

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Electroporation Based Drug Delivery and Its Applications

Tuhin Subhra Santra, Pen-Cheng Wang and
Fang Gang Tseng

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/55369>

1. Introduction

When a certain strong electrical pulse applied across a cell or tissue, the structures of the cell or tissue would be rearranged to cause the permeabilization of the cell membrane, named in early 1980's "electroporation"[1]. The theoretical and experimental studies of electric field effects on living cells with their bilayer lipid membrane has been studies in 1960's to 1970's century [1-6]. During these years, the researches were primarily dealt with reversible and irreversible membrane breakdown in vitro. Based on these research, the first gene transfer by custom-built electroporation chamber on murine cells was performed by Neumann et al. in 1982 [7]. When electric field ($E \approx 0.2V$, Usually 0.5-1V) applied across the cell membrane, a significant amount of electrical conductivity can increase on the cell plasma membrane. As a result, this electric field can create primary membrane "nanopores" with minimum 1 nm radius, which can transport small amount of ions such as Na^+ and Cl^- through this membrane "nanopores". The essential features of electroporation included (a) short electric pulse application (b) lipid bilayer charging (c) structural rearrangements within the cell membrane (d) water-filled membrane structures, which can perforate the membrane ("aqueous pathways" or pores) and (e) increment of molecular and ionic transportation [8]. In conventional electroporation (Bulk electroporation) technique, an external high electric field pulses were applied to millions of cells in suspension together in-between two large electrodes. When this electric field was above the critical breakdown potential of the cell, a strong polarization of the cell membrane occur due to the high external electric field. Applying a very high electric field could be resulted in the formation of millions of pores into the cell membrane simultaneously without reversibility [9]. Several methods other than electroporation can be used for gene transfer like microprecipitates, microinjection, sonoporation,

endocytosis, liposomes, and biological vectors [10-16]. But electroporation have some advantages when compared to other gene transfer methods such as, (a) easy and rapid operation with high reproducibility due to control of electrical parameters (b) higher transformation efficiency when compared to CaCl_2 and PEG mediated chemical transformation (c) controllable pore size with variation of electrical pulse and minimizing effect of cytosolic components, and (d) easy to uptake DNA into cells with smaller amount, when compared to other techniques [17-19]. For bulk electroporation, drug delivery can be performed in homogeneous electric field, whereas as single cell electroporation (SCEP), can introduce an inhomogeneous electric field focused on targeted single adherent or suspended cell without affecting other neighboring cells. Both techniques can deliver molecules such as DNA, RNA, anticancer drugs into cells in-vitro and in-vivo. However SCEP is more advanced technique compared to the bulk electroporation technique. Recently researchers are concentrating on more advanced research area, such as localized single cell membrane electroporation (LSCMEP), which is an efficient and fast method to deliver drugs into single cell by selective and localized way from millions of cells. This LSCMEP can judge cell to cell variation precisely with their organelles and intracellular biochemical effect. This process can deliver more controllable drug delivery inside the single cell with application of different pulse duration. Both single cell electroporation (SCEP) and localized single cell membrane electroporation (LSCMEP) can provide high cell viability rate, high transfection efficiency, lower sample contamination, and smaller Joule heating effect in comparison with bulk electroporation (BEP) process.

2. Electroporation conditions

To achieve excellent gene delivery into the cells, several electroporation conditions will be accomplished during experiment. Also these electroporation conditions depend upon cell to cell variation. Generally these conditions can be divided into three categories (a) cellular factors (b) physiochemical factors and (c) electrical parameters.

