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Electroporation for Electrochemotherapy and Gene Therapy

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The new avenue in treatment of tumors is discussed in this chapter. The possibilities for electroporation of tissues in order to enhance the drug and gene delivery are presented from both basic science and clinical application points of view. In a condensed way, the authors present the journey from first basic experiments with electroporation of single cells through electroporation in cell suspension and in tissue. Discussion of mechanisms of electroporation is followed by discussion of electroporation for electrochemotherapy and electrogenetransfer. A brief review of experimental conditions is linked to electrode requirements and to available commercial devices for electroporation in laboratory and clinical conditions.

I. INTRODUCTION

A. Description of the Phenomenon

When a cell is exposed to an electric field, a transmembrane voltage is induced on the membrane. If this voltage exceeds a certain value, this leads to a significant increase of the electric conductivity and the permeability of the membrane. Typically, each increases by several orders of magnitude. Formation of a state of increased permeability of the membrane caused by an exposure to the electric field is called *electroporation* (also *electropermeabilization*).

As the result of the increased permeability of the membrane, molecules that are otherwise deprived of transport mechanisms can be transported across the membrane. With appropriate duration and amplitude of the electric field, the membrane returns into its normal state after the end of the exposure to the electric field (reversible electroporation). However, if the exposure is too long or the amplitude of the electric field is too high, the membrane does not reseal after the end of the exposure, leading to cell death (irreversible electroporation).

Reversible "electrical breakdown" of the membrane has first been reported by Stämpfli in 1958, but for some time this report has been mostly unnoticed. Nearly a decade later, Sale

and Hamilton have reported on nonthermal electrical destruction of microorganisms using strong electric pulses. In 1972, Neumann and Rosenheck have shown that electric pulses induce a large increase of membrane permeability in natural vesicles. This report has motivated a series of further investigations, and from this time on, the data started to accumulate more rapidly and systematically. Most of the early work was done on isolated cells in conditions in vitro, but it is now known that many applications are also successful in situation in vivo. Using electroporation, both small and large molecules can be introduced into cells and extracted from cells, and proteins can be inserted into the membrane and cells can be fused. Due to its efficiency, electroporation has rapidly found its application in gene transfection, preparation of monoclonal antibodies, and electrochemotherapy of tumors. Nowadays, it is finding its way into many fields of biochemistry, molecular biology, and medicine and is becoming an established method used in oncology for treatment of solid tumors. It also holds great promises for gene therapy (1).

B. Schwan's Equation

Although a biological cell is not perfectly spherical, in theoretical treatments, it is usually considered as being such (Fig. 1). Also, the plasma membrane of the cell has a very low conductivity with respect to the intracellular and extracellular environment, and in an approximation it can be considered nonconductive. When a single cell is placed into a homogeneous electric field, the voltage induced on the membrane can be determined by solving Laplace's equation. For a spherical cell with a nonconductive membrane, the solution of Laplace's equation is a formula often referred to as Schwan's equation,

$$\Delta\Phi_m = \frac{3}{2} ER\cos\varphi$$

where $\Delta\Phi_m$ is the induced transmembrane voltage, E is the electric field in the region where the cell is situated, R is the cell radius, and φ is the polar angle measured from the center of the cell with respect to the direction of the field. This formula tells that the maximum voltage

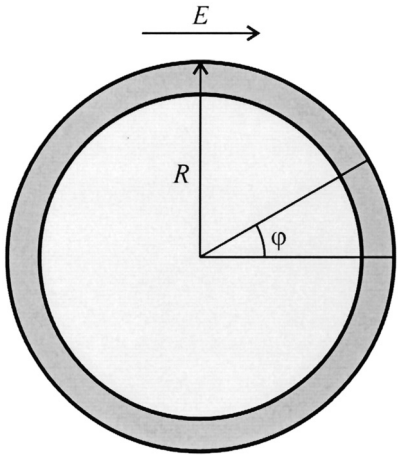

Figure 1 A spherical cell with a nonconductive membrane and the parameters of Schwan's equation: cell radius (R), electric field (E), and the angle measured with respect to the field (φ).

is induced at the points where the electric field is perpendicular to the membrane, i.e., at $\varphi = 0°$ and $\varphi = 180°$, the points we shall refer to as the "poles" of the cell, and varies proportionally to the cosine of the angle in between these poles. Also, the induced voltage is proportional to the applied electric field and to the cell radius.

Schwan's equation as given above describes only the static situation, which is typically established several microseconds after the onset of the electric field. Since durations of exposure to electric field used for electroporation are typically in the range of hundreds of microseconds up to tens of milliseconds, Schwan's equation can safely be applied in electroporation.

C. Electroporation of a Single Cell

Most experimental data suggest that electroporation is a threshold phenomenon—if the induced membrane voltage in a region of the membrane exceeds certain critical value, this leads to electroporation in this region. From Schwan's equation one can deduce that for a single spherical cell (e.g., a cell floating in a medium), electroporation occurs at the caps around the two poles (see Sec. I.A), and it is through these caps that the transport will be established. For stronger fields, the area of these caps gets larger.

A voltage in the range of tens of millivolts is always present on the cell membrane. When a cell is exposed to an electric field, this voltage (the resting transmembrane voltage) combines with the induced voltage. Since the resting voltage is the same all over the membrane, while the induced voltage varies proportionally to the cosine of the angle with respect to the direction of the field, the resultant transmembrane voltage is actually somewhat higher on one pole of the cell than on the other. Since typical transmembrane voltages leading to electroporation are in the range of hundreds of millivolts, this asymmetry is not large. Still, when using bipolar pulses, which compensate for this asymmetry, electroporation is obtained at lower pulse amplitudes than unipolar ones (see Sec. III).

Schwan's equation only describes the transmembrane voltage induced on a spherical cell with a nonconductive membrane. However, more general formulae exist that are valid for spherical cells with a membrane of non zero conductivity (2) and such that apply to spheroidal and ellipsoidal cells (3,4).

D. Electroporation in Cell Suspensions

When cells in suspension are exposed to an electric field, applying Schwan's equation to determine the induced transmembrane voltage is in general not valid. This is due to the fact that the field outside a cell is not homogeneous, as it is distorted by the presence of other cells in the suspension. For suspensions in which the cells represent less than 1% of the total suspension volume, the deviation of the actual induced transmembrane voltage from the one predicted by Schwan's equation is practically negligible. As the volume fraction occupied by the cells gets larger, the prediction obtained from Schwan's equation gets less and less realistic (Fig. 2). For volume fractions over 10% 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 (5,6).

In conclusion, the induced transmembrane voltage depends not only on the geometrical and electrical properties of the cell but also on the density of the cells in suspension.

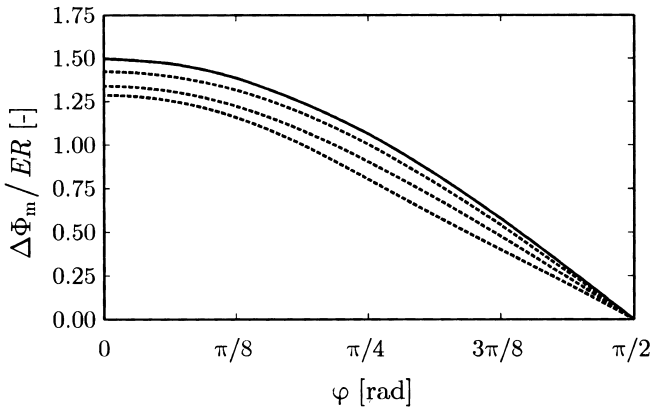

Figure 2 Induced transmembrane voltage normalized to electric field and cell radius. Solid: prediction of Schwan's equation. Dashed: numerical results for cells arranged in a face-centered cubic lattice and occupying (from top to bottom) 10%, 30%, and 50% of the total suspension volume. (Adapted from Ref. 6 with the permission of the authors.)

