trigger IFN-γ response in treated patients, leading to prolonged disease-free period and survival of treated animals [74].

Perspectives

In oncology, local ablative therapies are very effective, but they lack a systemic component. Therefore, great efforts are being made to develop treatments that are systemic or that add a systemic component to local treatment. With the advancement of knowledge in tumor immunology, new immunomodulatory therapies have been developed to treat cancer and are currently being combined with standard treatment with great success. DNA vaccines and immune gene therapies with cytokines aim to stimulate antitumor immunity and are therefore good candidates for combination with local ablative therapies [75,76].

Several studies combining electrochemotherapy or radiotherapy with gene electrotransfer have been studied preclinically. The most promising immune gene therapy that has already reached clinical trials in veterinary and human oncology is gene electrotransfer from IL -12. In the preclinical studies IL -12, gene electrotransfer has been combined with electrochemotherapy and radiotherapy in various tumor models. Intramuscular gene electrotransfer of IL -12 in combination with electrochemotherapy with cisplatin increased the percentage of complete regression of fibrosarcoma SA -1 tumors to 60% compared to 17% complete regression after electrochemotherapy alon [77]. When combined with radiotherapy even 100% complete response of LPB tumors was obtained [78]. Intratumoral IL-12 gene electrotransfer resulted in ~2.0 radiation dose modifying factor [79].

Clinically, several studies were performed in client owned dogs, combining electrochemotherapy with either bleomycin or cisplatin and intratumoral or peritumoral application of IL-12 gene electrotransfer [73–76]. Recently, a study compared the use of canine IL-12 administered either peritumorally or intratumorally in combination with electrochemotherapy with electrochemotherapy alone. The study in mast cell tumors showed significant superiority of the intratumoral approach, resulting in prolonged disease-free and progression-free survival. The percentage of complete remissions at the end of the observation period (24 months) was 69% after electrochemotherapy, 89% after electrochemotherapy combined with peritumoral application of plasmid DNA encoding canine IL-12, and 94% after intratumoral application [84]. These clinical trial results are very promising and further studies, hopefully in human oncology, are foreseen.

Gene electrotransfer holds great potential for further developments that could lead to new clinical trials in both DNA vaccination and gene therapy applications. Plasmid design is critical for appropriate production and action of therapeutic proteins. Therefore, research is focused on codon

optimization, the use of different promoters (tissue-specific and inducible), the incorporation of different immunostimulatory motifs into the plasmid sequence, and the use of plasmids without antibiotic resistance genes, which is in line with regulatory authorities. In addition, physical factors such as increased temperature can also lead to improved gene transfer. Furthermore, new types of electrodes such as microneedles and non-invasive multi-electrode arrays with carefully selected parameters of electrical pulses and real-time measurement of impedance are being evaluated, which will lead to efficient gene electrotransfer with minimal side effects and inconvenience for the patients.

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Chapter 9

Prophylactic and Therapeutic Applications of Gene Electrotransfer

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Abstract: Gene-based medicine has the potential to be used in multiple applications for the prevention or treatment of disease. Significant progress is being made including several therapies gaining regulatory approvals. However, there are still areas that need to be improved including to efficacy and toxicity. A key hurdle that still needs to be overcome is delivery. While viral vector-based delivery has dominated the gene therapy field, there have been major advances in non-viral delivery in recent years. Successful protocols, particularly for nucleic acid vaccines using lipid nanoparticles as the delivery vehicle have been accomplished. Physical methods of nucleic acid delivery have also seen major advances. These approaches include hydrodynamic delivery, ultrasound and electroporation. Of the physical delivery approaches, electroporation also referred to as gene electrotransfer (GET) has made the most progress. GET has been tested in clinical trials for multiple indications, but primarily as a tool for delivery of DNA vaccines or for immunotherapy. The major indications have been for cancer and infectious diseases. A major advantage of GET is the versatility of delivery which allows for the development of delivery protocols that can achieve expression profiles that can achieve the desired therapeutic or protective response. As this approach advances, it is anticipated that in the not-too-distant future there will be approved therapies using GET for gene-based therapeutics.

Gene Transfer/Therapy

Therapeutics are typically delivered in the form of recombinant proteins. Since the concentration of the therapeutic molecule is known as well as the

pharmacodynamics and pharmacokinetics of the protein, dosing can be more efficiently controlled. However, since most of these proteins have short half-lives, therapeutic dosing typically requires higher dosing and more frequent dosing to maintain therapeutic levels. This can lead to adverse effects. In addition, since these proteins are typically produced in non-human cells there can be an induced immune response following administration. Utilizing a gene-based approach can poentially overcome these issues. Following delivery of the gene, the protein can be continually produced by the patient's cells. This reduces the frequency of treatment and reduces unwanted reactions since the protein is produced by the patient's own cells and modified correctly. However, since dosing is based on the level of the nucleic acid delivered, the levels of the expressed protein can be variable.

Nucleic acid-based approaches can be used for a wide variety of applications and include both therapeutic and prophylactic approaches. Applications include therapeutic protocols for cancer, cardiovascular deficiencies, metabolic disorders and pulmonary diseases. Vaccine approaches can be for both prophylactic and therapeutic and are predominately designed for infectious diseases or cancer.

Recently there have been major advances in moving from gene transfer protocols to gene therapy. Although clinical trials testing various gene-based approaches have been tested for over 40 years, it has not been until the last 10-15 years that these approaches have gained regulatory approval. As of March, 2023, there are 46 nucleic acid-based therapies that have been approved by one or more regulatory agencies [1]. Twenty-four of these utilize DNA and include modified cell therapies. Twenty-two of these utilize RNA. Many of these are vaccines for CoVID-19.

Protocol Development

A key component for developing a successful nucleic acid-based approach is designing an effective delivery protocol. A critical element of the delivery is to achieve expression of the effector molecule in the right location and achieving the appropriate expression that will result in the desired clinical outcome with minimal adverse effects.

As mentioned above, effective delivery is an important aspect to developing an effective gene therapy protocol. Delivery can be divided into two broad categories, biological and non-biological. Biological delivery utilizes a biological carrier/vector to transport the gene into the cell. Viruses, bacteria and phages have been used for this purpose although viral

vectors are the predominant carrier utilized. Non-biological (non-viral) utilizes plasmid DNA. In this case, delivery is done via simple injection into the tissue of choice or facilitated by using chemical or physical assistance. Factors to be considered when deciding how to perform the delivery include efficiency, potential efficacy and potential adverse effects.

Many gene therapies require long-term expression for protein replacement or gene correction, in these therapeutic approaches it is typical to utilize viral vectors [2-4]. In contrast, when the transgene encodes a toxic molecule or the expressed protein will modulate an immune response or requires low or short-term expression, it is typical to utilize a non-viral delivery approach [5-15]. Recently as non-viral approaches have become more efficient and technologies have been developed to enable longer term expression there is less of a clear distinction between viral and non-viral approaches. The improvements to non-viral delivery make it a more attrative option due to the better safety profile as well as reduced risk of genomic integration, less potential for immunogenicity and reduced risk for environmental spread.

While biological vector-based delivery has been the predominant delivery approach utilized for gene therapy, there have been major advances in non-biological delivery in recent years. Certainly, the success of lipid nanoparticles in recent years, particularly for delivering mRNA-based vaccines, is the most prominent example [16-19]. However, other delivery approaches have also made major advances such as physical methods including hydrodynamic delivery, ultrasound and electroporation [13, 20-29]. Electroporation of plasmid DNA now referred to as gene electrotransfer (GET) was initially tested in skin, liver, muscle, and tumors [24,26-31]. Since then, this technique has been applied to many other tissues and animal models [13,33,34]. Currently, there are over 130 clinical trials listed at clinicaltrials.gov using electroporation [35,36].

Of the physical delivery methods, GET has made the most progress. A key difference between protein therapy and gene therapy is dosing. Controlling the dose of the administered transgene is certainly achievable and can be redily documented, but controlling the levels of the expressed protein is more elusive. One way to exert control is through the use of inducible promoters [37,38]; however, for applications that require short term or strict control of protein expression levels, control through the delivery method would be more desirable. Utilizing GET has the potential to achieve this goal. GET parameters including electric field strength, pulse duration, pulse number, electrode geometry and configuration can be manipulated to some extent in order to control onset, level, and duration of protein expression of the transgene [39,40].

The characteristics of expression necessary for the specific therapeutic outcome should be determined first when designing a GET protocol. Pulse parameters are not the only characteristics to take into account when designing the protocol. Other factors include the tissue target, plasmid construct and electrode design. The tissue target can influence many aspects including the level and kinetics of expression as well as if you want local or systemic expression. Most GET protocols have used muscle, skin and tumors within the skin as the tissue target [13-15,27,32,37,39]. However, other tissue targets such as liver, lung, heart, brain, kidney and spleen have also been part of GET protocols [13,15,25-34]. Muscle is a major target for achieving high and long expression and to achieve systemic expression. Although, recent studies have shown that skin could also be used to achieve systemic expression as well [14,24,39-51]. A key criterion for any tissue tartget is being able to access it and inject the plasmid and deliver the pulses.

The electrode design facilitates delivery to tissues. The two main categories of electrodes are penetrating and nonpenetrating. Electrode designs have also become more sophisticated and can be more than just two plates or two needles. Arrays can be utilized that have all or some of the electrodes active during each pulse [52-57]. These arrays can be designed to enable rotation of the field, which can influence the level and efficiency of expression. For treating internal tissues, catheter electrodes have been developed to enable minimally invasive delivery approaches to be accomplished [58-62]. See also Chapter 11.

