Cell Membrane Electroporation—Part 2: The Applications

Key words: microbial deactivation, electroextraction, electrochemotherapy, gene electrotransfer, nonthermal tissue ablation

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Electroporation can be used as a tool for extracting or introducing molecules from or into a cell. The most important and promising applications of electroporation in medicine and biotechnology are described.

Introduction

Each new experimental technique takes time to develop, further time to understand the mechanisms underlying the data, and even more time to have it adopted in routine industrial or medical practice. Because of lack of industrial interest, high initial investment costs, or concern over personnel safety, many proposed new techniques are abandoned. However, that has not been the case with electroporation.

The influence of an electric field on the node of Ranvier was described in 1958 [1], and in 1972 the first observations of transient permeability changes in the vesicle membranes as a consequence of vesicle exposure to an external electric field were reported [2]. The method was termed electroporation, and two branches were developed, namely reversible electroporation in which the treated cells survived and irreversible electroporation in which they did not.

If a cell is exposed to a sufficiently high electric field, its membrane becomes temporarily permeable to molecules that otherwise cannot pass through it. This process has been used as a tool for introducing foreign substances such as exogenous DNA into cells (gene electrotransfer) [3] or for introducing membrane-impermeant drugs in order to kill cancer cells (electrochemotherapy) [4], [5].

Irreversible electroporation occurs when the electric field applied results in leakage of cellular components, which leads to cell death. The method was used in microbiology in order to kill bacterial cells (microbial deactivation) [6], [7] and in medicine to ablate tissue nonthermally [8].

It should be emphasized that membrane electroporation leading to increased permeability of the membrane to specific molecules is nonselective, i.e., molecules can be driven into or out of the cell, depending on their concentration gradients across the membrane [9]–[11].

This article is the second in a series of three on electroporation. The first article [12] dealt with the phenomenon itself and its manifestations on the molecular and cellular level. In this article its most widely established and promising applications are presented, specifically its use in biotechnology for microbial

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deactivation in food and water, and for extraction of molecules from cells, and its utilization in medicine for electrochemotherapy, gene therapy through electrotransfer, DNA vaccination, and tissue ablation. The third article will focus on the associated hardware, standards, safety, and certification.

Applications in Biotechnology

Microbial Deactivation

Bacterial, viral, unicellular organism pathogens, and their by-products (enzymes and toxins) in food or water can represent serious threats to human health. One of the first steps toward microbial deactivation in food was made by Appert at the beginning of the 19th century [13]. Following heat deactivation the food was safe for consumption for a longer period of time, but it often suffered loss of flavor, color, and texture or change of chemical composition. Preservation of food by electroporation maintains color and flavor, and the antioxidant levels are unaffected. The procedure was introduced more than 40 years ago [6] and is now a promising nonthermal food processing method [14], competing with ultrasound and high-pressure methods. It does not generate by-products and only mild heating occurs. During the last few years microbial and enzyme deactivation using electric pulses has been achieved in a variety of foods and beverages [15]. The effectiveness of the procedure depends on several parameters, e.g., pulse amplitude, duration, frequency and polarity, and temperature [15]. Optimization of such parameters is the subject of several ongoing studies.

Pathogenic microbes in water have long been a concern to the public. Microbial deactivation in water can be achieved by various methods, the most common being chlorine, ozone, and ultraviolet treatments. Although these methods have been extensively used, many of them are not as effective as electroporation, or result in the formation of hazardous by-products [16]. Electroporation has been demonstrated as a promising method of deactivation of microorganisms in fresh, waste, sea, or oilfield reinjection water [17]–[20].

The main effects of electric pulses on a microorganism are shown in Figure 1 [21]. Synergism of electroporation with other water treatment methods [22] and with moderate heating [23], [24] has also been demonstrated. Some problems remain to be solved, e.g., deactivating more resistant microbial species, reducing the initial investment cost, and standardizing treatment procedures.