E. Electroporation in Tissue

In tissues, additional difficulties arise in the analysis of transmembrane voltage induced by an exposure to electric field. First, unlike suspended cells, the cells in tissue are far from spherical. Second, also on a larger scale, most tissues are not homogeneous, e.g., due to the presence of blood vessels. Third, electrical properties of different tissues can differ considerably. Due to these facts, the distribution of electric field in tissues can be quite intricate (7). In addition, after certain regions of tissue are electroporated, this causes a redistribution of electric field, which in general becomes higher around nonporated regions. As a consequence, some of these regions may get electroporated subsequently, and this again leads to a rearrangement of the electric field. Thus, electroporation in a tissue can proceed in a domino-effect manner, with poration of certain regions giving rise to poration of other regions. The actual situation is therefore a dynamical one, and to correctly describe electroporation in a tissue, one must account for ongoing rearrangements of the electric field throughout the exposure. An example is tumor treatment with pulses delivered to the surface electrodes placed on the skin, where the skin gets electroporated first, then the tissue underneath it, and so on (Fig. 3).

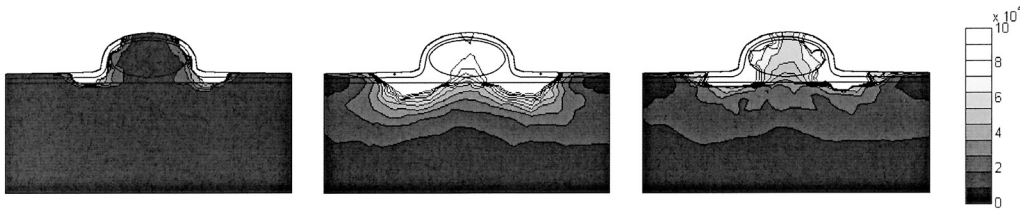

Figure 3 Numerical evaluation of the time course of electric field distribution during electroporation in tissue with a pulse of 1000 V amplitude delivered to plate electrodes placed on the skin at a distance of 8 mm. Units on the scale are in volts per meter (V/m).

F. Mass Transport

There are three general mechanisms of transmembrane transport by which the molecules can pass through an electroporated membrane:

1. Diffusion, driven by the molecular concentration difference across the membrane
2. Electrophoresis, driven by the electric potential difference across the membrane
3. Osmosis, driven by the osmotic pressure difference across the membrane

Most experimental studies imply that diffusion is the main component of transport of small molecules through an electroporated membrane (8–10). On the other hand, it is known that for macromolecules, diffusion itself is often insufficient for adequate uptake into a cell, and the presence of electrophoretic forces can improve such uptake significantly (11,12).

The dependence of the efficiency of transport mechanisms on the size of the transported molecule led to a relatively sharp distinction between the protocols of electroporation used for smaller molecules and those for macromolecules. For smaller molecules, electroporation and a concentration gradient suffice for the transport to occur, and pulses with durations of tens of microseconds up to several milliseconds successfully achieve this aim. On the other hand, electrophoretic transport of macromolecules mostly proceeds during the pulse, and to achieve sufficient uptake of molecules such as DNA, pulses of typical durations of milliseconds to tens of milliseconds are used (11,13). As an alternative, pulses similar to the ones used with smaller molecules are used to obtain electroporation, and a longer pulse with a lower amplitude is applied subsequently to sustain the electrophoretic movement (12,14).

G. Mechanisms of Electroporation

The theory of formation of aqueous pores in the membrane is widely considered as the most convincing theoretical explanation of electroporation (15). According to this theory, a sufficiently long and strong exposure to an electric field leads to a formation of hydrophilic (aqueous) pores, in which the lipids adjacent to the aqueous inside of the pore are reoriented in a manner that their hydrophilic heads are facing the pore, while their hydrophobic tails are hidden inside the membrane. The hydrophilic state of the pore can only be reached through a transition from an initial, hydrophobic state in which the lipids still have their original orientation (Fig. 4). As electric field amplitude increases, the presence of hydrophilic pores becomes energetically ever more favorable, which leads to the formation of pores with an average radius corresponding to the minimum of the free-energy curve (Fig. 5, the upper curve). Further increase of the field amplitude pushes this curve downward, and eventually the energy minimum disappears (Fig. 5, the lower curve), resulting in a complete breakdown of the membrane, which corresponds to irreversible electroporation.

Several studies have recently added to the credibility of the theory of formation of aqueous pores. The measurements of the optical properties of the membrane have shown that during electroporation, the lipid molecules are reoriented, and water penetrates into the bilayer (16). Existence of transient metastable aqueous pores has also been observed in a molecular dynamics simulation of the lipid bilayer formation (17). In a recent experiment, we have also observed that the threshold of irreversible electroporation is lowered if molecules of octaethyleneglycol-dodecylether ($C_{12}E_8$) are incorporated into the membrane (18). These molecules form conical inclusions in the membrane, which makes the reorientation of lipids that is expected to occur in hydrophilic pores more energetically favorable and the pores more stable.

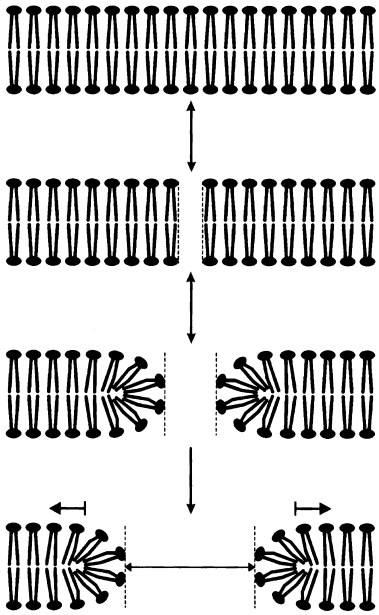

Figure 4 Formation of an aqueous pore. The situation is shown for transmembrane voltage increasing from top to bottom: a nonporated membrane, formation of a hydrophobic pore, transformation into a hydrophilic pore (reversible electroporation), and enlargement beyond the stable size (irreversible electroporation).

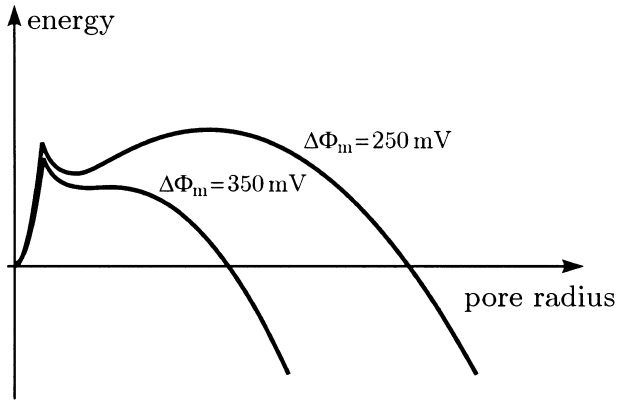

Figure 5 A schematic representation of the amount of energy required for formation of an aqueous pore of a given radius for a transmembrane voltage that yields reversible (the upper curve) and irreversible electroporation (lower curve). The sharp local maximum corresponds to the transition from hydrophobic to hydrophilic state. The local minimum, if it exists, corresponds to the radius at which the pore stabilizes.