The plasmid construct can be designed to influence expression [63-64]. The promoter can be selected to target expression to a specific tissue or cell type such as keratinocytes or dendritic cells which would enable targeting the expression to the skin [13,65]. While this approach can be used to target expression, the level of expression is usually lower with targeted promoters and the targeting may not be as specific as required. Inducible promoters can also be used to potentially control expression levels and kinetics. When utilizing an inducible promoter, a specific ligand or specific molecule is used after delivery to turn expression on and once the stimulus is expended the expression is turned off [66, 67]. One also needs to be aware that there could be some baseline expression present even in the absence of the stimulus. Studies have also been conducted that have demonstrated that targeted expression can be performed using DNA nuclear targeting sequences which can target expression to specific cell types [68-70]. Electrotransfer of plasmid DNA will typically result in transient expression. If long-term expression or gene correction is desired then one can include transposons, integrase from phage phiC31 or use a CRIPR Cas system [71-82].

There are many aspects of the pulse parameters that should be considered when designing an electrotransfer protocol. The applied voltage or nominal field strength (defined as volts/distance between positive and negative electrodes) is typically one of the main characteristics to determine. An arbitrary dividing line between low and high voltage pulses is 700 V/cm. The second critical characteristic is pulse width which is typically in the range of microseconds to milliseconds. Microsecond pulses are usually delivered with high voltage pulses and millisecond pulse with low voltage pulses. The other major characteristic is pulse number and pulse repetition rate (when multiple pulses are used). There are times when a single pulse will be effective, but most often protocols will use multiple pulses. Protocols can be designed using one pulse type or combination of pulse types and field strengths [83]. Manipulation of these core characteristics can influence the expression levels. For most tissue targets, when using microsecond pulses at high field strength will yield low to moderate expression while using millisecond pulses at low field strength will yield high expression. There are tissues where the opposite is true, so it is important to take the tissue target into consideration when choosing pulse parameters.

Preclinical studies for translation of electrically mediated gene delivery

A major step in testing a GET protocol is to determine the efficacy which is typically performed in an appropriate animal model. The model and the tissue target would be dependent on the disease or condition that the therapy is being designed for. Prior to initiating these studies, it is important to determine what level and kinetics of expression are required to achieved the desired response. There are times that a protocol can be first tested using a reporter gene to test the expression. While this will provide some important basic information, it is important to note that if the therapeutic gene is in a different plasmid construct, it is possible that the expression may vary from what was seen with the reporter plasmid. Multiple tissues have been utilized for GET approaches. While other tissues have been utilized, most protocols have been developed for delivery to muscle, skin and tumors.

To move a potential gene therapy into a clinical trial and eventually achieve regulatory approval there are multiple steps that need to be accomplished. Efficacy and safety of the approach needs to be demonstrated. This would need to be done in an appropriate model and in

some cases may require multiple preclinical models. In addition, because a drug (plasmid DNA encoding a transgene) and a device (pulse generator and electrode) are both being used, additional elements for approval may be required as it will be evaluated as a combination therapy. Investigators would need to show efficacy and safety of both the drug and device. In some cases, this may require two animal models. Safety studies may also require performing a full toxicity study and potentially may also require performing a biodistribution study to determine location and persistence of the plasmid [84]. Once the preclinical studies are completed and both safety and efficacy are demonstrated, investigators can seek approval to initiate clinical trial.

Delivery to muscle

Muscle has been a common target for many GET protocols. Because the cells are long-lived it is possible to achieve long-term expression when delivering to the muscle. It is also a highly vascularized tissue which makes it an excellent target for protocols requiring the expressed protein to be in the blood circulation [85,86]. Delivery can also be achieved without exposing the tissue, using minimally invasive approaches. Delivery can be accomplished with either penetrating or non-penetrating electrodes [32,41]. In addition to potential protein replacement protocols, muscle has been tested quite extensively for delivery of DNA vaccines [36,87-94]. There have been several reviews that have been published documenting the use of muscle for GET [93,95-98].

Delivery to skin

Skin is the largest organ and is highly accessible which has made it a rapidly emerging target for gene therapy protocols [99-105]. Similar to muscle, delivery to skin can be done in a non-invasive or minimally invasive approach using either penetrating or non-penetrating electrodes. Skin is an interesting target for protocol development as delivery can be targeted to different layers within the skin. Superficial delivery can be achieved to the epidermis, but deeper penetration can also be achieved to the dermis or subdermis. The target withing the skin would be dependent on the specific application. Skin also has the advantage of enabling easy observation of the injection site and placement of electrodes. A distinct advantage of skin is the presence of antigen presenting cells, which can be

utilized to enhance the effectiveness of DNA vaccines delivered to the skin using GET. Most DNA vaccine protocols have been targeted against infectious disease, but there have also been protocols evaluated for the treatment of cancer [54,57,106-111]. Delivery to skin has also been an effective delivery target for enhancing wound healing and treating ischemic tissue [112-117].

Delivery to tumors

There has been extensive work developing GET protocols for the potential treatment of various cancer types. As mentioned above both muscle and skin have been utilized as targets for these protocols. However, direct delivery to tumors is a viable option and can result in very effective protocols. Successful protocols have been developed that have utilized either a direct killing of tumor cells with the delivered agent or an indirect approach in which the delivered agent stimulates a reaction against the tumor cells or a combination of the two. GET can be a valuable tool to deliver immunostimulatory agents because by modulating the various pulse parameters, one can have better control of the expression pattern which is critical when stimulating an immune response. This approach is also important when delivering a transgene encoding a toxic protein. In both of these cases expression levels need to be controlled to achieve the desired response with minimal adverse effects. Multiple strategies have been utilized to effectively cause regression of solid tumors such as immune stimulation, delivery of toxic molecules, induction of apoptosis or inducing anti-angiogenesis [13,118,119]. Predominately, protocols have targeted cutaneous or subcutaneous due to the accessibility allowing more controlled injection of the plasmid and placement of electrodes. Treatment of internal tumors, while more of a challenge, has been accomplished either through the combination of surgery or the use of transcutaneous electrodes that are placed through imaging. As the technology has advanced electrodes have been incorporated into catheters and scopes to enable a less invasive approach [120-125].

Delivery to other tissues

GET protocols can be developed to target almost any tissue. The key is determining the best way to access the tissue and developing the appropriate electrode to use for the delivery. In general, as was true with

muscle, skin and tumors, either penetrating or non-penetrating electrodes can be utilized. For internal tissues access can be obtained either through surgery or by the use of catheters or scopes. Once access is achieved the same principles apply with respect to injection of the plasmid and manipulation of GET parameters to achieve the appropriate expression profile. For all tissues, it is important to understand the unique properties of that tissue to select the correct delivery parameters and develop the appropriate electrode design.

The three tissues that have been targeted the most outside of muscle, skin and tumors, are the liver, lung and heart. Liver has been evaluated for a variety of applications, but predominately has been used as a target to test potential treatments for metabolic diseases and cancer. Two approaches have been tested, injecting the plasmid DNA through the vasculature or by injecting directly into the liver. A variety of differently designed electrodes have been tested [24,126-129]. Lung is an interesting tissue for gene therapy applications as there are several genetic and acquired diseases associated with the lung. Gene therapy approaches for the lung have primarily utilized viral vectors. The major obstacle for the use of GET is design of appropriate electrodes as well as proper placement of the electrodes. There has been work done that has demonstrated that successful delivery to the lungs can be accomplished using GET [45,46]. In small animal models it was possible to use plate electrodes placed across the chest combined with inhalation of a plasmid DNA solution [46]. More elaborate designs were utilized when this work moved to larger animals such as sheep and pigs [47,130,131]. Applications have been tested for potential treatment of tumors, reduction of inflammation or to improve lung function [132-134]. One aspect that these studies also addressed was potential disruption of cardiac function. However, in the studies conducted thus far, no signs of cardiac dysfunction have been observed [45,46,135].

While potential disruption of cardiac function including damage or fibrillation was a concern with gene delivery to the lung, it is especially a concern when delivery is targeted directly to the heart. There have been several studies that have evaluated using GET to deliver directly to the heart. It has been demonstrated in isolated cells and utilizing Langendorff-perfused whole heart that GET could be a useful tool to deliver plasmid DNA directly to cardiomyocytes. [42,43,136-138]. Studies have also been conducted demonstrating that the approach could be used to deliver plasmid DNA *in vivo* to the beating heart [138]. In moving studies to larger animals, it was determined that to achieve successful delivery and to prevent fibrillation it was critical to synchronize the applied pulses with the electrocardiogram. Pulses must be applied at the beginning of the R wave and be completed prior to the initiation of the T wave [42]. As with all GET

applications it is important to carefully select the electrode design, pulse parameters and delivery site [43,139]. One application tested with GET delivery to the heart has been as a potential treatment for ischemia and to reduce the size of or to treat an infarct by delivering plasmids encoding angiogenic factors [4243,139-141]. This approach was also shown to induce production of new cardiomyocytes [142].

Delivery to these internal tissues using GET demonstrated that the procedure could be performed effectively and safely. This opens up the possibility of treating disease within these tissues. While only muscle, skin, tumor, live, lung and heart have been mentioned, GET has been tested in several other tissues. These include, blood vessels, cornea, retina, sub-retinal space, brain, spleen and kidney [48,50,143-146].

Clinical Studies Utilizing GET

There have been many advances in gene therapy approaches over the past 4-5 decades. More than 3,800 clinical trials have been either initiated or completed for a variety of indications including cancer, monogenetic diseases, infectious disease, cardiovascular disease, neurological disease, ocular disease and inflammatory disease and cardiovascular diseases [35,118,147-150]. The majority of gene therapy clinical trials (56%) are Phase I safety and tolerability studies with an additional 23% Phase I/II (https://a873679.fmphost.com/fmi/webd/GTCT). Less than 15% of trials move to Phase II and less than 5% move to Phase III. Many trials to not make it past Phase I due to either adverse reactions or low efficacy.