Extraction of Biomolecules From Microorganisms and Plants

Cost-effective protein production in recombinant bacteria is important in industry and in medicine [25]. In order to extract cell content, mechanical disintegration (homogenization) and chemical extraction (such as alkaline lysis) are the principal methods. The main disadvantage of both is that cellular organelles (which are special subunits within a cell with specific functions, such as cell breathing, protein production, DNA maintenance) are also destroyed, and residual cell husks are not easily removed. During mechanical disintegration all intracellular molecules are released from the cell, including those in cell organelles, and therefore additional steps are necessary in order to purify the target molecule. Electroporation shows great potential in this context because it causes reversible damage to the membrane but does not affect cell organelles [3]. The pulses would need to be much shorter, and generate much greater field strengths, than those used in classical plasma membrane electroporation, in order to affect cell organelles [26]. Classical pulse lengths are in the microsecond to millisecond range and generate electric fields around 1 kV/cm. Adjustment of these parameters has been shown to release intracellular proteins selectively (Figure 2), and only a very short time (up to 10 s) is needed to gain maximal protein concentration from cells [25].

Electroporation has proved effective in releasing proteins from bacteria, yeast, and cells that contain complex structures enclosed within membranes [10], [25], [26]. Thus it can extract DNA molecules from bacteria cells, with a yield comparable to that obtained by alkaline lysis [27].

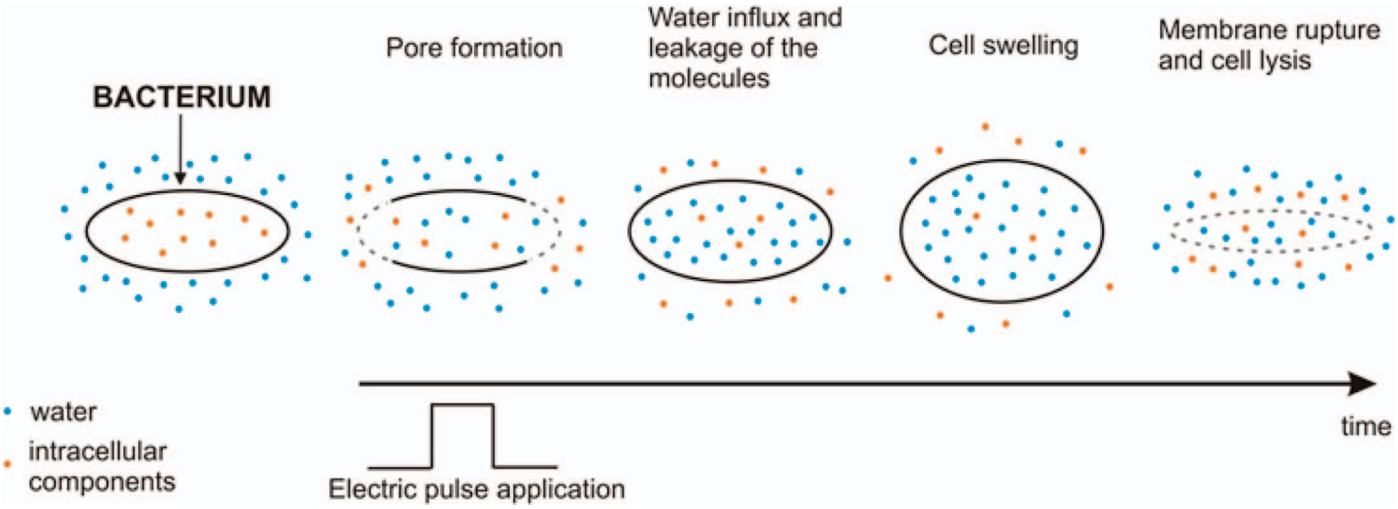

Figure 1. Microbial deactivation of a microorganism using electric pulses. The cells are electroporated, and various small molecules can then leak into or out of the cell.