II. APPLICATIONS

There are many prospects for application of electroporation in biochemistry, molecular biology, and above all in various fields of medicine. Sections II.A and II.B are devoted to a more detailed description of the two applications that are already finding their way into practice—electrochemotherapy (ECT) and electrogenetransfection (EGT). Here, we outline some other applications.

ELECTROSTERILIZATION. Irreversible electroporation can also be exploited with the aim of sterilization, i.e., killing of bacteria or other microorganisms (19–21). Other techniques of sterilization, such as by antibiotics, detergents, or exposure to radiation, in general lead to some kind of contamination, which is not the case with electrosterilization. However, this approach has proved rather costly with respect to other techniques, and it has not yet found a way into practical applications.

ELECTROINSERTION. Another application of electroporation is insertion of molecules into the cell membrane. As the membrane reseals, it entraps some of the transported molecules, and if these molecules are amphipathic (constituted of both polar and nonpolar regions), they can remain stably incorporated in the membrane. Electroinsertion was observed with several transmembrane proteins, such as CD4 receptors (22) and glycophorin A (23), and could prove valuable in the research of the role of various transmembrane proteins.

ELECTROFUSION. Under appropriate experimental conditions, delivery of electric pulses can lead to the merger (fusion) of membranes of adjacent cells. Electrofusion has been observed between suspended cells (24,25), between suspended cells and cells in tissue (26) and between cells in tissue (27). For successful electrofusion in suspension, the cells must previously be brought into close contact, for example, by dielectrophoresis (24). Electrofusion has proved to be a successful approach in production of vaccines (28,29) and antibodies (30).

TRANSDERMAL DRUG DELIVERY. Application of high-voltage pulses to the skin causes a large increase in ionic and molecular transport across the skin (31). This has been applied for transdermal delivery of drugs, such as metoprolol (32), and also works for larger molecules, for example, DNA oligonucleotides (33).

A. Electrochemotherapy (ECT)

In cancer treatment, some of the drugs aim at damaging DNA. Chemotherapy based on these drugs is only effective for those that readily permeate through the cell membrane and act cytotoxically when reaching their intracellular targets. Unfortunately, some of the very cytotoxic chemotherapeutic drugs permeate the plasma membrane very slowly, or practically not at all. These drugs are good candidates for electrochemotherapy (ECT), which combines chemotherapy and electroporation. The delivery of electric pulses at the time of when a chemotherapeutic drug reaches its highest extracellular concentration considerably increases the transport through the membrane towards the intracellular targets of cytotoxicity.

Two chemotherapeutic drugs, bleomycin and cisplatin, have proven to be much more effective in electrochemotherapy than alone when applied to tumor cell lines in vitro, as well as in vivo on tumors in mice (34–36). Cytotoxicity of bleomycin was shown to be increased for several 100-fold, and of cisplatin up to 70-fold when cells were electroporated. Sarcomas, carcinomas, or melanoma tumors responded with high percentage of complete responses when the drugs were injected intravenously or intratumorally. These experiments provided

sufficient data to demonstrate that ECT with either bleomycin or cisplatin is effective in treatment of solid tumors.

Based on these preclinical data, ECT with bleomycin and cisplatin entered clinical trials. Both drugs have proved their value in clinical ECT protocols with cutaneous and subcutaneous tumour nodules of various malignancies in cancer patients (Fig. 6). Most of the treated nodules responded with objective responses in 60–100% (37,38). In the protocols, both intravenous and intratumoral drug administration were used. Current knowledge about antitumor effectiveness of ECT considers this therapy as local treatment being effective on most tumor types tested so far. Since ECT can be performed using surface electrodes, it does not lead to scaring, which is unavoidable with surgical procedures. In addition, the high local concentration of the chemotherapeutic drug in the tumor allows to overcome the developing resistance to the drug (39).

Antitumor effectiveness of ECT is considered to be mainly due to the increased drug uptake into the tumor cells, caused by electroporation. However, other mechanisms, such as prolonged drug entrapment in the tumors due to decrease in tumor blood flow caused by electric pulses and vascular-targeted effects, may also contribute to the effectiveness of ECT (40–42).

The results indicate that electrochemotherapy with bleomycin is equally effective when the drug is given intravenously or intratumorally, while ECT with cisplatin is more efficient when the drug is given intratumorally than when given intravenously. The advantage of electrochemotherapy with cisplatin is that the drug itself, without application of electric pulses, may exert considerable antitumor effect.

It is difficult to foresee all the clinical applications of electrochemotherapy. In the first step, more controlled clinical trials are needed evaluating treatment response of different tumor types. So far, only percutaneously accessible tumor nodules have been treated in the clinical trials, but with development of new electrodes it will become possible to treat tumors in internal organs.

In its concept, electrochemotherapy is a local treatment, and therefore approaches must be exploited to add a systemic component, either by adjuvant immunotherapy or in combination with other systemic treatments. Some chemotherapeutic drugs, including bleomycin and cisplatin, also interact with radiation therapy (43).

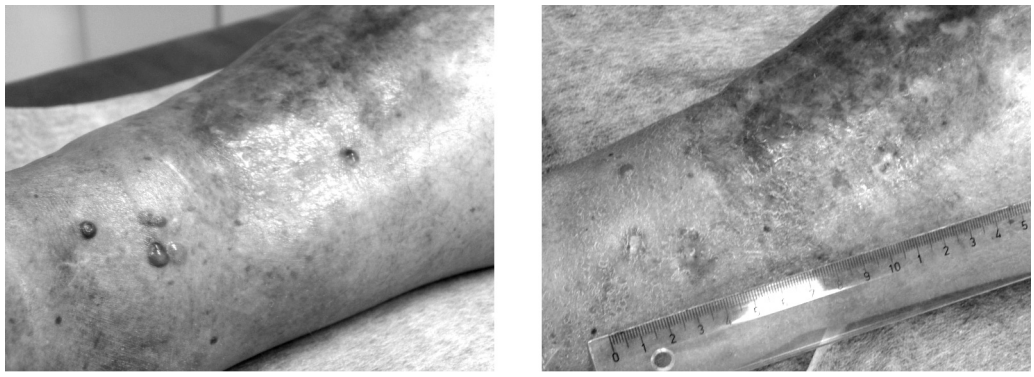

Figure 6 ECT treatment of skin metastases of malignant melanoma with cisplatin: left, before the treatment; right, 1 year after the treatment. The treatment is described in detail in (38). (The photographs were kindly provided by prof. Zvonimir Rudolf and prof. Gregor Serša.)

B. Electrogenetransfection (EGT)

Unlike electrochemotherapy, application of electroporation for transfer of DNA molecules into the cell, often referred to as *electrogenetransfection* (EGT), has not yet entered clinical trials. Nevertheless, EGT is devoid of the health risks which are present in viral gene transfection, and it is presently considered to have large potential as a method for gene therapy aimed at correcting genetic diseases.

It has been shown that an injection of naked DNA into a skeletal muscle in itself results in an expression of the injected DNA. The gene expression can last up to several months, which makes the muscle a promising target for gene therapy, but the obtained levels of expression are low and extremely variable, which makes the results of such an approach unpredictable and hence unsuitable for clinical application. However, when DNA injection into the muscle is combined with electroporation, the gene expression is increased by two or three orders of magnitude, and the variability between muscle fibers is significantly reduced (44–46).