Since the first clinical trial utilizing GET was initiated in 2004 [151], the number of clinical trials utilizing GET has been steadily increasing. There have now been over 130 clinical trials using GET to deliver plasmid DNA that are either completed or ongoing. The advances seen with GET can be attributed to the increased reproducibility, high efficiency, potential effectiveness and relatively low adverse effects compared to other delivery approaches. As mentioned in the previous section, GET can be used to effectively deliver plasmid DNA to multiple different tissue targets. Thus far the majority of GET clinical trials have focused on delivery to muscle, skin and tumors. With the increasing number of preclinical studies targeting other tissues, it will not be too long before clinical trials targeting other tissues will be initiated.

As mentioned above there are 131 clinical trials that have used GET as the delivery tool (clinicaltrials.gov). Ninety-six of these studies used muscle as the target an additional sixteen delivered directly to tumor and nine used

skin as the delivery target. There were also ten *ex-vivo* studies that transformed cells and then injected those cells. Within the 121 *in vivo* clinical trials, ninety-eight were designed to deliver vaccines with sixty-four targeting infectious agents and thirty-four designed for cancer therapy. The other twenty-three trials were designed to stimulate an immune response.

The initial GET clinical trial was designed to evaluate a cancer immunotherapy for melanoma. The approach was designed to deliver a plasmid encoding interleukin-12 (IL-12) directly into melanoma tumors. See also Chapter 8. The safety was documented in the Phase I trial as there were no systemic treatment related adverse events observed. In addition, over 70% of the treated lesions completely regressed. Interestingly, three patients of the nineteen patients with additional sites of disease outside the treated lesions showed complete regression of all metastases, both treated and untreated [151]. A Phase II clinical trial confirmed the results of the Phase I study. This approach has also been tested with other tumor types including Merkel cell carcinoma, Triple Negative Breast Carcinoma and Head and Neck cancer [153-155]. Due to the observation that patients who did not respond to the IL-12 gene therapy had elevated tumor levels of PD1 and PDL1 [156-157], a clinical trial was initiated that combined delivery of plasmid IL-12 with checkpoint inhibitors [156]. The Phase II combination trial resulted in minimal to low adverse events and objective responses were observed in 9 of 22 patients (41%) and complete response rate (all tumors responding) was 36% [156].

DNA vaccines delivered using GET have been tested as potential therapies for cancer. The concept is to stimulate an immune response against existing disease [14,35,118]. One study evaluated delivering a plasmid encoding tyrosinase and demonstrated stimulation of an immune response against melanoma tumor with minimal toxicity [158]. Another approach tested utilized a plasmid that encoded the tetanus toxin domain fused with prostate specific membrane antigen as a possible treatment for prostate cancer. This study also demonstrated an immune response with minimal toxicity [159,160]. This first prostate trial utilized an intramuscular approach and a second trial tested an intradermal approach and in this 2nd trial there was only a limited specific response against the encoded prostate specific antigen [161]. Another set of studies evaluated a DNA vaccine delivered via the intramuscular route evaluating a potential therapy for cervical intraepithelial neoplasia grade 2 or 3. In this study, specific T-cell responses were observed with no dose-limiting toxicities as well as a reduction in disease progression [162-167]. Other cancer vaccines have made it into clinical trials as well [168-170]

Infectious diseases have been a major target for DNA vaccines. There are distinct advantages using a nucleic acid approach including not having to

cultivate the pathogen and using nucleic acid significantly reduces the potential of infection as opposed to using attenuated or live virus as is done in some conventional vaccines. A nucleic acid vaccine can also be produced more rapidly and can be designed for a specific clone of the pathogen. The key for the use of nucleic acid-based vaccines is delivery. GET has made major contributions to resolving this issue and has been broadly tested in clinical trials [13,149-150].

The majority of GET vaccine trials have been designed to protect against viral infections. Of these studies most are evaluating vaccines for HIV. The major route of administration in these trials has been intramuscular. The HIV trials have explored a variety of antigens and results were encouraging as all showed increased immunogenicity and no significant adverse effects were observed [171-175]. Other vaccines have been tested for the following viruses: Hepatitis B, Hepatitis C, Dengue, Hantaan, Puumala, Ebola, and influenza [176-182]. In these studies, as with the HIV studies, good immune stimulation was observed with minimal or no significant adverse effects. Recently, this approach was tested for delivering a vaccine for SARS-CoV-2 [183-186].

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Chapter 10

Electrochemotherapy from bench to bedside: principles, mechanisms and applications

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Abstract: Electrochemotherapy consists of administration of the chemotherapeutic drug followed by application of electric pulses to the tumour, in order to facilitate the drug uptake into the cells. Only two chemotherapeutics are currently used in electrochemotherapy, bleomycin, and cisplatin, which both have hampered transport through the plasma membrane without electroporation of tumours. Besides these two drugs also calcium is used and is termed calcium electroporation. Preclinical studies elaborated on the treatment parameters, route of drug administration and proved its effectiveness on several experimental tumour models. Based on the known mechanisms of action, electrochemotherapy has been successfully tested in the clinics and is now in standard treatment of cutaneous tumours and metastases. Electrochemotherapy as a platform technology is now being translated also into the treatment of bigger and deep-seated tumours. With new electrodes and new electric pulse generators, clinical trials are on-going for treatment of liver metastases and primary tumours, of pancreas, bone metastases and soft tissue sarcomas, as well as brain metastases, tumours in oesophagus or in rectum.

Introduction

Electrochemotherapy consists of administration of the chemotherapeutic drug followed by application of electric pulses to the tumour, in order to facilitate the drug uptake into the cells. Electrochemotherapy protocols were optimized in preclinical studies *in vitro* and *in vivo*, and basic mechanisms elucidated, such as electroporation of cells, tumour drug

entrapment (vascular lock), vascular-disrupting effect and involvement of the immune response. Based on all these data, electrochemotherapy with bleomycin and cisplatin was promptly evaluated in clinical trials. Up today, electrochemotherapy has spread in Europe into 160 cancer centers. The timeline of electrochemotherapy development presents the milestones of its development, with the first multicentric study – ESOPE, and the first SOP, to development of new electrodes and inclusion in recommendations for treatment of tumors in different countries in Europe (Fig. 1). Several reviews electroporation technology and its applications in biomedicine and clinical practice [1–4].

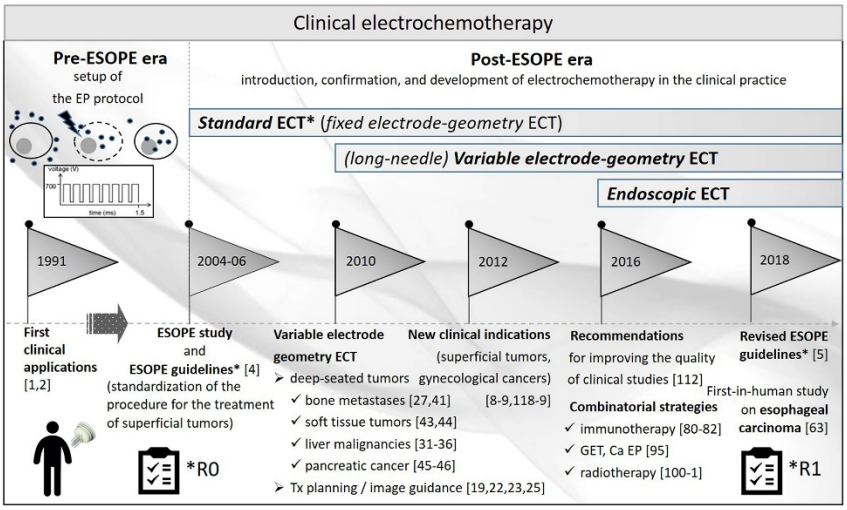

Figure 1: Development and timeline of clinical electrochemotherapy. Legend: EP = electroporation; ESOPE = European Standard Operating Procedures of ECT; GET = gene electrotransfer; Tx = treatment. *With permission from* (4).

PRECLINICAL STUDIES

In vitro studies

Electroporation proved to be effective in facilitating transport of different molecules across the plasma membrane for different biochemical and pharmacological studies. However, when using chemotherapeutic drugs, this facilitated transport increases intracellular drug accumulation with the aim to increase their cytotoxicity. Since electroporation can facilitate drug

transport through the cell membrane only for molecules which are poorly permeant or non-permeant, suitable candidates for electrochemotherapy are limited to those drugs that are hydrophilic and/or lack a transport system in the membrane. Several chemotherapeutic drugs were tested *in vitro* for potential application in combination with electroporation of cells. Among the tested drugs, only two were identified as potential candidates for electrochemotherapy of cancer patients. The first is bleomycin, which is hydrophilic and has very restricted transport through the cell membrane, but its cytotoxicity can be potentiated up to several 1000 times by electroporation of cells. A few hundred internalized molecules of bleomycin are sufficient to kill the cell. The second is cisplatin, whose transport through the cell membrane is also hampered. Early studies suggested that cisplatin is transported through the plasma membrane mainly by passive diffusion, while recent studies have demonstrated that transporters controlling intracellular copper homeostasis are significantly involved in influx (Ctr1) and efflux (ATP7A and ATP7B) of cisplatin [5]. Electroporation of the plasma membrane enables greater flux and accumulation of the drug in the cells, which results in an increase of cisplatin cytotoxicity by up to 80-fold [6–8]. This promising preclinical data obtained *in vitro* on a number of different cell lines has paved the way for testing these two drugs in electrochemotherapy *in vivo* on different tumor models. Recently calcium has been demonstrated to be suitable drug for electrochemotherapy. Its cytotoxicity is enhanced and the method is called calcium electroporation [9].

In vivo studies

Bleomycin and cisplatin were tested in an electrochemotherapy protocol in animal models *in vivo* (Fig. 2). Extensive studies in different animal models with different types of tumors, either transplantable or spontaneous, were performed [6–8,10].