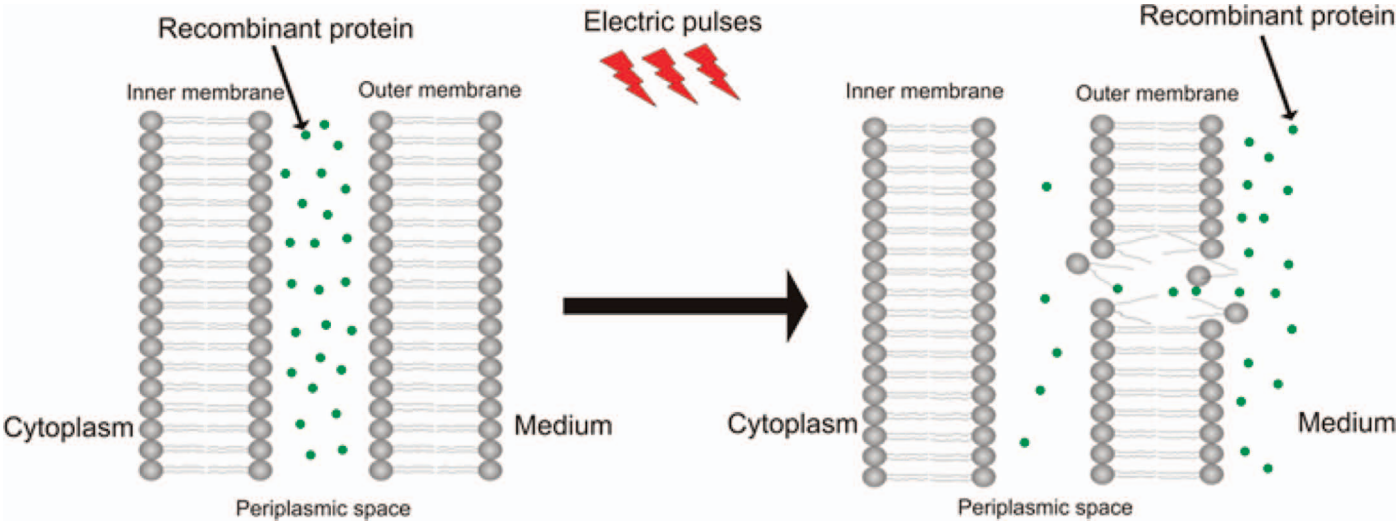

Figure 2. Selective release of bacteria proteins. Using electroporation with appropriate electric pulse parameters, only the outer membrane of bacteria with two membranes is permeabilized. As a consequence, proteins located in the space between the two membranes are released into the media surrounding the bacteria cells and can be collected.

Electroporation is also being used for extracting intracellular components from plants. It has been shown that the quantity and purity of extracted components can be greatly increased relative to those achieved using conventional methods. One of the most promising industrial-scale applications is in sugar extraction from sugar beets (Figure 3) [11], [28].

Electroporation has also proved to be an energy-efficient drying method for green biomass, which serves as a source material for biofuel. Electroporation treatment prior to conventional pressing reduces the required drying energy by more than 50%. The equipment and procedures have to be tailored to each application; the equipment used for various biotechnological applications has been reviewed recently [28].

Applications in Medicine

Electrochemotherapy

Chemotherapy is a widely accepted cancer treatment using chemotherapeutic drugs. Since most of these drugs act on dividing cells, they also affect normal tissues and so have undesired

Figure 3. Sugar extraction from sugar beets. Left: More efficient extraction of juice from sugar beet using electroporation (EP) rather than conventional pressing. Right: Demonstration plant with mass flow of 10 ton/hour and 2 Marx-generators (1.2 kJ/pulse, 20 Hz). Up to 30% less energy is required when electroporation extraction is used.

side effects. Alternative treatment approaches are therefore being sought, and electrochemotherapy (ECT) is among the most promising. Some chemotherapeutic drugs targeting intracellular material have poor membrane permeability, requiring high doses for antitumor effectiveness, and therefore produce pronounced side effects. Since electroporation increases the permeability of the cell membrane for such drugs, access to the cell interior is much easier. Hence the dose of the chemotherapeutic agent can be lowered, and the severity of side effects is reduced. Electrochemotherapy is thus a local antitumor treatment in which electric pulses are applied to the tumor after injection of a membrane-impermeant anticancer drug (Figure 4) [29].