Expression of the therapeutic gene coding for erythropoietin has already been reported in animals, and elevated values of erythropoietin have been observed for long periods after the gene transfer (47,48). These results suggest that cell transfection by EGT could be the appropriate method for correction of genetic diseases, vaccination, and cancer treatment. Clinical trials, which can be expected to start in the near future, thus hold great promises for these areas of medicine.

III. PULSE PARAMETERS AND EXPERIMENTAL CONDITIONS

For the large majority of applications in vitro, the efficiency of electroporation is determined by the fraction of reversibly porated cells with respect to the whole treated cell population. In the optimization of electroporation, one thus searches for pulse parameters and other experimental conditions that yield the highest fraction of porated cells that survive the treatment. In addition, for the treatment to serve its purpose, it is often necessary that a certain quantity of exogenous molecules enters into each cell, and in these cases optimal pulse parameters should also ensure a sufficient molecular uptake per cell.

For these reasons, the role of pulse parameters and experimental conditions is usually investigated using a combination of tests, estimating the fraction of porated cells, the fraction of cells surviving the treatment, the average amount of exogenous molecules introduced into the cell, and sometimes also the time of recovery of the cells back into the nonporated state.

A. The Role of the Amplitude, Duration, and Number of Pulses

The role of parameters of rectangular pulses in the efficiency of electroporation was investigated in a number of studies (11,14,49–52). These studies show that poration becomes detectable as the pulse amplitude exceeds a certain critical value. Above this value, with further increase of pulse amplitude, the percentage of porated cells increases, while the percentage of cells surviving the treatment decreases. As a function of pulse amplitude, the percentage of porated cells approximately follows an ascending sigmoidal curve, while the percentage of viable cells resembles a descending sigmoidal curve (Fig. 7). Similar results have been obtained with exponentially decaying pulses (53), where the time constant of pulse decay was used instead of pulse duration.

In a study performed on a number of different cell lines, Èemaar and co-workers have shown that both poration and cell survival as functions of pulse amplitude vary significantly between various types of cells (52). Though some of the observed differences can be

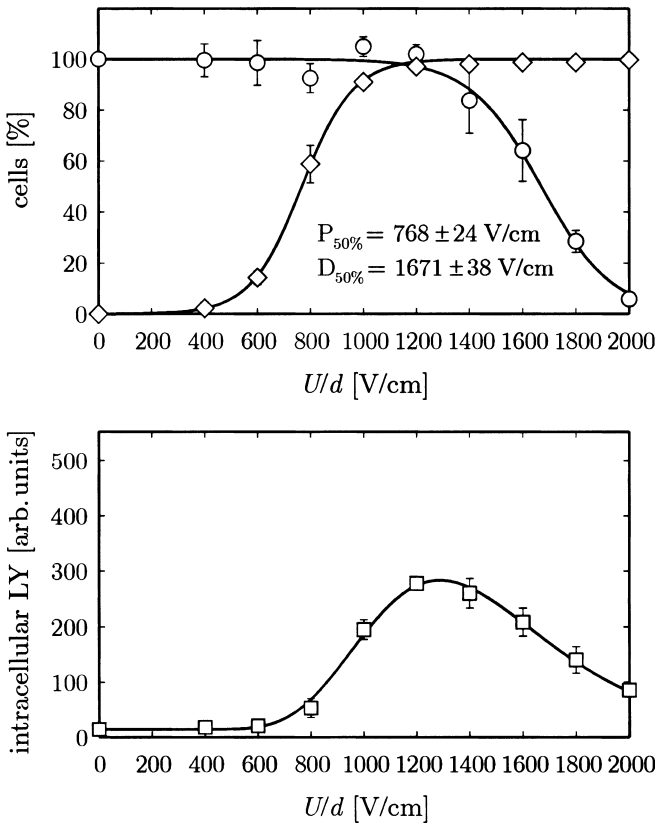

Figure 7 Top: Percentages of porated (diamonds) and viable cells (circles) as functions of pulse amplitude (the ratio between the voltage and the electrode distance). Bottom: uptake of lucifer yellow (LY) into the cells. DC-3F cells (spontaneously transformed Chinese hamster fibroblasts) were porated with eight unipolar rectangular 100-μs pulses delivered in 1-s intervals. $P_{50\%}$ and $D_{50\%}$ denote pulse amplitudes which lead to poratation and death, respectively, of 50% of the cells. Extracellular concentration of LY was 1 mM.

attributed to differences in cell size, these results imply that the differences in membrane composition and structure also play an important role.

Experiments show that the critical pulse amplitude of electroporation is lowered if the number and/or duration of the pulses is increased (11,49) (compare Figs. 7 and 8). If the values of these two parameters are not too large, the average amount of molecules introduced into a cell also increases with an increase of the number of pulses. Using four or more pulses, a pronounced peak of molecular uptake is obtained.

Several studies have demonstrated that in the case of macromolecules, electrophoresis plays an important role in the transport of molecules across the membrane, and sufficiently long pulse duration is crucial for adequate uptake (11,14,50). Typically, pulse durations for the uptake of smaller molecules are in the range of hundreds of microseconds, while for macromolecules, durations from several milliseconds to several tens of milliseconds are usually required.

In a study utilizing a broad range of rectangular pulse parameters, Maèek-Lebar and co-workers have shown that the total energy of a train of pulses is not a crucial parameter in

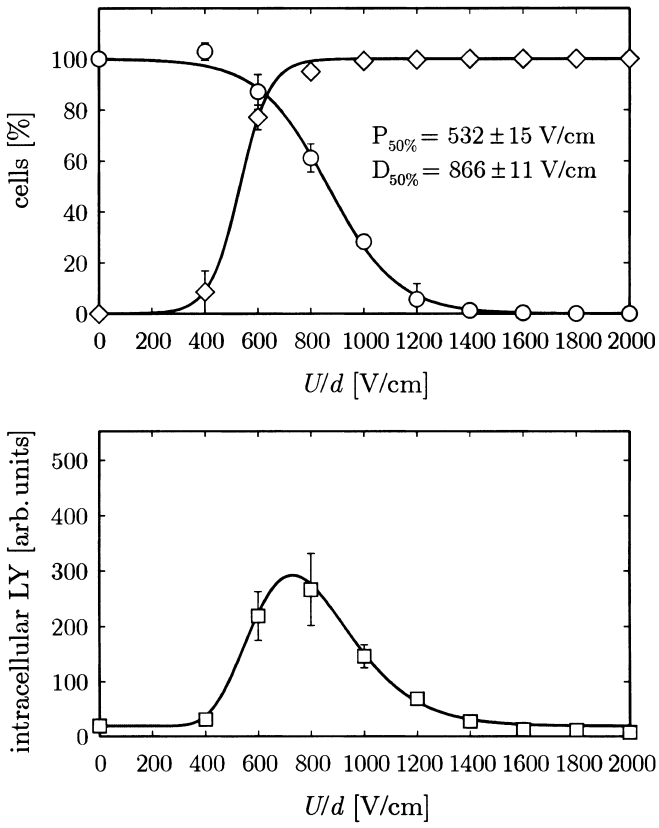

Figure 8 Electroporation and survival of DC-3F cells (top) and uptake of LY (bottom) for eight unipolar rectangular 1-ms pulses delivered in 1-s intervals.

either drug uptake or cell survival. On the contrary, a significant difference was observed in the uptake induced by different trains of the same total energy (51).