2.1. Cellular factors

The gene delivery by electroporation into living cells should take place with high transfection efficiency and high cells viability in a physiological unperturbed state, so that, the effect of gene on a specific cellular function can be measured. The transformation efficiency can be influenced by growth phase of the cells, cell density, cell diameter, cell rigidity etc. The growth period of the cells in higher transformation success can be achieved from early to middle phase [20]. For electroporation, two main parameters needed to be optimized, one is electric field strength and the other is the pulse duration of electric field. When we apply voltage between two electrodes (this two electrodes maintain some distance), the pulse is generally an exponentially decayed signal with a time constant given by the product of the capacitance and resistance of the buffer solution. For any kind of field strength and pulse duration, the extent of macromolecular entry and degree of mortality will vary among different cell lines [21]. If transmembrane potential (TMP) difference is proportional to the cell size, the electric field

strength will be more sensitive for larger cells compared to smaller cells [22]. Also it has been reported that, transmembrane potential difference is related to cell angles and the directions of applied electric fields, where TMP values linearly proportional to the external applied electric field and cell diameter [23]. For the detection of specific effect of electroporated antibody, cellular function can depends on many variables, such as (a) concentration and affinity of introduced antibody into the target cell (b) restriction of antibodies to bind by target molecules (c) antibody can effect by intracellular concentration of target molecules (d) target molecules cellular factor such as epitopes(s) which can recognize the antibodies are unable to bind with target molecules (e) the cellular distribution of target molecules is accessible or not for antibody [21]. The cell viability during electroporation is also an important factor. Several literatures reported that nucleic acid molecules can be delivered in a highly efficient manner by optimizing the electroporation parameters, and the optimized electroporation conditions using a fluorescently labeled transfection control siRNA resulted in 75% transfection efficiency for Neuro-2A, 93% for human primary fibroblasts, and 94% for HUVEC cells, as analyzed by flow cytometry [24]. Saunders et al. have shown the successful uptake of trypan blue and FDA in cells, protoplasts and pollen from different plants using variety of pulse generator for optimizing the electroporation conditions [25].

2.2. Physiochemical factors

Physiochemical factors are more important for electroporation. This phenomena can occur during tissue development which contain the transportation, consumption of nutrients and oxygen, waste generated by cells, mechanical loading of tissue or cells, electromechanical phenomena (piezoelectricity), chemomechanical phenomena (swelling), electrochemical phenomena (Debye length) or osmotic phenomena (transport through the cell membrane). During cell culture stage, cells have to proliferate, colonize homogeneously in porous scaffolds and synthesized extracellular matrixes [26]. Different type of molecules or elements can interact with cells during cell culture [27]. Among all of the soluble elements, oxygen molecules possess the major importance for tissue growth particularly for osteoarticular system [28-29]. The magnitude of cell local oxygen consumption could be affected by cell concentration and temperature. The oxygen molecules passes through the cell membrane subject to enzymatic chemical, which is familiar as fundamental in enzymology. The oxygen consumption (R_s) per unit area of cell layer with surface density σ_{cell} can be described as the following expression

$$R_s = \sigma_{\text{cel}} \times V_{\text{max}} \times \frac{C}{C + K_M} = -R_{\text{max}} \times \frac{C}{C + K_M} \quad (1)$$

Where K_M is the Michaelis constant, C is the nutrient molecular concentration and the negative sign indicated that all cell layers have a sink effect. The maximum oxygen consumption rates V_{max} depends upon cell types and it can vary several order of magnitude. The oxygen consumption in unit volume such as porous substrate as written as

$$R_V = S_V \times R_S \quad (2)$$

This law also can be utilized for other biological phenomena such as cell population growth, drug uptake by tumor cells or absorption of biochemical molecules within kidney [26]. The electroporation efficiency can be affected by ionic composition of buffer solution. The resistivity and RC time constant of the electric pulse can be determined by ionic concentration of the buffer as written as [20]