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

- The drugs can be given by different *routes of administration*, they can be injected either intravenously or intratumourally. The prerequisite is that, at the time of application of electric pulses to the tumour, a sufficient amount of drug is present in the tumour. Therefore, after intravenous drug administration into small laboratory animals (for example 4 mg/kg of cisplatin or 0.5 mg/kg bleomycin), only a few minutes interval is needed to reach the maximal drug concentration in

the tumours. After intratumoural administration, this interval is even shorter and the application of electric pulses has to follow the administration of the drug as soon as possible (within a minute) [6–8].

- ❖ Good antitumor effectiveness may be achieved by good tissue electroporation. Electroporation of the plasma membrane is obtained if the cell is exposed to a sufficiently high electric field. This depends on the *electric field distribution in the tissue* which is controlled by the electrode geometry and tissue composition. The electric field distribution in the tissue and cell electroporation can be improved by rotating the electric field. Surface tumours can be effectively treated by plate electrodes, whereas appropriate electric field distribution in the deeper parts of the tumour is assured by using needle electrodes [11–13].
- ❖ The antitumor effectiveness depends on the *amplitude, number, frequency, and duration of the electric pulses applied*. Several studies in which parallel plate electrodes were used for surface tumours showed that amplitude over distance ratio above 1000 V/cm is needed for tumour electroporation and that above 1500 V/cm, irreversible changes in the normal tissues adjacent to the tumour occurred. For other types of electrodes, the electric field distribution and thus, also the necessary amplitude of electric pulses, need to be determined by numerical calculations. *Repetition frequencies of the pulses* for electrochemotherapy are either 1 Hz or 5 kHz with equal effect if the concentration of drug present in the tumour is high enough. The minimal number of pulses used is 4; most studies use 8 electric pulses of 100 μs [12,14–16].

All the experiments conducted *in vivo* in animals provided sufficient data to demonstrate that electrochemotherapy with either bleomycin or cisplatin is effective in the treatment of solid tumours, using drug concentrations which have no or minimal antitumor effect without application of electric pulses. A single treatment by electrochemotherapy already induces partial or complete regression of tumours, whereas treatment with bleomycin or cisplatin alone or application of electric pulses alone has no or minimal antitumour effect.

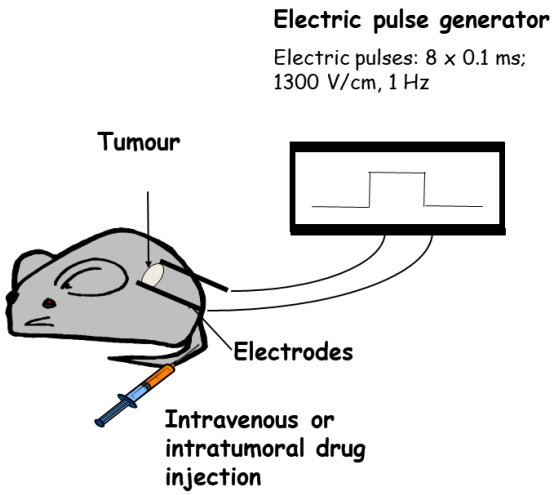

Figure 2: Protocol of electrochemotherapy of experimental tumours presented schematically. The drug is injected either intravenously or intratumourally at doses which do not usually exert an antitumour effect. After an interval which allows sufficient drug accumulation in the tumours, electric pulses are applied to the tumour either by plate or needle electrodes. The electrodes are placed in such a way that the whole tumour is encompassed between the electrodes, providing good electric field distribution in the tumours for optimal electroporation of cells in the tumours.

Mechanisms of action

The principal mechanism of electrochemotherapy is *electroporation* of cells in the tumours, which increases the drug effectiveness by enabling the drug to reach the intracellular target. This was demonstrated in studies which measured the intratumoural drug accumulation and the amount of drug bound to DNA. Basically, the amounts of bleomycin and cisplatin in the electroporated tumours were up to 2-4 fold higher than in those without application of electric pulses [6–8] Fig. 3. Besides membrane electroporation, which facilitates drug transport and its accumulation in the cell, other mechanisms that are involved in the antitumor effectiveness of electrochemotherapy were described. The application of electric pulses to tissues induces a transient, but reversible *reduction of blood flow* [17,18]. Restoration of the blood flow in normal tissue is much faster than that in tumours [19,20]. The vascular lock in the tumour induces *drug entrapment* in the tissue, providing more time for the drug to act.

The cytotoxic effect of electrochemotherapy is not limited only to tumour cells in the tumours. Electrochemotherapy also acts on stromal cells, including endothelial cells in the lining of tumour blood vessels, which undergo cell death [20]. Consequently, by vascular-disrupting action of electrochemotherapy, a cascade of tumour cell death occurs due to long-lasting hypoxia in the affected vessels. This represents yet another mechanism involved in the antitumour effectiveness of electrochemotherapy, i.e. a *vascular-disrupting effect* [21–23]. This vascular-disrupting action of electrochemotherapy is important in clinical situations where haemorrhagic tumour nodules need to be treated [24].

A difference in the antitumor effectiveness of electrochemotherapy was observed between immunocompetent and immunodeficient experimental animals, indicating on involvement of the *immune response* in antitumour effectiveness [25]. Due to massive tumour antigen shedding in organisms after electrochemotherapy, systemic immunity can be induced and also up-regulated by additional treatment with biological response modifiers like IL-2, IL-12, GM-CSF and TNF-α [25–30]. Recent study by has shown that the response of expereimental tumors in mice to electrochemotherapy is dependent on the immunogenicity of the tumors; i.e. more immunogenic tumors respond better. However, the adjuvant effect of IL-12 stimulation to electrochemotherapy is better in less immunogenic tumors [31].

To sum up, the electrochemotherapy protocol was optimized in preclinical studies *in vitro* and *in vivo*, and basic mechanisms were elucidated. In addition to the electroporation of cells, vascular lock leading to drug entrapment in tumours, a vascular-disrupting effect and involvement of the immune response were also demonstrated. Based on all this data, electrochemotherapy with bleomycin and cisplatin was promptly evaluated in clinical trials and is now in routine use in human and veterinary oncology.

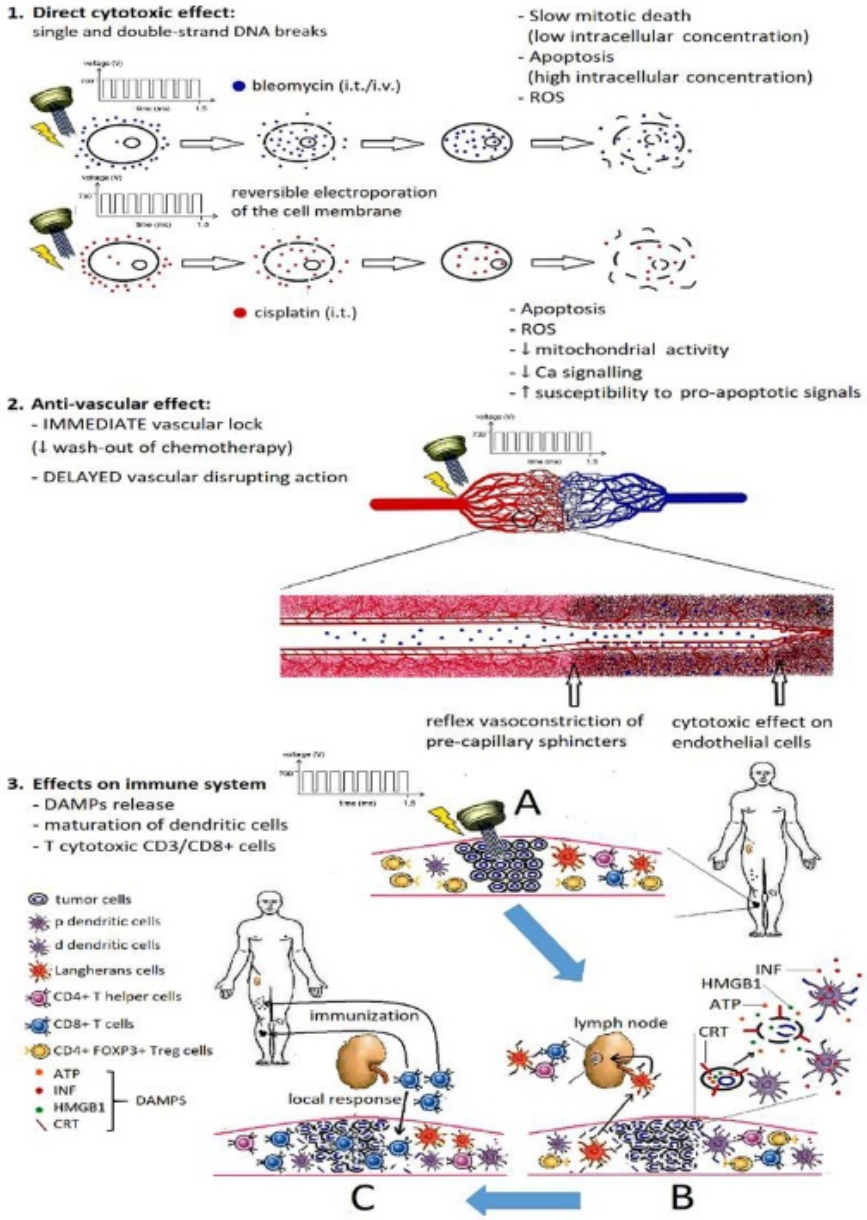

Figure 3: Basic mechanisms of electrochemotherapy; direct cytotoxic effect and indirect ones, vascular disrupting and immunomodulating. With permission from [3].