B. The Role of Pulse Shape

Because commercially available pulse generators with sufficient voltages for electroporation of cells in suspension are mostly limited to rectangular and exponentially decaying pulses, relatively few studies have dealt with the role of pulse shape in the efficiency of cell electroporation. Chang and co-workers have reported that the efficiency of poration was increased when a sine wave of 30–200 kHz amplitude was superimposed onto a rectangular pulse, though the amplitude of the sine wave was only about 5% of the total pulse amplitude (54,55). Tekle and co-workers found that the efficiency of DNA transfection in vitro was significantly higher with a bipolar 60-kHz square wave of 400-μs duration than with a unipolar wave of the same frequency and duration (56). Schoenbach and co-workers have reported on electropermeabilization with ultrashort (60 ns) pulses (57). In a study comparing unipolar and bipolar rectangular pulses, we have shown that with bipolar pulses, the critical voltage of electroporation is lowered considerably, while cell viability remains practically unaffected (cf. Figs. 8 and 9); at the same time, the peak of the uptake increases with respect to the one obtained by unipolar pulses (58).

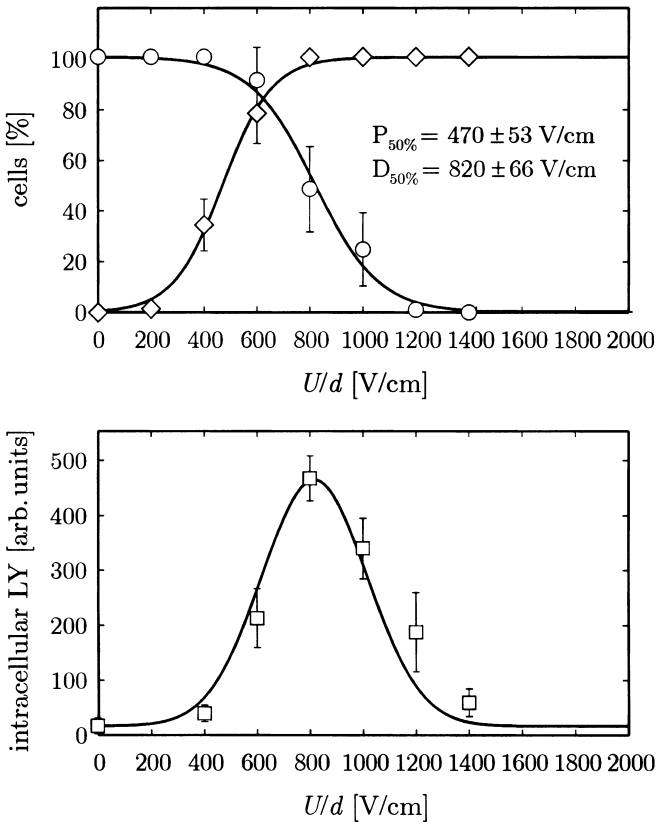

Figure 9 Electroporation and survival of DC-3F cells (top) and uptake of LY (bottom) for eight bipolar rectangular 1-ms (500 μs + 500 μs) pulses delivered in 1-s intervals.

In addition, we have shown that with both aluminum and stainless steel electrodes, the release of metal ions from the electrodes into the cell suspension is reduced significantly if bipolar pulses are used (59). This reduces the electrolytic contamination of the cell suspension and also prolongs the lifetime of the electrodes.

Comparing the results obtained with pulses having 1-ms amplitude duration, but with rise and fall times ranging from 2 μs to 100 μs, we found no detectable differences between the efficiencies of these pulses. Thus, it seems that the rise and fall times of the pulses do not play a significant role in the efficiency of electroporation.

C. Pulse Repetition Frequency

In general, patients find electrochemotherapy tolerable, in spite of unpleasant sensations associated with contraction of muscles located beneath or in the vicinity of the electrodes. These contractions are due to the intensity of the electric pulses required for effective electropermeabilization of tumor cell membranes. Since a train of eight electric pulses with repetition frequency of 1 Hz is usually applied to the tumors, each pulse in the train excites underlying nerves and provokes muscle contractions. The use of pulses with repetition frequency higher than the frequency of tetanic contraction would therefore cause a reduced number of muscle contractions and associated unpleasant sensations. In a recently

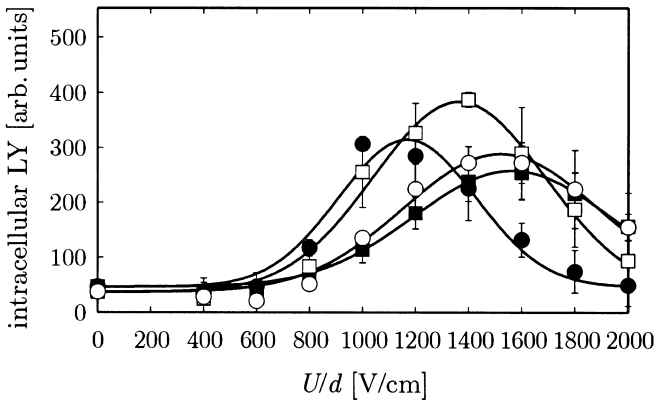

Figure 10 Uptake of LY for eight unipolar rectangular pulses 100-μs pulses delivered with repetition frequencies of 1 Hz (●), 10 Hz (□), 1 kHz (○), and 2.5 kHz (■).

performed study, we have shown that for repetition frequencies ranging from 1 Hz to 8.3 kHz, the uptake of LY into electroporated cells in vitro stays at similar levels (60). Part of these results is shown in Fig. 10. In an ongoing study, similar results are being obtained in vivo. This suggests that there are prospects for efficient use of pulses with high repetition frequency also in clinical electrochemotherapy.

D. Other Experimental Conditions

Besides the pulse parameters, the efficiency of poration also depends on many physical and chemical parameters. An important role is played by the properties of the extracellular medium: its ionic strength and composition (61–63), osmotic pressure (64,65), and its temperature before and after poration (66). In addition, as described in more detail in Sec. IV.B, for successful electroporation in vivo, uniformity of the electric field in the tissue is also important (7,67).

E. Recommendations for the Choice of Pulse Parameters

Based on the studies discussed above, some general advice in the design of experiments involving electroporation can be made. Pulse amplitude (voltage-to-distance ratio) should typically be in the range from 200 V/cm up to 2000 V/cm. Pulse durations should be in the range of hundreds of microseconds for smaller molecules and from several milliseconds up to several tens of milliseconds for macromolecules such as DNA fragments (in the latter case, due to the very long pulse duration, optimal pulse amplitude can even be lower than 100 V/cm). If the equipment allows, bipolar pulses should be used instead of unipolar ones. Bipolar pulses yield a lower poration threshold, higher uptake, and an unaffected viability compared to unipolar pulses of the same amplitude and duration.

These guidelines should provide a starting point for a design of experiments involving electroporation. Still, the optimal values of pulse parameters strongly depend on the cell type used, on the molecule to be introduced, and on specific conditions under which the experiment is performed. Therefore, for best possible results, pulse parameters should be optimized under specific experimental conditions before the actual study is initiated.