The efficacy of the method is increased by three additional effects. These are (i) vascular lock after electric pulse application, which decreases tumor blood flow and thus causes the drug to be retained in the tumor for longer periods of time; (ii) vascular disruption, causing blood flow within the tumor to be reduced; and (iii) immune response, i.e., due to enormous tumor substance shedding in the organism after ECT, the immune system is evoked and antibodies are produced [30], [31].

Following promising results of numerous preclinical studies of ECT, the first clinical studies were initiated. The first report of clinical phase I-II trial was presented in the early 1990s [32]. Head and neck squamous cell carcinoma was treated using intravenous administration of bleomycin, followed by administration of electric pulses on tumor nodules [32]. The authors reported significant tumor size reduction after treatment. Electrochemotherapy using the drug cisplatin was established a few years later [32]–[34]; effective eradication of tumor nodules in patients with malignant melanoma, or squamous and basal cell carcinoma, was reported. Since then several clinical studies have been launched, and in 2002 the European Standard Operating Procedures for Electrochemotherapy and Electrogenetherapy (ESOPE) project was set up to define standard operating procedures. Treatment modalities were proposed to ensure patient safety and to achieve optimum treatment results with respect to (i) selection of chemotherapeutic drug, (ii) drug delivery route (intravenous or intratumoral), (iii) electrode shape (plate or needle), and (iv) pulse parameters (usually eight pulses 100 μs in duration, 1-Hz to 5-kHz pulse repetition frequency) [35]. Electrochemotherapy using bleomycin or cisplatin on malignant melanoma nodules yielded 73.7% complete tumor eradication [36]. No local or general side effects were observed during treatment; only temporary flushing and fluid accumulation occurred around the treated areas [37].

Electrochemotherapy is successfully used today in clinical practice for treatment of cutaneous and subcutaneous tumors, especially melanoma nodules (Figure 5), and is being developed for treatment of deep-seated tumors (Figure 6) [38], [39] and chest-wall breast cancer [31]. The number of patients benefitting from ECT treatment is rapidly increasing, with more than 1,500 patients treated in 2011 in more than 100 hospitals around the world.