Clinical studies

The first clinical study was published in 1991 on head and neck tumour nodules [31], which was thereafter followed by several others [2]. These clinical studies demonstrated the antitumor effectiveness of electrochemotherapy using either bleomycin or cisplatin, given intravenously or intratumourally. In addition to single or multiple cutaneous or subcutaneous melanoma nodules, a response was demonstrated in breast and head and neck cancer nodules, as well as Kaposi's sarcoma, hypernephroma, chondrosarcoma and basal cell carcinoma. However, these clinical studies were performed with slightly variable treatment protocols, different electrodes and different electric pulse generators. Thus, there was a need for a prospective multi-institutional study, which was conducted by a consortium of four cancer centers gathered in the ESOPE project funded under the European Commission's 5th Framework Programme. In this study, the treatment response after electrochemotherapy according to tumour type, drug used, route of administration and type of electrodes, was tested [32]. The results of this study can be summarized as follows:

- An objective response rate of 85% (73.7% complete response rate) was achieved for electrochemotherapy-treated tumour nodules, regardless of tumour histology and drug or route of administration used.
- At 150 days after treatment, the local tumour control rate for electrochemotherapy was 88% with bleomycin given intravenously, 73% with bleomycin given intratumourally and 75% with cisplatin given intratumourally, demonstrating that all three approaches were equally effective in local tumour control.
- Side effects of electrochemotherapy were minor and tolerable (muscle contractions and pain sensation).

The results of the ESOPE study confirmed previously reported results on the effectiveness of electrochemotherapy and Standard Operating Procedures (SOP) for electrochemotherapy were prepared [33].

The ESOPE study set the stage for the introduction of electrochemotherapy in Europe. After the encouraging results of the ESOPE study, several cancer centers have started to use electrochemotherapy and reported the results of their studies. Collectively, the results were again similar as reported in the ESOPE study. However, some advances in the treatment were reported. Predominantly it was reported that tumours bigger than 3 cm in diameter can be successfully treated by electrochemotherapy in successive electrochemotherapy sessions

[34,35]. In general, electrochemotherapy provides a benefit to patients especially in quality of life [35].

Clinical use and treatment procedures for electrochemotherapy

Based on all these reports, electrochemotherapy has been recognized as a treatment option for disseminated cutaneous disease in melanoma, and accepted in many national and also international guidelines for treatment of melanoma [36].

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

- Effective in treatment of tumours of different histology in the cutaneous or subcutaneous tissue.
- Palliative treatment with improvement of patient's quality of life.
- Treatment of choice for tumours refractory to conventional treatments.
- Cytoreductive treatment before surgical resection in an organ sparing effect.
- Treatment of bleeding metastases.

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

In summary, electrochemotherapy has been recognized as a valid treatment approach; over 160 cancer centers have started to use it and have reported positive results. So far the effectiveness of the therapy is on case based evidence and further controlled and randomized studies are needed for the translation of this technology into broader and standard clinical practice. For further acceptance of electrochemotherapy in medical community, the first important step has been made, since electrochemotherapy for treatment of melanoma skin metastases and for treatment of primary basal cell and primary squamous cell carcinoma was recently listed in NICE guidelines.

Recently all published studies up to 2012 on electrochemotherapy in treatment of superficial nodules were reviewed in systematic review and meta-analysis [37]. Data analysis confirmed that electrochemotherapy had a significantly ($p<0.001$) higher effectiveness (by more than 50%) than

bleomycin or cisplatin alone, where only 8% of the tumours were in CR. After a single electrochemotherapy, the treatment can be repeated with similar effectiveness. The overall effectiveness of electrochemotherapy was 84.1% objective responses (OR), from these 59.4% complete responses (CR).

A multicentric study on patients in InspECT registry has evaluatedthe response rate of the electrochemotherapy treated patients on 2482 tumor nodules of different histologies. The study has shown good response rate of all the treated tumors, on average 86% objective response rate and 71% complete response rate. The best responses have been on Caposi sarcoma and the worst on melanoma [39].

Based on the clinical prognostic factors there are three: tumor histotypes, tumor size and previous treatment. However, we need to explore more in-depth biological factors responsible for electrochemotheray, in order to be able to do selection of patients for electrochemotherapy or combined treatment with other modalities and improve the response rate (Fig. 4) [40].

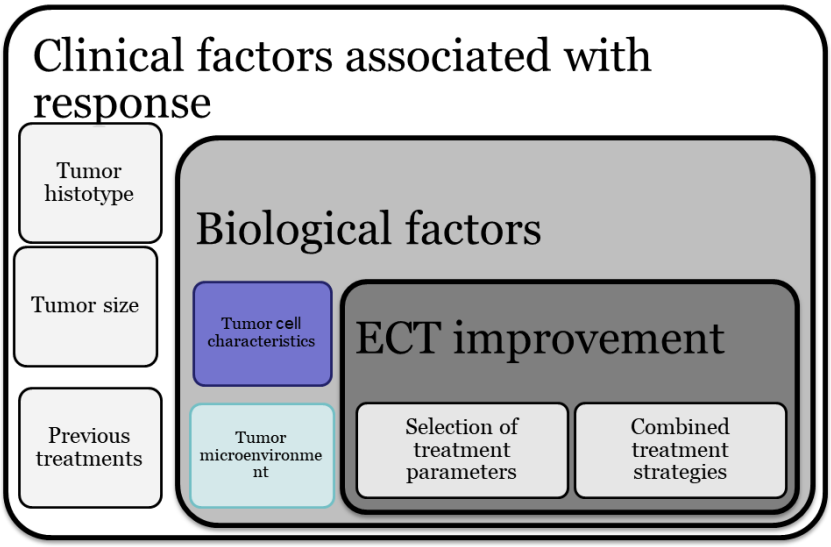

Figure 4. Clinical and biological factors affecting the response rate of tumors to electrochemotherapy (With permission from [40].

Based on broad acceptance of electrochemotherapy as effective local ablative technique, the group of experts that has been involved in preparation of the first SOP has prepared an updated version of SOP [38].

Described are indications, potential side effects and procedures for safe and effective execution of electrochemotherapy. The choice of the drug administration and anesthesia depends on the size and the number of tumour nodules to be treated (Fig. 5).

	Consider local anesthesia / local drug injection	Consider general anesthesia / intravenous drug injection
Tumour size	≤ 3 cm	> 3 cm
Tumour count	≤ 7	> 7
Region suitable for local anesthesia	yes	no

Figure 5: Decision on treatment strategy based on number and size of the tumors to be treated. With permission from [38].

New clinical applications of electrochemotherapy

Based on clinical experience that electrochemotherapy can be effectively used in treatment of cancer with different histology, when appropriately executed, the treatment could be used also for treatment of deep seated tumours. Prerequisite for that is further development of the technology in order to reach and effectively treat the tumours located either in the muscle, liver, bone, oesophagus, rectum, brain or other internal organs.

The first reports have already been published in treatment of colorectal liver metastases (Fig. 6) [39], hepatocellular carcinoma [40], pancreatic tumours, bone metastases, colorectal tumours [4]. These approaches have been undertaken during open surgery, however the future directions are in percutaneous treatment. Some attempts have been already published as case reports [41–43].

New major clinical indication in treatment is head and neck tumours. In this indication is the highest number of recently treated tumours [44,45].

Electrochemotherapy is also gaining importance in treatment of basal cell carcinoma, where the highest (>90%) complete responses are obtained [44].

The future of electrochemotherapy is also in the combined treatment with immunotherapy. Electrochemotherapy induces immunological cell death, that can serve as *in situ* immunization for the combination with immune checkpoint inhibitors. This concept is already being verified in the clinics [26,46]. The study combining electrochemothetapy with pembrolizumab, a immune checkpoint inhibitor, has shown tha electrochemotherapy is safe when combined with pembrolizumab, and has *in situ* vaccination effect, since it reduces progression of the disease (melanoma metastases) and increases overall survival of the patients [53]. Another approach is also to combine it with gene therapy, for instance with gene electrotransfer of plasmid coding for IL12 [47]. This concept has already been tested pre-clinical, but awaits verification in human oncology as well.

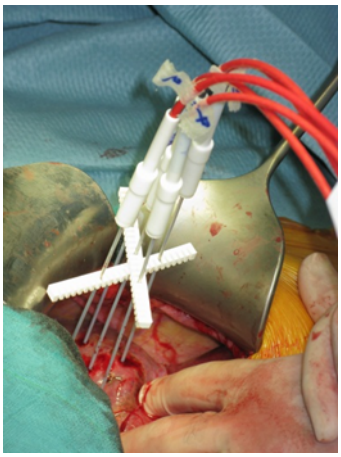

Figure 6: Electrochemotherapy of liver metastasis. Electrodes were inserted into the tumour and around the tumour in healthy liver tissue and connected to the electric pulse generator. Electric pulses were delivered between the pairs of electrodes according to the treatment plan.

Conclusion

Electrochemotherapy is one of the biomedical applications of electroporation. Its development has reached clinical application and is an example of successful translational medicine. However, its development is not finished yet; new technical developments will certainly enable further clinical uses and eventually clinical benefit for the patients. Another

application of electroporation is still awaiting such translation, gene therapy based on gene electrotransfer.

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Chapter 11

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

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

Development of electrochemotherapy

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

A very active field in cancer therapy in the 1970s and 80s was resistance to drug therapy, and there was great optimism that understanding resistance

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

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

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

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

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

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

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

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

In other words, there was agreement about the overall purpose, but three different approaches. The ESOPE study [13] brought these three

approaches together, and on the technical side, the three different electrodes were manufactured, and the final conclusion of the different methods and electrodes were defined in collaboration.

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

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

Implementation

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

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

as the integration of electrochemotherapy into specific guidelines (see e.g. [17]) are very important for the improving accessibility to treatment.

Research

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

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

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Chapter 12

Development of devices and electrodes

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

Introduction

Since first reports on electroporation (both irreversible and reversible), a number of applications has been developed and list of applications which are based on electroporation is constantly increasing. First pulse generators have been simple in construction and have provided an exponentially decaying pulse of up to several thousands of volts. Also the electrodes were very simple in their design – usually parallel plate electrodes with couple of millimeters distance between them was used, and cells in suspension were placed in-between [1]. Later, new pulse generators were developed which were/are able to provide almost every shape of pulse, and the electrodes available for purchase are also extremely diverse [2–6]. It is important to note that most often nowadays devices that generate rectangular pulses are being used. In addition, high-frequency pulses, nanosecond pulses, and

bipolar/biphasic pulses are now used for their many advantages in improved electric field homogeneity, reduced muscle contractions, pain, and electrochemical reactions [7-10].