$$V = V_0 \exp\left(\frac{-t}{RC}\right) \quad (3)$$

$$\tau = RC \quad (4)$$

where, V is the voltage across the pulsing chamber, V_0 is initial voltage, t is the time after starting of the pulse, R is the resistance of suspension, C is the capacitor of the capacitance, and τ is the time constant. The salt concentration of the electroporation buffer as well as pH of the buffer solution can affect the electroporation efficiency [30-31]. Generally the pH value 7.2 can be considered as an appropriate value for electroporation condition. The permeability of the cell membrane depends upon the solubility properties (such as salt composition, pH), charges or chemistry and solute size. The water molecule can transport inside and outside by osmotic balance. Osmosis can maintain the turgor pressure of the cells, across the cell membrane between the cell interior and relatively hypotonic environment [32]. The swelling properties of biological tissues can be explained by osmotic disjoining pressure [33]. Also the electroporation efficiency is much better, by introducing gene into cells at (0-4 °C) compared to elevated temperature during electroporation experiment [34-35]. This low temperature helps to protect the rapid resealing of the pores and enhance the uptake efficiency of gene inside the cell [17]. It has been reported that high transformation efficiency can be achieved by cell suspension of slow growing mycobacteria at elevated temperature [36]. Regarding the transfer of DNA into cells, it has been shown that cooling at the time of permeabilization and subsequent incubation (37 °C), can enhance the transformation efficiencies and cell viability [37]. Some of the authors has reported that, the use of low conductivity medium for DNA transfer, can increase the cell viability and transformation efficiency [37]. Increasing the amount of DNA into the pulse chamber can increase linearly transfection level [38-39]. However the toxic effect can be observed for high DNA concentration [39-40]. It is generally considered the use of calcium in the medium during electroporation for not causing high intracellular level of electrolyte. However some researchers use calcium and magnesium into the buffer solution for performing DNA transformation into the cell. In such a condition, DNA with calcium ions can act as positively charged 'glue' and attracted by the negatively charged ions on the exterior cell membrane, as a result, DNA molecules are approximating to the membrane before the electroporation process [41-42].

2.3. Electrical parameters

Electrical parameters are the most important factors to achieve high transformation efficiency and high cell viability during successful gene transfer into living cells. The electrical parameters mainly depend upon electric field strength, pulse length, number of pulses, time between two pulses and etc. Cell plasma membrane always have a tendency to protect the cytoplasmic volume from outside of any exogenous molecules. Cell membrane also continuously prevent cell to cell fusion. However, if we apply external electric field pulses and if this electric field just surpasses the capacitance of the cell membrane, then transient electroporpermabilized state can occur, which allow the delivery of various extracellular molecules, such as drugs, antibodies, DNA, RNA, dyes, tracers and oligonucleotides from outside of the cell to inside of the cell. If the molecular size is small, it can enter inside the cell membrane by diffusion after electroporpermabilization. However if the size is large, the molecules can enter into the cell through electrophoretically driven process as like DNA transferring into the cell membrane. Previously it was reported that, short and strong electric field pulses can make the membrane permeable in a spontaneously reversible way [43]. Also, it was reported, an extremely short pulse in nanosecond range with very high voltages, cellular organelles can be electroporated without cell membrane permeabilization [44]. The cell membrane permeabilization area can be controlled by pulse amplitude. By this permeabilization area, diffusion can take place into the cell membrane [45]. The degree of permeabilization can be controlled by the pulse duration and pulse number, where the longer the pulse, the greater the perturbation of the membrane in a given area [46]. Also it has been reported that area of the membrane being permeabilized is larger on the pole facing positive electrode, but degree of permeabilization is greater on the cell, where pole facing negative electrode [47]. However high transformation efficiency can be obtained, when three successive pulses with two intermittent cooling steps of one minute in each or single pulse without cooling for transformation of *Enterococcus faecalis*, *E. coli* and *Pseudomonas putida* [38]. Kinetic study of electroporpermabilization leads to 5 steps.