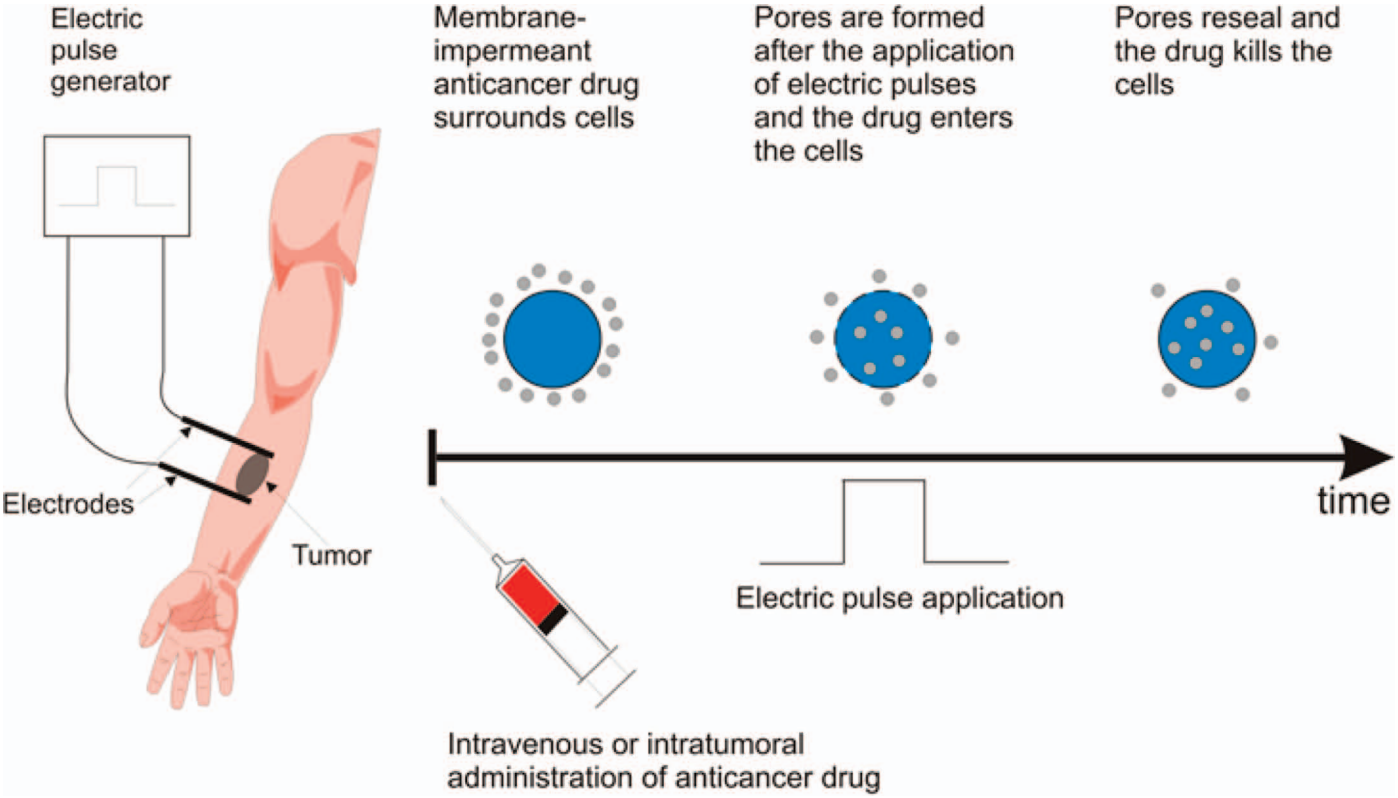

Figure 4. Electrochemotherapy stages. Anticancer drug administration (intravenous or intratumoral) is followed by local electric pulse application. The electric pulses reversibly permeabilize the cell membrane, and the poorly permeant drug enters the cell and kills it.

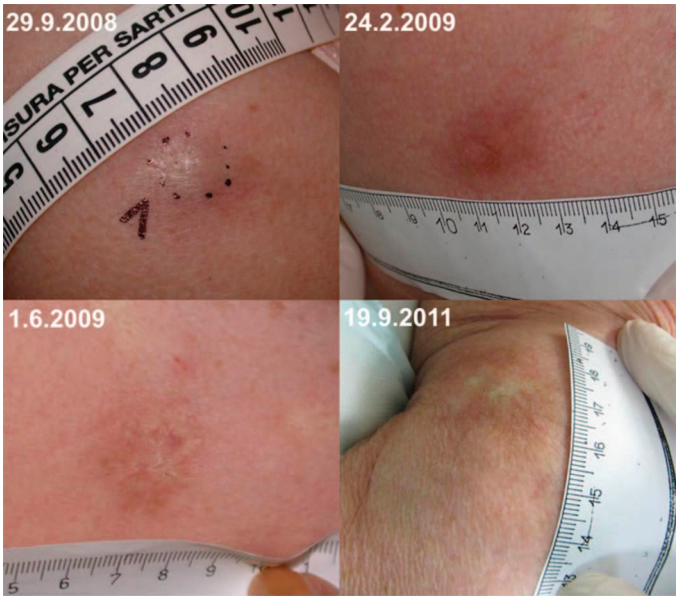

Figure 5. Electrochemotherapy of melanoma tumor nodule. A subcutaneous tumor nodule was treated by electrochemotherapy using intravenous injection of bleomycin followed by several electric pulses delivered to the nodule using plate electrodes. Within a few months the tumor regressed completely, without damage to the surrounding skin, and stayed in complete remission for three years after treatment, i.e., for the entire observation time.

Gene Electrotransfer for Gene Therapy and DNA Vaccination

Transfer of genes into cells in order to change their biological function was first used in the treatment of genetic defects of the immune system [40]. Since then many gene-transfer techniques have been tested, among them gene electrotransfer (GET), which is a nonviral method for delivery of DNA molecules into cells by means of electric pulses. Several biochemical methods also allow the introduction of genetic material into cells, but many of them are less effective than GET or have undesirable side effects resulting from the introduction of chemical or viral additives [41], [42]. The first transfer of DNA into cells cultivated *in vitro* by application of electric pulses was reported in 1982 [3]. In the same year general guidelines for the determination of optimum conditions were proposed [43]. Several later studies have also emphasized the influence of parameter choice (temperature, electric pulses, etc.) on the efficacy of GET [44]–[46].