The amplitude of pulses and their duration depend strongly on biotechnological/biomedical application. For electrochemotherapy most often a number of 1000 V pulses of 100 μs duration are needed. For effective gene transfer longer pulses 1-50 ms pulses but of lower amplitude, or a combination of short high- and longer low-voltage pulses are used. For other applications like tissue ablation by means of irreversible electroporation, or liquid-food or water sterilization, thousands of volts pulses are needed. In addition to the pulse amplitude and duration, an important parameter to be taken into account is also the power and energy that need to be provided by the generator.

The energy to be provided is determined by the voltage, current, and pulse duration and/or number of pulses. The current at a given voltage is determined by the load, and the load is determined by the geometry of the tissue/cell sample and its electrical conductivity. The geometry of the tissue to be exposed to the electrical pulses is determined primarily by the shape of the electrodes, the distance between them, and the depth of penetration of the electrodes into the sample. The electrical conductivity of the tissue/cell suspension depends on the type of tissue or the properties of the cell sample and can be significantly increased if the tissues/cells are exposed to electrical pulses of sufficient amplitude. From another point of view, the current is determined by the applied voltage and the volume, surface area and conductivity of the exposed tissues/cells.

Based on the above considerations not a single pulse generator will fit all applications and all needs of a researcher [11]. One can either seek for a specialized pulse generator which will only provide the pulses for a specific biotechnological or biomedical application, or for a general purpose pulse generator which will allow to generate “almost” all what researcher may find interesting in his/her research. Irrespective of the pulse generator choice, it has to be linked also to the electrodes choice [12–14].

Therapeutic and technological applications of electroporation

Nowadays electroporation is widely used in various biological, medical, and biotechnological applications (Fig. 1) [15–22]. Tissue ablation by irreversible electroporation is relatively new, but its efficacy is promising, especially in the treatment of nonmalignant tissues [23], in the field of water

treatment, where the efficacy of chemical treatment is enhanced by electroporation, and in food preservation, where electroporation has been shown to be as effective as pasteurization in some cases [24]. In contrast, applications based on reversible electroporation are currently more widespread and established in different experimental and/or practical protocols. Probably the most important of them is the introduction of definite amount of small or large molecules to cytoplasm through the plasma membrane. Furthermore, slight variation of electric field parameters results in an application where molecules can be directly inserted into the plasma membrane or cells can be effectively fused.

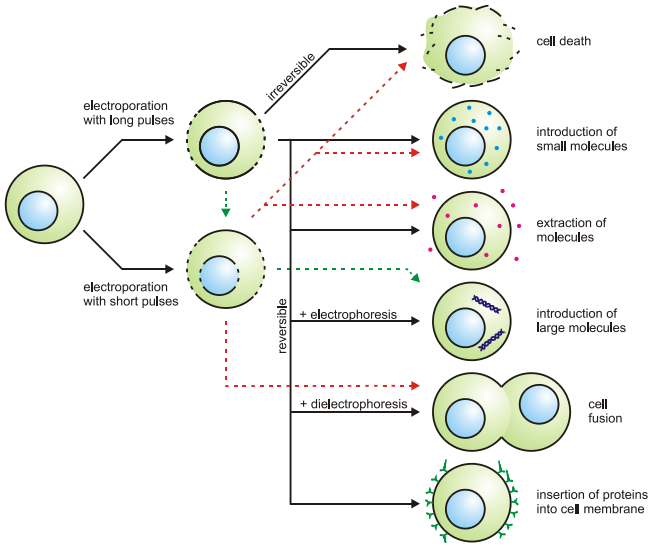

Figure 1: Exposure of a cell to an electric field may result either in permeabilization of cell membrane or its destruction. In this process the electric field parameters play a major role. If these parameters are within certain range, the permeabilization is reversible; therefore it can be used in applications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

Electrochemotherapy

The most representative application of delivery of small molecules through electroporated membrane is electrochemotherapy. It was demonstrated in several preclinical and clinical studies, both on humans and animals, that electrochemotherapy can be used as treatment of choice in local cancer treatment [25]. Most often a number of short rectangular 100 μs long pulses

with amplitudes up to 1000 V, are applied. Number of pulses that are usualy delivered is 8. These can be delivered at pulse repetition rate of 1 Hz or 5 kHz [26]. New technological developments were made ECT available for treating deep seated tumours, where 3000 V, 50 A and 100 μs pulses are being delivered [27]. Recent advances in treating liver metastasis, bone metastasis and soft tissue sarcoma have been reported [27–30]. Recently, it has been shown that *in vitro* electrochemotherapy is possible with high-frequency bipolar electroporation pulses that are 1-1-1-1 μs long (positive pulse – pause - negative pulse - pause) and have an amplitude 2.5 times higher than 100 μs pulses [31]. In addition, nanosecond electric pulses have been shown to be as effective as microsecond pulses in electrochemotherapy with cisplatin [32].

Tissue Ablation by Non-thermal Ireversible electroporation (IRE)

The ablation of undesirable tissue through the use of irreversible electroporation has recently been suggested as a minimally invasive method for tumor removal but could also be used in cardiac tissue ablation instead of RF heating tissue ablation or other tissue ablation techniques [16,23,33,34]. Similarly as in electrochemotherapy pulses of 50 or 100 μs with amplitudes up to 3000 V are used [35]. The number of pulses delivered to the target tissue is however considerably higher. If in electrochemotherapy 8 pulses are delivered, here 90 or more pulses are used. Pulse repetition rate needs to be low 1 or 4 Hz in order to avoid excessive heating [36]. To avoid muscle contraction and pain sensation during IRE, it has been suggested to use high-frequency electroporation pulses (H-FIRE) [8,9]. High-frequency pulses are of 0.2-10 μs duration with a pause between pulses in a similar range. The duration of H-FIRE pulses is tipically around 1 μs, and around 2 times higher amplitudes or treatment duration are used than for 50 or 100 μs pulses. H-FIRE shows high effectiveness for *in vivo* tumor ablation [37].

Gene ElectroTransfer

Exogenous genetic material can be delivered to cells by using non-viral methods such as electroporation [38]. Electrotransfection can be achieved using: exponentially decaying pulses; square wave pulses with superimposed RF signals; or simply using long square wave pulses up 50

ms and with amplitudes ranging from 200 to 400 V [39]. Although no consensus is reached yet, it can however be stated that longer pulses are generally used in gene transfection than in electrochemotherapy with few exceptions [40]. Furthermore, two distinct roles of electric pulses were described. In experiments where several short high voltage pulses (e.g. 8 × 100 μs of 1000 V) were followed by long low voltage pulses (e.g. 1 × 100 ms of 80 V) [41]. It was demonstrated that short high voltage pulses are permeabilizing the membrane while the longer lower voltage pulses have an electrophoretic effect on DNA itself facilitating interaction of plasmid with the membrane [42]. Recently, it has been shown that nanosecond or high-frequency bipolar pulses can also be used for gene electrotransfection [43].

Electrofusion

So far we have presented applications of electroporation that are used to introduce different molecules either to the cytosol or to the cell plasma membrane. But electroporation of cell plasma membrane can also result in fusion of cells. This process has been termed electrofusion. First reports of *in vitro* electrofusion of cells date back into 1980s. In these reports it has been shown that fusion between two cells can proceed only if the cells are in contact prior or immediately after electroporation. The contact between the cells can be achieved either by dielectrophoretically connecting neighboring cells, which is followed by electroporation or by centrifugation of cell suspension after exposure to electric field. In both cases cells must be reversibly permeabilized, otherwise they lose viability and there is no electrofusion [44]. Electrofusion in *in vitro* environment is possible due to high possibility of cell movement while cells in tissues are more or less fixed, nevertheless *in vivo* electrofusion has been observed in B16 melanoma tumors as well as cells to tissue fusion [45,46]. Electrofusion of cells of different sizes can be achieved by nanosecond pulsed electric fields [47].

Electroextraction

Electroporation can be used to extract substances (e.g. juice, sugar, pigments, lipid and proteins) from biological tissue or cells (e.g. fruits, sugar beets, microalgae, wine and yeast). Electroextraction can be more energy and extraction efficient, and faster than classical extraction methods

(pressure, thermal denaturation and fermentation) [48–52]. An economic assessment of microalgae-based bioenergy production was recently made [53] as well as protein extraction from E. coli [54]. Due to the enormous differences in substrates and electroporation parameters, as well as the variability of results obtained in different laboratories, there are recommendations guidelines on the most important information to be reported in biotechnological studies [55].

Electro-pasteurization and sterilization

Irreversible electroporation can be used in applications where destruction of microorganisms is required, i.e. food processing and water treatment [56]. However, the use of irreversible electroporation in these applications means that the material under treatment is exposed to a limited electric field, since it is desirable that no changes occur in the treated material (e.g., change in food flavor) and that no byproducts result from exposure to the electric field (e.g., byproducts resulting from electrochemical reactions at the electrode-electrolyte interface). This is one of the reasons why short pulses (in comparison to medical applications) in the range of 1-3 μs are used. Especially industrial scale batch or flowthrough exposure systems may require huge power generators with amplitudes up to 40 kV and peak currents up to 500 A. Although batch and flow-through processes are both found on industrial scale, flow-through is considered to be superior as it allows treatment of large volumes. However, this mode requires continuous operation, which requires higher output power from the pulse generators [17,57].