Time dependent electroporpermabilization		
Trigger	The external field induces an increase in the transmembrane potential up to the critical permeabilizing threshold	μm
Expansion	A Time dependent membrane transition occurs as long as the field is maintained at a overcritical value	ms
Stabilisation	A dramatic recovery of the membrane organization take place as soon as the field is subcritical	ms
Resealing	The annihilation of leaks is slow	s
Memory	Cell viability is preserved but membrane structural (flip flop) and physiological properties (macropinocytosis) recover on a much longer time	h

Table 1. Time dependence of electroporpermabilization. Permission to reprint obtained from Elsevier [50].

Table-1, illustrates the five steps where “Induction step” describes the field induced membrane potential increase which provides local defects, when it reached to a certain critical value (above 200mV). Here mechanical strength of the cell membrane depends upon buffer composition. The “Expansion step” comes when field presents with a strength larger than a critical value. In this case electromechanical stress present. “Stabilization step” indicates, field intensity is lower than threshold value, a stabilization process will take place in a few milliseconds. As a result membrane will be permeabilized for small molecules. “Resealing step” demonstrates a slow resealing on a scale of seconds and minutes. The “Memory effect” comes due to some changes of the membrane properties for longer time, such as an hours, but cell behavior is still normal [48-50]. Table-2 demonstrate electroporation conditions of various cell types [51], where electric field strength, pulse length, no of pulses, time between two pulses vary in each different type of cells.

Cell type	Voltage (Volt)	Pulse length (μS)	Number of pulses (sec)	Time between pulses (second)	Number of cells
HMSC	700V	90	5sec	0.1	75,000
HUVEC	250V	150	-	-	75000
RPTEC	300V	300	-	0.1	75000
Human T-Cells	300V	400	-	0.1	200000
NHDF-neo	900V	70	5sec	5	75000
PC-12	450V	200	-	-	75000
Rat astrocytes	300V	90	0.1sec	0.1	75000
NHA	450V	120	0.1sec	0.1	75000
K562	350V	130	0.1sec	0.1	150000

Table 2. Electroporation conditions for various cell types. Permission to reprint obtained from RNA society [51].

3. Single cell electroporation

3.1. Prospect of SCEP over Bulk Electroporation (BEP)

For single cell electroporation (SCEP), the electric field parameters can be controlled to avoid cell death. In SCEP, where an inhomogeneous electric field is applied locally surrounding the single cell adhesion or suspension, whereas in bulk electroporation (BEP), a homogeneous electric field is applied to suspension of millions of cells together. Fig.1. shows two types of conventional bulk electroporation(BE) chamber, to apply electric field with suspension of millions of cells together for vitro experiment. Both figures has shown the cross sectional view with two metal electrodes.