In spite of its extensive use, the precise molecular mechanism of GET has not yet been fully elucidated. Thus the process of DNA entry to the cell is more complex than simple diffusion of DNA through pores created by electric pulse application. The principal steps in GET are shown in Figure 7 [44].

Almost 10 years after the first GET into cells cultivated *in vitro*, the first GET into tissue was demonstrated [47]. Since then many researchers have investigated its use on muscle [48], tumors [49], liver [50], skin [51], lung [52], heart [53], cornea [54], and the central nervous system [55]. The main obstacle, especially in its application to tumors, is the slow diffusion of DNA through the extracellular matrix. Several solutions have been proposed, e.g., the use of enzymes in order to cause partial degradation of the extracellular matrix [56]. Also crucial for efficacious GET in tissues are the delivery mode (local injection), electrode size and shape, electric pulse parameters, and DNA design. Nevertheless, GET efficacy has reached levels sufficient to justify clinical trials. The Phase I clinical trial of GET of DNA with interleukin-12 in patients with metastatic melanoma was completed in 2008 and showed GET to be effective, safe, and accurately controllable [57]. DNA vaccination using electric pulses also shows great promise in clinical practice [58]. Currently more than 20 Phase I or II clinical studies in which DNA vaccine is administered using electric pulses are registered [59]. Therapeutic and prophylactic vaccinations are under active investigation.

Nonthermal Tissue Ablation by Means of Irreversible Electroporation

Irreversible electroporation is a biophysical process in which electric fields applied across a cell cause extensive or permanent permeabilization of the cell membrane, which eventually leads to cell death because the interior of the cell becomes unstable due to the leakage of ions [60]. This phenomenon may have been observed in the middle of the 18th century, when damage to skin from the discharge of a static electricity generator was studied [61]. Electric fields produce, simultaneously, several biophysical effects, one of which is Joule heating. The use of Joule heating for tissue ablation is now an important medical technology, using radiofrequency, microwave, or ac or dc electrical energy sources. However, since heating causes cell death through inactivation of biological molecules in the heated volume, most of the molecules in that volume, including the extracellular matrix,

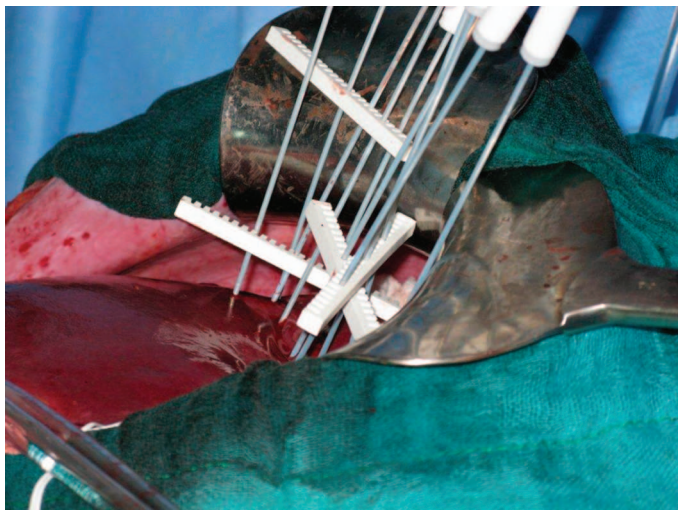

Figure 6. Electrochemotherapy treatment of deep-seated tumors. Electrodes are inserted into the liver during surgery. Anticancer drug is administered intravenously, and electric pulses are applied. The position of the electrodes and the choice of pulse parameters are determined in treatment planning (image courtesy of Tomaž Jarm, University of Ljubljana, Faculty of Electrical Engineering).