Electric field distribution *in vivo*

In most applications of tissue permeabilization it is required to expose the volume of tissue to electric field intensities between the two "thresholds" i.e. to choose in advance a suitable electrode configuration and pulse parameters for the effective tissue electroporation [58]. Therefore electric field distribution in tissue has to be estimated before the treatment, which can be achieved by combining results of rapid tests or *in situ* monitoring [59] with models of electric field distribution [60–64]. However, modeling of electric field distribution in tissue is demanding due to heterogeneous tissue properties and usually complex geometry. Analytical models can be employed only for simple geometries. Usually they are developed for 2D

geometries and tissue with homogenous electrical properties. Therefore in most cases numerical modeling techniques are still more acceptable as they can be used for modeling 3D geometries and complex tissue properties. For that purpose mostly finite element method and finite difference method are applied. Both numerical methods have been successfully applied and validated by comparison of computed and measured electric field distribution. Furthermore, advanced numerical models were developed, which take into consideration also tissue conductivity increase due to tissue or cell electroporation. These advanced models describe E distribution as a function of conductivity σ(E). In this way models represent electroporation tissue conductivity changes according to distribution of electric field intensities [65,66]. In addition, complex multi-tissue and multi-scale models have been developed to simulate electroporation in complex organs such as skin and anisotropic muscle [67,68].

Electrodes for *in vitro* and *in vivo* applications

Effectiveness of electroporation in *in vitro*, *in vivo* or clinical environment depends on the distribution of electric field inside the treated sample. Namely, the most important parameter governing cell membrane permeabilization is the local electric field to which the cell is exposed [58,69]. To achieve this we have to use an appropriate set of electrodes and an electroporation device – electroporator that generates required voltage or current signals. Although both parts of the mentioned equipment are important and necessary for effective electroporation, electroporator has a substantially more important role since it has to be able to deliver the required signal to its output loaded by impedance of the sample between electrodes.

Nowadays there are numerous types of electrodes that can be used for electroporation in any of the existing applications [70–75]. According to the geometry, electrodes can be classified into several groups, i.e. parallel plate electrodes, needle arrays, wire electrodes, tweezers electrodes, coaxial electrodes, etc (Fig. 2). Each group comprises several types of electrodes that can be further divided according to the applications, dimensions, electrode material etc. In any case selection of electrode type plays an important role in characterization of the load that is connected to the output of the electroporator. During the design of the electroporator load characterization represents the starting point and represents a considerable engineering problem, because electrical characteristics of material between electrodes (e.g. cell suspension, tissue, etc.) vary from experiment to

experiment and even during the course of experiment. In general the load between electrodes has both a resistive and a capacitive component. The value of each component is defined by geometry and material of electrodes and by electrical and chemical properties of the treated sample. Under *in vitro* conditions, these parameters affecting the impedance of the load can be well controlled since the size and geometry of the sample are known, especially if cuvettes are used. In addition, by using specially prepared cell media, the electrical and chemical properties can be defined or measured. However, it must be mentioned that the cell/electroporation medium can influence the response of the cells and the results of the experiments [76,77]. On the other hand, in *in vivo* conditions, size and geometry can still be controlled to a certain extent but electrical and chemical properties can only be estimated, especially if needle electrodes are used that penetrate through different tissues. However, even if we manage to reliably define these properties during the development of the device, it is practically impossible to predict changes in the electrical and chemical properties of the sample due to exposure to high-voltage electric pulses [78–80]. Besides electropermeabilization of cell membranes which increases electrical conductivity of the sample, electric pulses also cause side effects like Joule heating and electrolytic contamination of the sample [81], which further leads to increased sample conductivity [82,83].

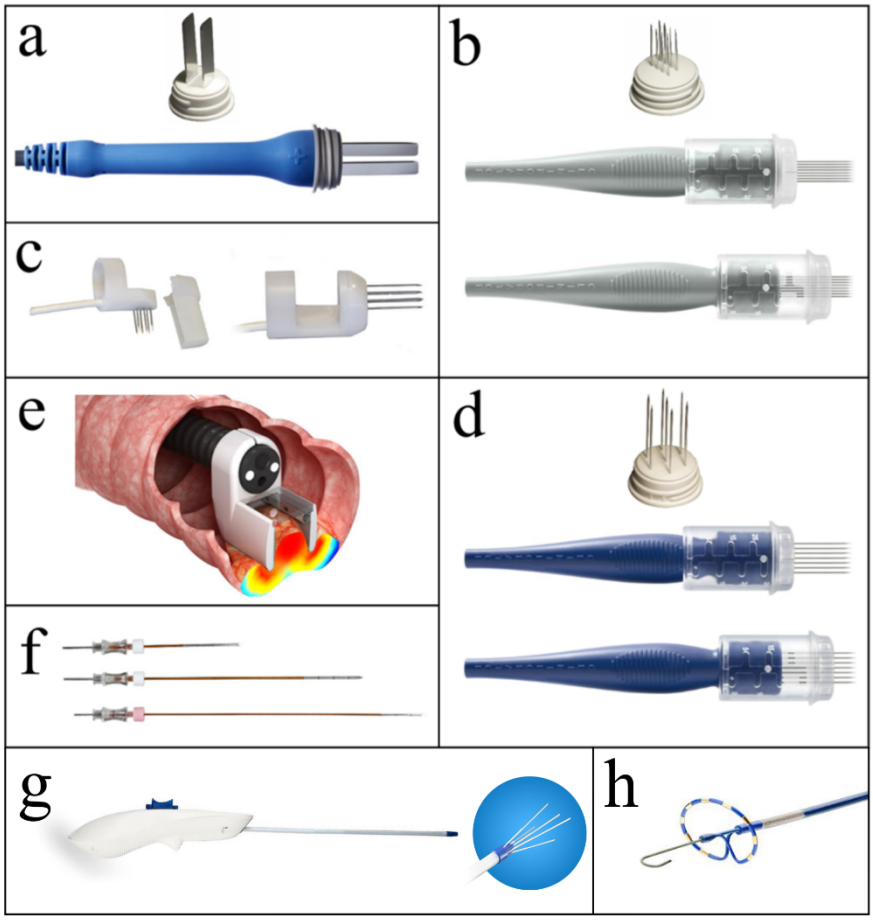

Figure 2: Examples of commercially available electrodes for electropermeabilization. a) Plate electrodes (by IGEA S.p.A.). b) Linear (parallel needle) array electrodes (first top image), adjustable linear needle electrodes with needle-length adjustment with 5 mm increment (bottom two images). c) Finger electrodes with orthogonal linear needles (left) and longitudinal linear needles (right). d) Hexagonal needle electrodes (first top image), adjustable hexagonal configuration needle electrodes with needle-length adjustment with 5 mm increment (bottom two images) [40,72]. e) Endoscopic electrode EndoVE (Endoscopic Vacuum Electrode) which is mounted at the head of an endoscope and utilizes a vacuum source to suck the tissue between the electrode. f) Individual (long) needle electrodes for variable electrode-geometry (from 2 to 6 electrodes with 16- to 30 cm long needle and active tip of 3 or 4 cm). g) Endoscopic and laparoscopic minimally invasive electroporation electrodes with 15 or 20 mm length of the active part. h) Catheter electrodes for the treatment of atrial fibrillation with pulsed field ablation.

Electric pulses

For better understanding and critical reading of various reports on electroporation phenomenon and electroporation based applications, complete disclosure of pulse parameters needs to be given. Electric pulses are never "square" or "rectangular", but they are characterized by their rise time, duration/width, fall time, pulse repetition rate, etc (Fig. 3). Rise time and fall time are determined as time needed to rise from 10% to 90% of the amplitude, drop from 90% to 10% of amplitude, respectively. Pulse width is most often defined as time between 50% amplitude on the rise and 50% amplitude on the fall. Pulse repetition rate is the inverse of the sum of pulse width and pause between two consecutive pulses. These may seem trivial when discussing pulses of 1 ms, but becomes an issue when discussing ns or even ps pulses [84]. The cell membrane damage and uptake of ions can be significantly reduced when using bipolar ns pulses instead of monopolar [85]. Shapes other than "rectangular" have been investigated with respect to electroporation efficiency [86], as well as sinusoidal signal [87]. It was suggested exposure of cells to pulse amplitudes above given critical amplitude and duration of exposure to this above critical value seem to be determining level of membrane electroporation irrespective of pulse shape. Exponentially decaying pulses are difficult to be considered as such but were predominantly used in 80s for gene electrotransfer. Their shape was convenient as the first part of the pulse i.e. the peak acts as the permeabilizing part, and the tail of the pulse acts as electrophoretic part pushing DNA as towards and potentially through the cell membrane [41]. Exponentially decaying pulses or, as they have recently been called, E2 pulses (electroporation and electrolysis) are being explored for tissue ablation [88].