IV. ELECTRODES

A. Electrode Designs

The electric field distribution in a cell suspension or in a tissue is to a large extent determined by the geometry of the electrodes. For electroporation of cells in a suspension, typical electrodes consist of two parallel plates at a distance of 1–4 mm. The commercially available electrodes are made of aluminum and usually mounted in a cuvette that also serves as a container for the cell suspension, while several groups also use parallel plate electrodes made of stainless steel or platinum. If the plates are sufficiently large with respect to the distance between them, this design provides a relatively homogeneous field in the suspension. Still, several practical problems arise with electroporation of cells in suspension.

First, it has been reported that with aluminum electrodes, voltage drop at the electrode–solution interface can represent a significant fraction of the total voltage delivered to the electrodes (68). In contrast, this drop is insignificant with stainless steel electrodes (69). Second, electric pulses cause a certain amount of metal ions to be released from the electrodes into the suspension. Aluminum ions released from the electrodes can significantly affect biochemical processes involving inositol phosphates (70). With stainless steel electrodes, which are often used in experimental setups, the release of iron ions is of similar magnitude as that of aluminum ions from aluminum electrodes (59). The problem of electrolytic contamination can be reduced by using bipolar charge-balanced pulses (59), but most commercially available devices for electroporation are unable to generate such pulses. Another option is to use platinum electrodes, but due to the cost of platinum this is not a viable option with experimental setups where the electrodes can only be used once (e.g., for sterile conditions).

A variety of electrode designs have been used for electroporation in vivo. The most widely used are the plate electrodes, either at a fixed or a variable distance between them. In the latter case, the electrode plates can be mounted on a caliper (Fig. 11A). This type of electrode is used for electrochemotherapy of cutaneous and subcutaneous experimental tumors, smaller tumors in patients, and gene delivery in rat mouse and subcutaneous

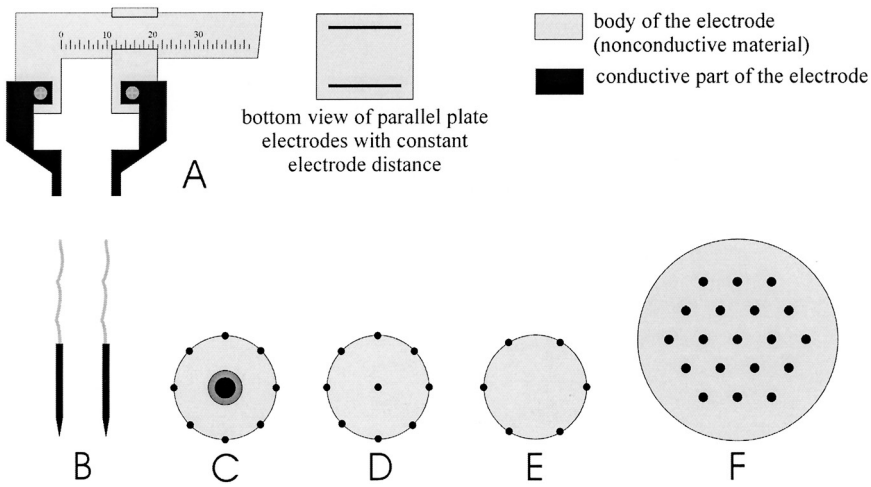

Figure 11 Electrode designs: (A) parallel plate electrodes, (B) simple needle electrodes, (C–F) multiple needles. (Adapted from Ref. 73 with the permission of the authors.)

tumors. Other types of electrodes (Fig. 11 B–E) have been used and compared in electrochemotherapy of experimental subcutaneous mice tumors with variable response (71). Another type of electrode, "honeycomb" (Fig. 11F), has been constructed and used for treatment of larger tumor volumes in rabbits (72). In the latter type, a division of volume to smaller fractions is introduced as pairs of needle electrodes are sequentially fired in a way that eventually the whole (arbitrarily large) volume is being permeabilized. The designs shown in Fig. 11 A and E have also been used in clinical trials (73).

B. Calculations

It is difficult to compare directly the effectiveness of different types of electrodes used in ECT, since they were in many cases used in different experimental setups, with different tumors, and with different voltages. However, it is possible to model numerically the electric field distribution in the tumor obtained with each electrode type. Such modeling clearly shows that the amplitude of electric field in the tumor plays the decisive role in the efficiency of ECT with a given electrode type (7,67). Another important factor to be considered is the homogeneity of the electric field in the tumor. The main reason for this is that in highly nonhomogeneous fields, in the regions with the weakest field, many cells are not electroporated at all, while in the regions where the field is the strongest, for many cells electroporation is irreversible. This is to some extent acceptable for ECT, but not for EGT, where all cells should survive the treatment.

C. Recommendations for the Choice of Electrodes

Mathematical modeling shows that a uniform coverage of a tumor with a sufficiently high electric field is necessary for good effectiveness of electrochemotherapy. This approach can be very useful in further search for electrodes that would make electrochemotherapy and other applications of electroporation in vivo more efficient. The objective of such studies would be to optimize electrode configuration in order to obtain above-threshold electric fields in the whole targeted tissue, e.g., tumor, with the least possible variation of the field amplitude.

V. GENERATORS

In the market, there are a number of pulse generators for electroporation. These devices are usually referred to as *electroporators*. An overview of commercially available electroporators is accessible on the World Wide Web (http://www.the-scientist.com/yr1997/july/shockjok.pdf). Electroporators for clinical use are offered or being developed by Genetronics (http://www.genetronics.com) and IGEA (http://www.igea.it), but the vast majority of electroporators were designed, and are sold, for applications in vitro. Some deliver unipolar rectangular (square wave) pulses, some exponentially decaying pulses, and some are able to deliver more intricate pulse shapes, such as sine-modulated pulses. The main problem, besides the fact that they are designed for in vitro environment, is the fact that the range of pulse parameters is not sufficiently flexible. One of the main reasons for a need of pulse flexibility lies partly in the fact that the exact mechanisms of electroporation are not yet fully known. Therefore, the user has to either rely on protocols and pulse parameters provided by the producers and on protocols from the literature or to develop his own. In the process of developing an efficient protocol and selecting the most effective pulse parameters for a specific need, flexibility is needed.

VI. CONCLUSIONS

Electroporation has been for decades used in cell biology and bioelectrochemistry laboratories for introducing genes into various types of cells. The physical nature of the phenomenon, i.e., increased permeability of plasma membrane, due to exposure to short high-voltage pulses, allows its use in plant, yeast, bacteria, and eukaryotic cells. In addition, electroporation can be used to introduce various sized molecules (from ions up to DNA) that otherwise can not or difficultly pass the membrane into the cells. It has also been demonstrated that proteins can be inserted into the membrane and cells fused, under appropriate experimental conditions.

In the last decade it has been successfully demonstrated by various groups that electroporation can be successfully applied also to cells in tissue in situation in vivo. In preclinical studies on many different tumor models, electropermeabilization has been combined with predominantly two antitumor agents: bleomycin and cisplatin. Both drugs have an intracellular target and the membrane represents a barrier for them. Therefore they are the prime candidates for electrochemotherapy.

In addition to its established role in electrochemotherapy, electroporation might soon become the method of choice for gene transfection in cell biology and bioelectrochemistry, and the existing body of experience and knowledge makes electroporation extremely interesting also as a nonviral method for introduction of therapeutic genes, for immunotherapy and DNA vaccination. As it has been demonstrated for electrochemotherapy, electroporation of cells in tissues in situ can be performed safely and effectively, and electroporation for clinical gene transfection is likely to have a bright future in the era of decoded human genome. It remains, however, extremely important that technology is developed and used with great care. We are just coming into the situation when electroporators and electrodes for clinical use are becoming available.