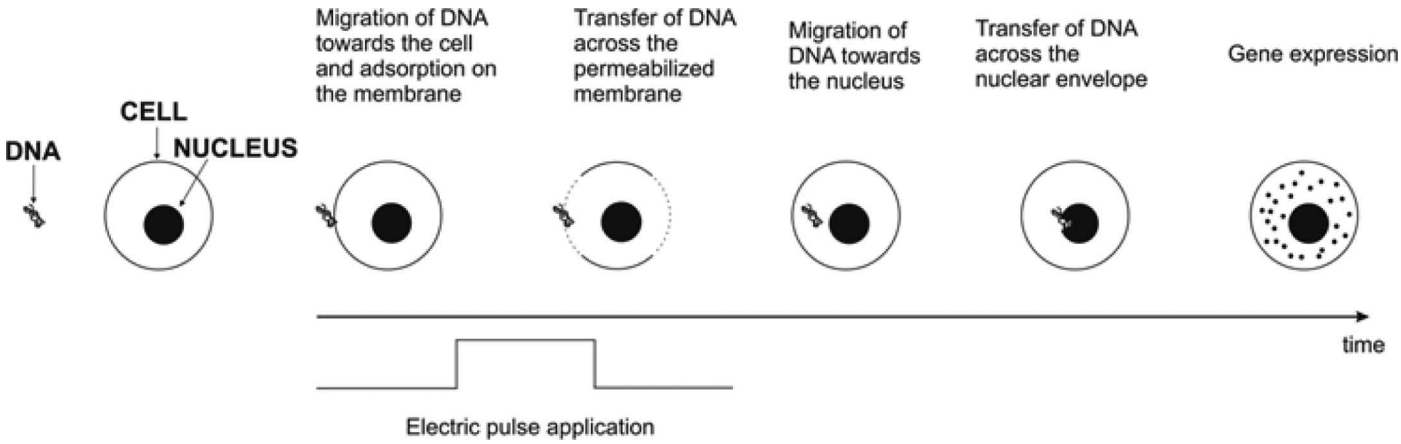

Figure 7. Steps involved in gene electrotransfer. The mechanism underlying transfer of DNA across the permeabilized membrane is still unclear.

blood vessels walls, and nerve conduits, are destroyed. This is a serious disadvantage.

The potential of irreversible electroporation in medicine was realized with the emergence of nonthermal irreversible electroporation [62]. Generation of electric fields producing irreversible electroporation, with minimal thermal damage, is at the center of a new molecularly selective tissue ablation modality called nonthermal irreversible electroporation (NTIRE) [8], [63]. Nonthermal irreversible electroporation has enabled the ablation of undesirable (malignant) tissue with minimal damage to blood vessels [8], [63] and nerve [64] conduits in the treated volume. Thus the activity of the immune system, which is connected to all parts of the ablated tissue through the large blood vessel scaffolds [63], is enhanced. Furthermore, the intact extracellular