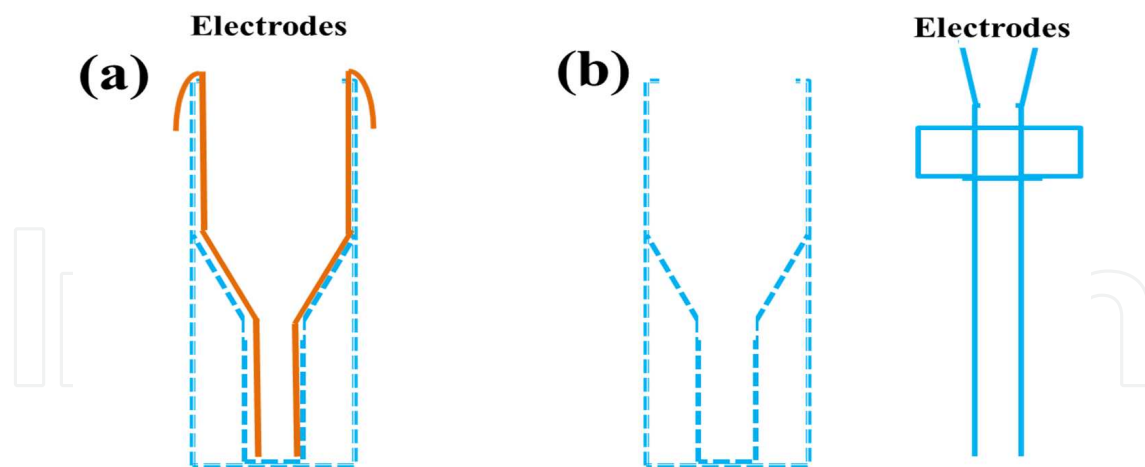


Figure 1. Bulk electroporation apparatus for vitro experiment. Two types of electroporation chamber, to apply an external electric field into the suspension of millions of cells together. Each chamber (a.b) consists cross sectional view of cuvette with two metal electrodes. Figure has redrawn with reprint permission [8].

Fig.2. demonstrates the single cell electroporation technique, where an external electric field is applied across the single cell membrane surface.

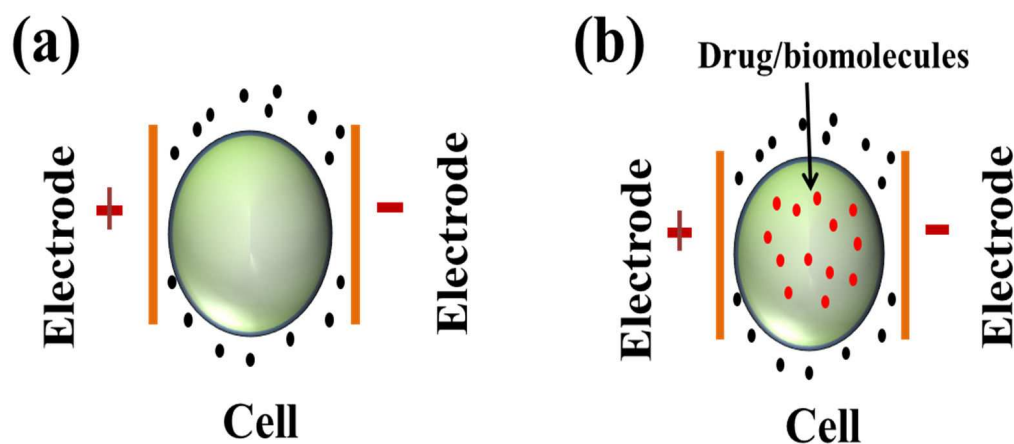


Figure 2. Single cell electroporation (SCEP) (a) Electric field was applied outside of the cell membrane (b) When external electric field reaches to a certain threshold value of the cell membrane, then cell membrane can permeabilized to deliver drug/biomolecules inside the single cell. After electroporation cell membrane reseal again.

When an external electric field beyond the certain threshold value of the cell membrane, then cell membrane can permeabilized to deliver biomolecules inside the single cell. The success rate like surviving cell for single cell electroporation is far better compared with bulk electroporation (BEP). This technique is faster and easy to perform with less toxicity and technical difficulty for application of wider tissues and cells. By this electroporation technique, the specific cell membrane region with small volume can be targeted to deliver the drugs, which can help to preserve expansive gene or molecules. Due to small volume of electroporation, different gene can be transferred in different electroporated time without cell damage. SCEP technique can provide precise temporal and spatial gene or dye delivery inside the cell. These processes are

affordable methods for fluorescently labeled and genetically manipulated individual cells [52]. This level of electroporation study is more convenient to understand molecular and genetic mechanisms with their biological functions and SCEP has ability to control temporally molecular biology of the cell, which was challenging task for transgenic model systems [52]. For bulk electroporation the required voltages are very high (10^3V) and this technique has little control of individual cell resulting in suboptimal parameters [53], as a result it is difficult to achieve reversible electroporation of all cells [54]. Moreover in single cell electroporation, there is good opportunity to observe the single cell response with specific cell size, shape, status and orientation of the electric field. SCE is useful for primary culture and heterogeneous culture such as brain tissue culture [55].