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REFERENCES

1. Mir LM. Therapeutic perspectives of in vivo cell electropermeabilization. Bioelectrochemistry 2001; 53:1–10.
2. Kotnik T, Bobanović; F, Miklavčič D. Sensitivity of transmembrane voltage induced by applied electric fields—a theoretical analysis. Bioelectrochem Bioenerg 1997; 43:285–291.
3. Kotnik T, Miklavčič D. Analytical description of transmembrane voltage induced by electric fields on spheroidal cells. Biophys J 2000; 79:670–679.
4. Gimsa J, Wachner D. Analytical description of the transmembrane voltage induced on arbitrarily oriented ellipsoidal and cylindrical cells. Biophys J 2001; 81:1888–1896.
5. Susil R, Šemrov D, Miklavčič D. Electric field induced transmembrane potential depends on cell density and organization. Electro Magnetobiol 1998; 17:391–399.
6. Pavlin M, Pavšelj N, Miklavčič D. Dependence of induced transmembrane potential on cell density, arrangement, and cell position inside a cell system. IEEE Trans Biomed Eng 2002; 49:605–612.
7. Miklavčič D, Beravs K, Šemrov D, Čemažar M, Demšar F, Serša G. The importance of electric field distribution for effective in vivo electroporation of tissues. Biophys J 1998; 74:2152–2158.
8. Tekle E, Astumian RD, Chock PB. Selective and asymmetric molecular transport across electroporated cell membranes. Proc Natl Acad Sci USA 1994; 91:11512–11516.
9. Neumann E, Toensing K, Kakorin S, Budde P, Frey J. Mechanism of electroporative dye uptake by mouse B cells. Biophys J 1998; 74:98–108.
10. Gabriel B, Teissié J. Time courses of mammalian cell electropermeabilization observed by millisecond imaging of membrane property changes during the pulse. Biophys J 1999; 76:2158–2165.
11. Rols MP, Teissié J. Electropermeabilization of mammalian cells to macromolecules: control by pulse duration. Biophys J 1998; 75:1415–1423.
12. Satkauskas S, Bureau MF, Puc M, Mahfoudi A, Scherman D, Miklavcic D, Mir LM. Mechanisms of in vivo DNA electrotransfer: respective contributions of cell electropermeabilization and DNA electrophoresis. Mol Ther 2002; 5:133–140.
13. Rols MP, Delteil C, Golzio M, Dumond P, Cros S, Teissie J. In vivo electrically mediated protein and gene transfer in murine melanoma. Nature Biotechnol 1998; 16:168–171.
14. Sukharev SI, Klenchin VA, Serov SM, Chernomordik LV, Chizmadzhev YA. Electroporation and electrophoretic DNA transfer into cells. Biophys J 1992; 63:1320–1327.
15. Weaver JC, Chizmadzhev YA. Theory of electroporation: a review. Bioelectrochem Bioenerg 1996; 41:135–160.
16. Kakorin S, Stoylov SP, Neumann E. Electrooptics of membrane electroporation in diphenylhexatriene-doped lipid bilayer vesicles. Biophys Chem 1996; 58:109–116.
17. Marinkk SJ, Lindahl E, Edholm O, Mark AE. Simulation of the spontaneous aggregation of phospholipids into bilayers. J Am Chem Soc 2001; 123:8638–8639.
18. Kandušer M, Fošnarič M. Šentjurc M. Kralj-Iglič; V, Hägerstrand H. Iglič A, Miklavčič D. Effect of surfactant polyoxyethylene glycol ($C_{12}E_8$) on electroporation of cell line DC3F Colloid Surf A. In press.
19. Sale AJH, Hamilton WA. Effects of high electric fields on microorganisms: I. Killing of bacteria and yeasts. Biochim Biophys Acta 1967; 148:781–788.
20. Hamilton WA, Sale AJH. Effects of high electric fields on microorganisms: II. Mechanism and action of the lethal effect. Biochim Biophys Acta 1967; 148:789–800.
21. Vernhes MC, Benichou A, Pernin P, Cabanes PA, Teissié J. Elimination of free-living amoebae in fresh water with pulsed electric fields. Water Res 2002; 36:3429–3438.
22. Mouneimne Y, Tosi PF, Barhoumi R, Nicolau C. Electroinsertion of full length recombinant CD4 into red blood cell membrane. Biochim Biophys Acta 1990; 1027:53–58.
23. Raffy S, Teissie J. Electroinsertion of glycophorin A in interdigitation-fusion giant unilamellar lipid vesicles. J Biol Chem 1997; 272:25524–25530.

24. Abidor IG, Sowers AE. Kinetics and mechanism of cell membrane electrofusion. Biophys J 1992; 61:1557–1569.
25. Sowers AE. Membrane electrofusion: a paradigm for study of membrane fusion mechanisms. Methods Enzymol 1993; 220:196–211.
26. Heller R. Spectrofluorometric assay for cell-tissue electrofusion. Methods Mol Biol 1995; 48:341–353.
27. Mekid H, Mir LM. In vivo cell electrofusion. Biochim Biophys Acta 2000; 1524:118–130.
28. Scott-Taylor TH, Pettengell R, Clarke I, Stuhler G, La Barthe MC, Walden P, Dalgleish AG. Human tumour and dendritic cell hybrids generated by electrofusion: potential for cancer vaccines. Biochim Biophys Acta 2000; 1500:265–267.
29. Orentas RJ, Schauer D, Bin Q, Johnson BD. Electrofusion of a weakly immunogenic neuroblastoma with dendritic cells produces a tumor vaccine. Cell Immunol 2001; 213:4–13.
30. Schmidt E, Leinfelder U, Gessner P, Zillikens D, Bröcker EB, Zimmermann U. CD19+ B lymphocytes are the major source of human antibody-secreting hybridomas generated by electrofusion. J Immunol Methods 2001; 255:93–102.
31. Prausnitz MR, Bose VG, Langer R, Weaver JC. Electroporation of mammalian skin: A mechanism to enhance transdermal drug delivery. Proc Natl Acad Sci USA 1993; 90:10504–10508.
32. Vanbever R, Lecouturier N, Preat V. Transdermal delivery of metoprolol by electroporation. Pharmacol Res 1994; 11:1657–1662.
33. Zewert TE, Pliquett U, Langer R, Weaver JC. Transport of DNA antisense oligonucleotides across human skin by electroporation. Biochim Biophys Res Commun 1995; 212:286–292.
34. Mir LM, Orlowski S, Belehradek J, Paoletti C. Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses. Eur J Cancer 1991; 27:68–72.
35. Mir LM, Orlowski S, Belehradek J Jr, Teissie J, Rols MP, Sersa G, Miklavcic D, Gilbert R, Heller R. Biomedical applications of electric pulses with special emphasis on antitumor electrochemotherapy. Bioelectrochem Bioenerg 1995; 38:203–207.
36. Serša G, Čemažar M, Miklavčič D. Antitumor effectiveness of electrochemotherapy with cis-diamminedichloroplatinum(II) in mice. Cancer Res 1995; 55:3450–3455.
37. Mir LM, Glass LF, Serša G, Teissié J, Domenge C, Miklavčič D, Jaroszeski MJ, Orlowski S, Reintgen DS, Rudolf Z, Belehradek M, Gilbert R, Rols MP, Belehradek J Jr, Bachaud JM, DeConti R, Štabuc B, Čemažar M, Coninx P, Heller R. Effective treatment of cutaneous and subcutaneous malignant tumors by electrochemotherapy. Brit J Cancer 1998; 77:2336–2342.
38. Serša G, Štabuc B, Čemažar M, Milklavčič D, Rudolf Z. Electrochemotherapy with cisplatin: clinical experience in malignant melanoma patients. Clin Cancer Res 2000a; 6:863–867.
39. Čemažar M, Serša G, Miklavčič D. Electrochemotherapy with cisplatin in the treatment of tumour cells resistant to cisplatin. Anticancer Res 1998b; 18:4463–4466.
40. Serša G, Čemažar M, Miklavčič D, Chaplin DJ. Tumor blood flow modifying effect of electrochemotherapy with bleomycin. Anticancer Res 1999a; 19:4017–4022.
41. Serša G, Čemažar M, Parkins CS, Chaplin DJ. Tumour blood flow changes induced by application of electric pulses. Eur J Cancer 1999b; 35:672–677.
42. Gehl J, Skovsgaard T, Mir LM. Vascular reactions to in vivo electroporation: characterization and consequences for drug and gene delivery. Biochim Biophys Acta 2002; 1569:51–58.
43. Serša G, Kranjc S, Čemažar M. Improvement of combined modality therapy with cisplatin and radiation using electroporation of tumors. Int J Radiat Oncol Biol Phys 2000b; 46:1037–1041.
44. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. Nat Biotechnol 1998; 16:867–870.
45. Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D. High efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci USA 1999; 96:4262–4267.
46. Durieux AC, Bonnefoy R, Manissolle C, Freyssenet D. High-efficiency gene electrotransfer into skeletal muscle: description and physiological applicability of a new pulse generator. Biochem Biophys Res Commun 2002; 296:443–450.