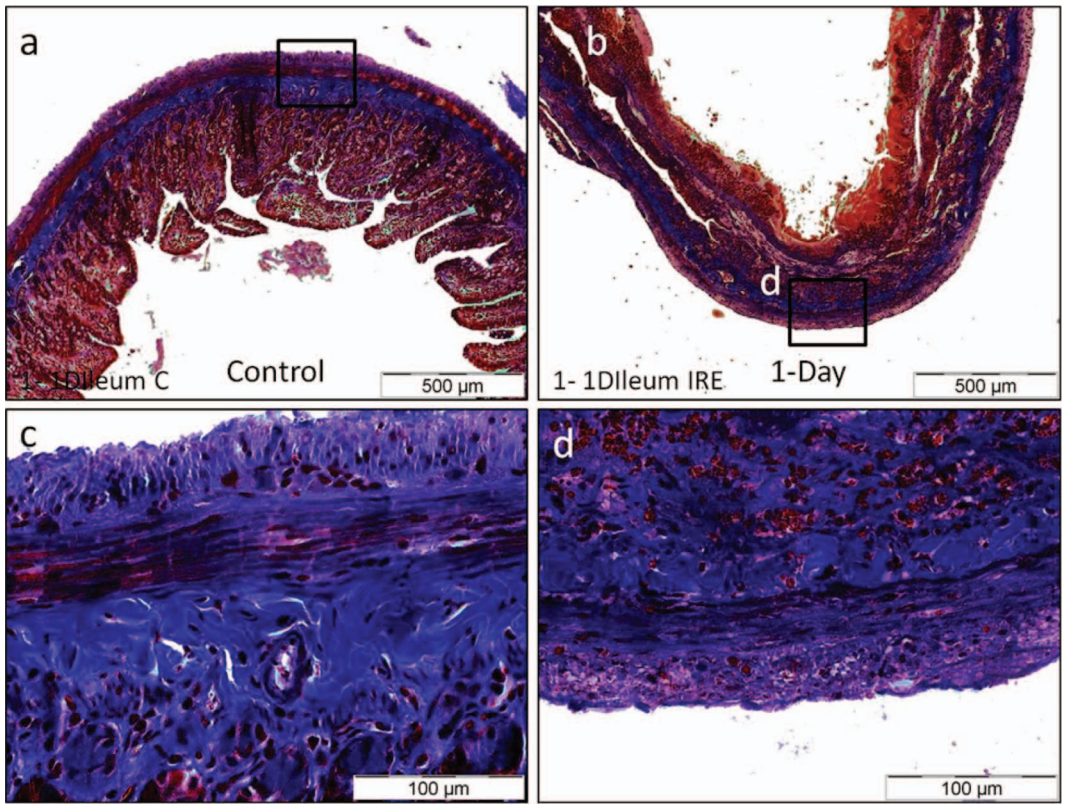

Figure 8. Effect of irreversible electroporation (IRE) on cell scaffold structure. Compared to the control (a), the cell scaffold remains intact although there is a loss of cellular architecture throughout the intestine one day after IRE treatment (b). Compared with the control (c), the blue collagen fibers are similar in morphology after IRE treatment (d) (image courtesy of Mary A. Phillips, University of California–Berkeley, Department of Mechanical Engineering, and R. Narayan, Pathology Research Laboratory Inc., Hayward, CA).

matrix facilitates regeneration of tissue in the liver [63], blood vessels [65], nerves [66], and small intestines [67].

The molecular selectivity of NTIRE is illustrated in Figure 8. The results are taken from the study reported in [67] and show that (i) immediately after NTIRE the scaffold remains intact while the cells are ablated and (ii) seven days after the tissue ablation, endothelial cells and muscle cells begin to regenerate and form new villi (thin protuberances of the small intestine, through which digested food passes into the blood). The molecular selectivity of NTIRE may find additional applications in tissue engineering [68].

In addition to molecular selectivity, NTIRE has several other advantages: (i) technically it is simple, requiring only the insertion of needle electrodes; (ii) it is very fast (less than one minute), with the advantages of reduced anesthesia time, reduced postablation pain and complications, and the possibility of more than one treatment at a time; and (iii) it can be monitored using conventional imaging modalities [63], [69]–[71].

The development of commercial irreversible electroporation technologies [72] has facilitated rapid clinical implementation of NTIRE to treat advanced malignancy of the liver, lung, kidney [73], brain [74], and pancreas [75]. The NTIRE field is proceeding rapidly, with more than 1,000 patients treated in more than 50 hospitals around the world during the last two years. However, much remains to be done in treatment planning, protocol design, safety, clinical techniques, device design, and fundamental research [76]–[78].

Conclusions

In this article we described some of the ways in which electroporation has been implemented in clinical and industrial practice. Other applications are under development, e.g., waste water treatment and pretreatment of excess sludge in water. Recently a project within the European Cooperation in Science and Technology network—COST Action TD1104—was launched, with the aim of optimizing existing electroporation-based technologies and treatments and exploring new applications in biology, medicine, pharmacy, and the food industry [79]. Since the exchange of knowledge between workers in the electroporation field is still inadequate, COST Action TD1104 is an ideal framework, funding cooperation between research groups working in electroporation.

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