The first single cell electroporation has been demonstrated by using two carbon fiber microelectrodes [46], where the electrodes ($2\text{ }\mu\text{m}$ to $5\text{ }\mu\text{m}$) was positioned from the boundary of the cell surface at an $0\text{-}20^\circ$ angle and $160\text{-}180^\circ$ angle with respect to the objective plane. The patch clamp technique demonstrated the single cell electroporation (SCE), where patch-clamp pipette was sealed on the cell at a 90° angle with respect to the microelectrodes [56]. Using this technique, from transmembrane current response, it was possible to determined electric field strength for ion permeable pore formation and kinetics of pore opening, closing as well as pore opening times [56]. The electrolyte-filled capillary (EFC) coupled with a high-voltage power supply has been used for single cell experiment [57]. For application of a large voltages across EFC, It causes the formation of pores in the cell membranes which induces an electroosmotic flow of electrolyte. Micropipettes filled with DNA or other molecules stimulated by electric field have been electroporate the single cell at the tip of the pipette and successfully deliver the molecules inside single cell [58]. Microfabricated chip was used to incorporated the biomolecules into live biological cells for single cell experiment [59]. To achieve successful single cell electroporation, cell must be isolated from its population or inhomogeneous electric field must be focused on a particular cell, leaving neighboring cells unaffected [60]. Microfabricated devices can fulfil both isolated single cell and focused the electric field on particular single cell. Also this technology can offer other functionalities into the chip. Nowadays, SCEP research is growing on rapidly for biomedical application in vivo and in vitro. However to allow selective manipulation of single organelles within a cell, the electrode size must be reduced to nanoscale level. Nanoelectrode can provide less toxicity with high cell viability during electroporation experiment. Thus the localized single cell membrane electroporation concept has come in several years [61]. Fig.3. shows the localized single cell membrane electroporation (LSCMEP) process, where electric field is applied very short region of the cell membrane.

As a result, due to permeabilization of the cell membrane, drug/biomolecules can be delivered precisely (through sub micrometer to nanometer region of the cell membrane surface) inside the single cell. By this technique selective manipulation of organelles and biochemical effects can be analyze more precisely of the individual cell and this technique have more advantage compared to SCEP. Also the cell rapture and cell death can be minimize because electric field can intense in localized region of the cell membrane compared to SCEP. But this technology is now in underdeveloped stage. Recently Boukany et al. suggested nanochannel electropo-

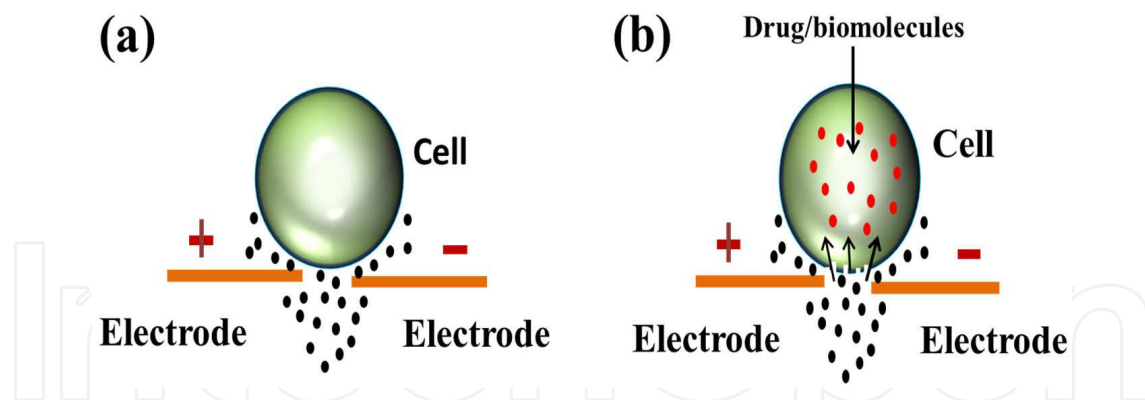


Figure 3. Localized single cell membrane electroporation (LSCMEP) technique, where drug/biomolecules can deliver precisely inside the single cell (a) Electric field was applied in a very small region of the cell membrane area (Localized way) (b) After electric field application, due to permeabilization of the cell membrane, drug/biomolecules can successfully deliver inside the single cell. Permission to reprint obtained from Springer [63].