47. Rizzuto G, Cappelletti M, Maione D, Savino R, Lazzaro D, Costa P, Mathiesen I, Cortese R, Ciliberto G, Laufer R, La Monica N, Fattori E. Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. Proc Natl Acad Sci USA 1999; 96:6417–6422.
48. Bettan M, Emmanuel F, Darteil R, Caillaud JM, Soubrier F, Delaere P, Branelec D, Mahfoudi A, Duverger N, Scherman D. High-level protein secretion into blood circulation after electric pulse-mediated gene transfer into skeletal muscle. Mol Ther 2000; 2:204–210.
49. Rols MP, Teissié J. Electropermeabilization of mammalian cells: quantitative analysis of the phenomenon. Biophys J 1990a; 58:1089–1098.
50. Wolf H, Rols MP, Boldt E, Neumann E, Teissié J. Control by pulse parameters of electric field-mediated gene transfer in mammalian cells. Biophys J 1994; 66:524–531.
51. Maček-Lebar A, Kopitar NA, Ihan A, Serša G, Miklavčič D. Significance of treatment energy in cell electropermeabilization. Electro Magnetobiol 1998; 17:253–260.
52. Čemažar M, Jarm T, Miklavčič D, Maček-Lebar A, Ihan A, Kopitar NA, Serša G. Effect of electric-field intensity on electropermeabilization and electrosensitivity of various tumor-cell lines in vitro. Electro Magnetobiol 1998a; 17:261–270.
53. Tomov TC. Quantitative dependence of electroporation on the pulse parameters. Bioelectrochem Bioenerg 1995; 37:101–107.
54. Chang DC. Cell poration and cell fusion using an oscillating electric field. Biophys J 1989; 56:641–652.
55. Chang DC, Gao PQ, Maxwell BL. High efficiency gene transfection by electroporation using a radio-frequency electric field. Biochim Biophys Acta 1991; 1092:153–160.
56. Tekle E, Astumian RD, Chock PB. Electroporation by using bipolar oscillating electric field: An improved method for DNA transfection of NIH 3T3 cells. Proc Natl Acad Sci USA 1991; 88:4230–4234.
57. Schoenbach KH, Peterkin FE, Alden RW, Beebe SJ. The effects of pulsed electric fields on biological cells: experiments and applications. IEEE Trans Plasma Sci 1997; 25:284–292.
58. Kotnik T, Mir LM, Flisar K, Puc M, Miklavčič D. Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses. Part I. Increased efficiency of permeabilization. Bioelectrochemistry 2001a; 54:83–90.
59. Kotnik T, Miklavčič D, Mir LM. Cell membrane electropermeabilization by symmetrical bipolar rectangular pulses. Part II. Reduced electrolytic contamination. Bioelectrochemistry 2001b; 54:91–95.
60. Pucihar G, Mir LM, Miklavčič D. The effect of pulse repetition frequency on the uptake into electropermeabilized cells in vitro with possible applications in electrochemotherapy. Bioelectrochemistry 2002; 57:167–172.
61. Rols MP, Teissié J. Ionic-strength modulation of electrically induced permeabilization and associated fusion of mammalian cells. Eur J Biochem 1989; 179:109–115.
62. Djuzenova CS, Zimmermann U, Frank H, Sukhorukov VL, Richter E, Fuhr G. Effect of medium conductivity and composition on the uptake of propidium iodide into electropermeabilized myeloma cells. Biochim Biophys Acta 1996; 1284:143–152.
63. Pucihar G, Kotnik T, Kanduser M, Miklavčič D. The influence of medium conductivity on electropermeabilization and survival of cells in vitro. Bioelectrochemistry 2001; 54:107–115.
64. Rols MP, Teissié J. Modulation of electrically induced permeabilizaiton and fusion of Chinese hamster ovary cells by osmotic pressure. Biochemistry 1990b; 29:4561–4567.
65. Golzio M, Mora MP, Raynaud C, Delteil C, Teissié J, Rols MP. Control by osmotic pressure of voltage-induced permeabilization and gene transfer in mammalian cells. Biophys J 1998; 74:3015–3022.
66. Rols MP, Delteil C, Serin G, Teissié J. Temperature effects on electrotransfection of mammalian cells. Nucleic Acids Res 1994; 22:540.
67. Miklavčič D, Šemrov D, Mekid H, Mir LM. A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy. Biochim Biophys Acta 2000; 1523:73–83.

68. Pliquett U, Gift EA, Weaver JC. Determination of the electric field and anomalous heating caused by exponential pulses with aluminum electrodes in electroporation experiments. Bioelectrochem Bioenerg 1996; 39:39–53.
69. Loste F, Eynard N, Teissié J. Direct monitoring of the field strength during electropulsation. Bioelectrochem Bioenerg 1998; 47:119–127.
70. Loomis-Husselbee JW, Cullen PJ, Irvine RF, Dawson AP. Electroporation can cause artefacts due to solubilizaion of cations from the electrode plates. Biochem J 1991; 277:883–885.
71. Gilbert RA, Jaroszeski MJ, Heller R. Novel electrode designs for electrochemotherapy. Biochim Biophys Acta 1997; 1334:9–14.
72. Ramirez LH, Orlowski S, An D, Bindoula G, Dzodic R, Ardouin P, Bognel C, Belehradek J, Munck JN, Mir LM. Electrochemotherapy on liver tumours in rabbits. Brit J Cancer 1998; 12:2104–2111.
73. Puc M, Reberšek S, Miklavčič D. Requirements for a clinical electrochemotherapy device—electroporator. Radiol Oncol 1997; 31:368–373.