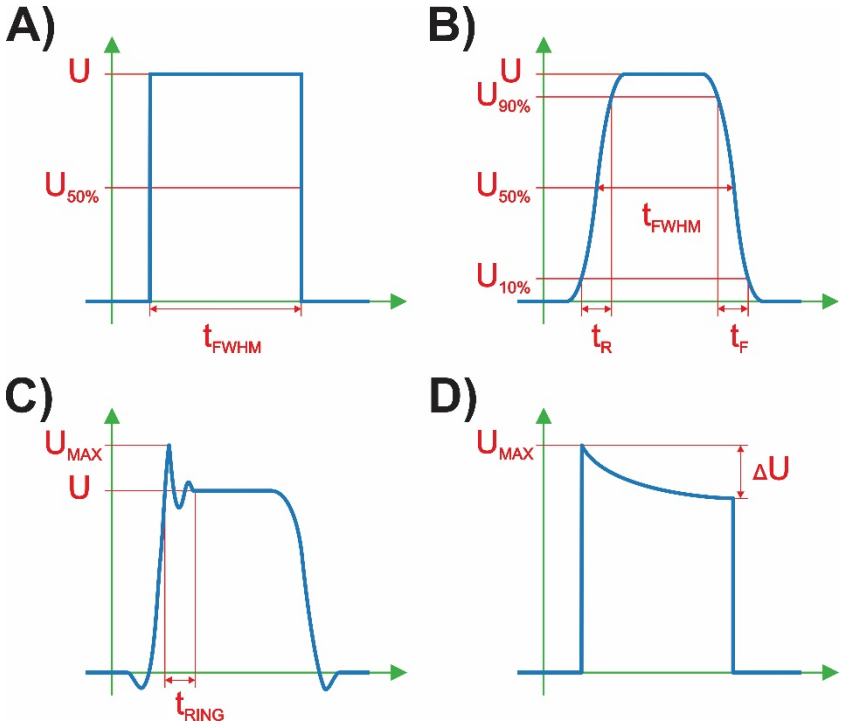

Figure 3: Time course and electrical parameters of square wave electroporation pulse. A) ideal square wave pulse, B) square wave pulse with slower rise and fall times, C) overshooting and ringing during voltage switching, and D) voltage drop during the pulse delivery. Square wave electroporation pulse can be characterized by: amplitude (U) which is the middle value of the pulse plateau; pulse duration (t_{FWHM}) which is the width of the pulse at 50% of the amplitude (U); rise time (t_R) in which the amplitude rises from 10% to 90% of its amplitude (U) and vice versa for the fall time (t_F); maximal amplitude (U_{MAX}); ringing time (t_{RING}) which is the time of amplitude oscillation; and voltage drop (ΔU) which is the drop of the pulse amplitude during the pulse delivery.

Electroporators – the necessary pulse generators

Electroporator is an electronic device that generates signals, usually square wave or exponentially decaying pulses, required for electroporation [1]. Parameters of the signal delivered to electrodes with the treated sample vary from application to application. Therefore, in investigating of electroporation phenomenon and development of electroporation based technologies and tretaments it is important that electroporator is able to deliver signals with the widest possible range of electrical parameters if

used in research (Fig. 4). If however used for a specific application only, e.g. clinical treatment such as electrochemotherapy, pulse generator has to provide exactly required pulse parameters in a reliable manner. Moreover, electroporator must be safe and easy to operate and should offer some possibilities of functional improvements. Clinical electroporators used in electrochemotherapy of deep-seated tumors or in non-thermal tissue ablation are also equipped with ECG synchronization algorithms which minimasizes possible influence of electric pulse delivery on heart function [89]. Clinical electroporators must comply with medical device standards and meet the requirements of local medical regulations in order to receive the CE certification mark in Europe and Food and Drug Administration (FDA) approval in the US to sell the device on the market [90].

Prices for electroporators can be considerable, but there are also some super-simple/cheap solutions, e.g., the "ElectroPen" [91]. There are also many "do-it-yourself" papers, but their safety and quality can be questioned and qualified engineers must also be available [11].

In principle, electroporators can be divided in several groups depending on biological applications, but from the electrical point of view only two types of electroporators exist: devices with voltage output (output is voltage signal U(t)) and devices with current output (output is current signal I(t)). Both types of devices have their advantages and disadvantages, but one point definitely speaks in favor of devices with voltage output. For example, if we perform *in vitro* experiments with parallel plate electrodes with plate sides substantially larger than the distance between them, the electric field strength E that is applied to the sample can be approximated by the voltage-to-distance ratio U/d, where d is the electrode distance and U the amplitude of applied signal obtained from an electroporator with voltage output. On the other hand, if an electroporator with current output is used, the same approximation could be used only if additional measurement of voltage difference between electrodes is performed or if the impedance Z of the sample is known, measured or approximated and voltage difference between electrodes is estimated using Ohm's law $U = I \bullet Z$. Nevertheless, there are several commercially available electroporator that fulfill different ranges of parameters and can be used in different applications [3,92]. A list of commercially available electrodes and electroporators was presented by Puc and colleagues in 2004 [93], and updated in 2010 [3] and in 2017 [94].

To be sure the applied pulses are adequate we have to measure the applied voltage and current during the pulse delivery [95].

In nanosecond applications rise time of the pulse is sometimes shorter than the electrical length (the time in which an electrical signal travels through the line) between the source and the load. In this case, the impedance of the load and the transmission line has to match the impedance

of the generator, so that there are no strong pulse reflections and consequently pulse prolongations.

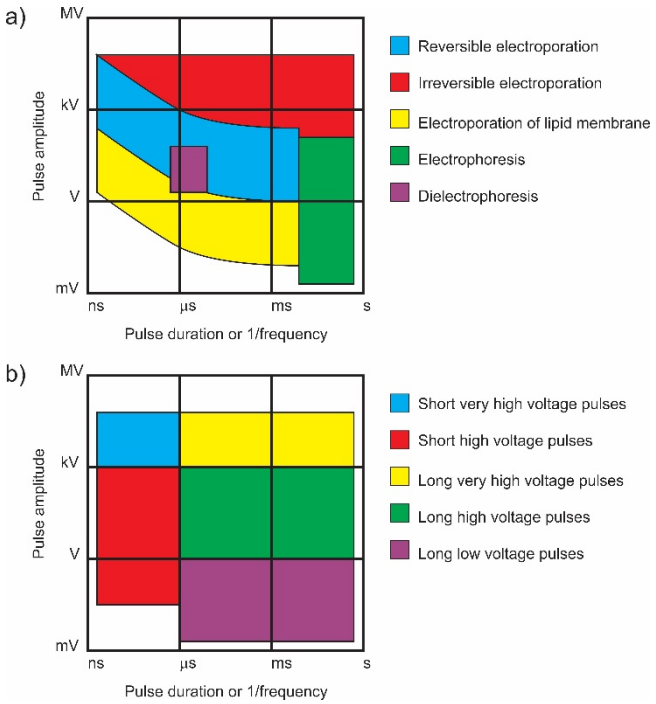

Figure 4: Areas of amplitude and duration of electrical pulses which are used in the research of electroporation and related effects (a). Five different areas of electroporation pulse generation (b). To amplify or to generate very-high-voltage electroporation pulses (over a few kV) spark gaps and similar elements are used, for high-voltage (a few V to a few kV) transistors and for low-voltage operational amplifiers are used. Nanosecond (short) pulses are generated with different techniques than pulses longer than 1 µs. Originally published in Advanced electroporation techniques in biology and medicine by Reberšek and Miklavčič 2010 [3].

Based on the studies reported in the literature it is very difficult to extract a general advice how to design experiments or treatments with electroporation. In principle we can say that pulse amplitude (voltage-to-distance ratio) should typically be in the range from 200 V/cm up to 2000 V/cm. Pulse durations should be in the range of hundreds of microseconds for smaller molecules and from several milliseconds up to several tens of milliseconds for macromolecules such as plasmid DNA (in the latter case, due to the very long pulse duration, optimal pulse amplitude can even be lower than 100 V/cm). Electroporators that generate bipolar pulses or have

the ability to change the orientation of the electric field in the sample are now readily available. These types of pulses/electroporators should be used because, compared to unipolar pulses of the same amplitude and duration, bipolar pulses result in a lower poration threshold, higher uptake, reduce electrolyte wear and electrolytic contamination of the sample. Better permeabilisation or gene transfection efficiency and survival can also be obtained by changing field orientation in the sample using special commutation circuits that commute electroporation pulses between the electrodes [70,72,94]. Short bipolar high-frequency electroporation pulses (HF-EP) were also investigated as they mitigate nerve/muscle stimulation and electric field distribution of such pulses in tissue is more homogeneous [96–98], and has less electrolytic contamination [10], but these pulses may already fall into vicinity of "cancellation effect" [98]. However, the development of high-frequency electroporators is much more challenging [99,100] and only a few commercial high-frequency electroporators are available [101], but the number of parameters that can influence the treatment outcome is (almost) unmanageable and the comparison of results from different studies almost impossible.

This general overview of electrical parameters should however only be considered as a starting point for a design of experiments or treatments. Optimal values of parameters namely also strongly depend on the cell type used, on the molecule to be introduced, and on specific experimental conditions. The pulse characteristics determined as optimal or at least efficient and the tissue/sample will than determine the architecture of the pulse generator, whether it will be a Marx generator, Blumlein, or... [11].

Conclusions

Electroporation has been studied extensively until now, and a number of applications has been developed. Electrochemotherapy has been demonstrated as an effective local treatment of solid tumors and is the most mature therapeutic application right now. Electroporation for gene transfection however has been long used in *in vitro* situation. With a hold on viral vectors electroporation represents a viable non-viral alternative also for *in vivo* gene transfection. Clinical applications and expansion of electrochemotherapy and tissue ablation have been hindered by the lack of adequate electroporators and their certification in Europe (CE Medical Device) and limited approval by FDA in USA [1]. Cliniporator (IGEA, s.r.l. Carpi, Italy) was certified in EU (CE mark) as a medical device and is offered on the market along with standard operating procedures for

electrochemotherapy of cutaneous and subcutaneous tumors. NanoKnife (AngioDynamics, Queensbury, USA) was certified in EU and approved by the FDA for surgical ablation of soft tissue. Some electroporators are now available under the license for clinical evaluation purpuses: Cellectra, Elgen, Medpulser, Cliniporator VITAE, BetaTech, DermaVax, EasyVax, Ellisphere, and TriGrid [4]. Farapulse, Affera, and Galaxy devices are also approved in the EU for cardiac ablation for the treatment of atrial fibrillation.

Development of new applications warrants further development of pulse generators and electrodes. Based on the above considerations however, a single pulse generator will not fit all applications and all needs of researchers. One can either look for a specialized pulse generator that provides only the pulses for one's specific biotechnological or biomedical application, scale up or scale down the technology, or a general purpose pulse generator that can be used to generate "almost" anything the researcher finds interesting/necessary for his/her research. Irrespective of the choice, this has to be linked also to the electrodes choice and tissue/sample conductivity.

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