ration with precise amount of biomolecules delivery by LSCMEP process. Where single cell has been positioned in one microchannel by optical tweezers and transfection agent was loaded to another microchannel. Two microchannel were connected by one nanochannel. Due to application of voltage between two microchannels, transfection agent was delivered through nanochannel using electrophoretically driven process and finally drugs delivered inside single cell through a very small area of the cell membrane [62]. Nawarathna et al. demonstrated localized electroporation technique using atomic force microscopy (AFM). Where modified AFM tip (0.5 μm) was used as a nanoelectrode, which was produced localized electric field into the cell membrane [61]. Fig 4.(a-h) shows the results of LSCMEP technique using AFM tip for electroporation process and Fig.4(i) demonstrated the AFM tip, which was positioned on top of the single cell for LSCMEP process.

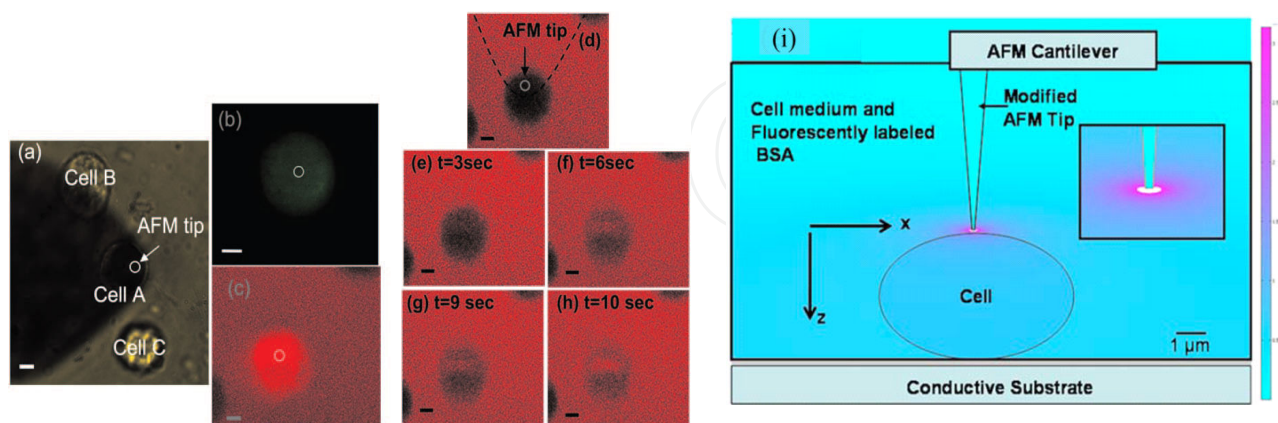


Figure 4. (a) Bright field image of AFM tip where the cell in the electroporation medium (cell A is electroporated while cell B and C are about 20 μm away from cell A). (b) Fluorescence image of rat fibroblast cell after electroporation. (c) Confocal fluorescence image of an electroporated cell. (d)-(h) Sequence of real time confocal fluorescence images of rat fibroblast cell after electroporation. (i) Calculated spatial distribution of electric field in the vicinity of the cell being electroporated. Permission to reprint obtained from American Institute of Physics (AIP) [61].

Chen et al. demonstrated localized single cell membrane electroporation (LSCMEP) by using microfluidic device. Where ITO thin film was used as microelectrode with 1 μm gap between two micro-electrodes. The ITO microelectrode with 100 nm thickness and 2 μm width intense electric field much more in between two microelectrode gap [63]. Fig.5. shows the device fabrication for localized electroporation experiment.

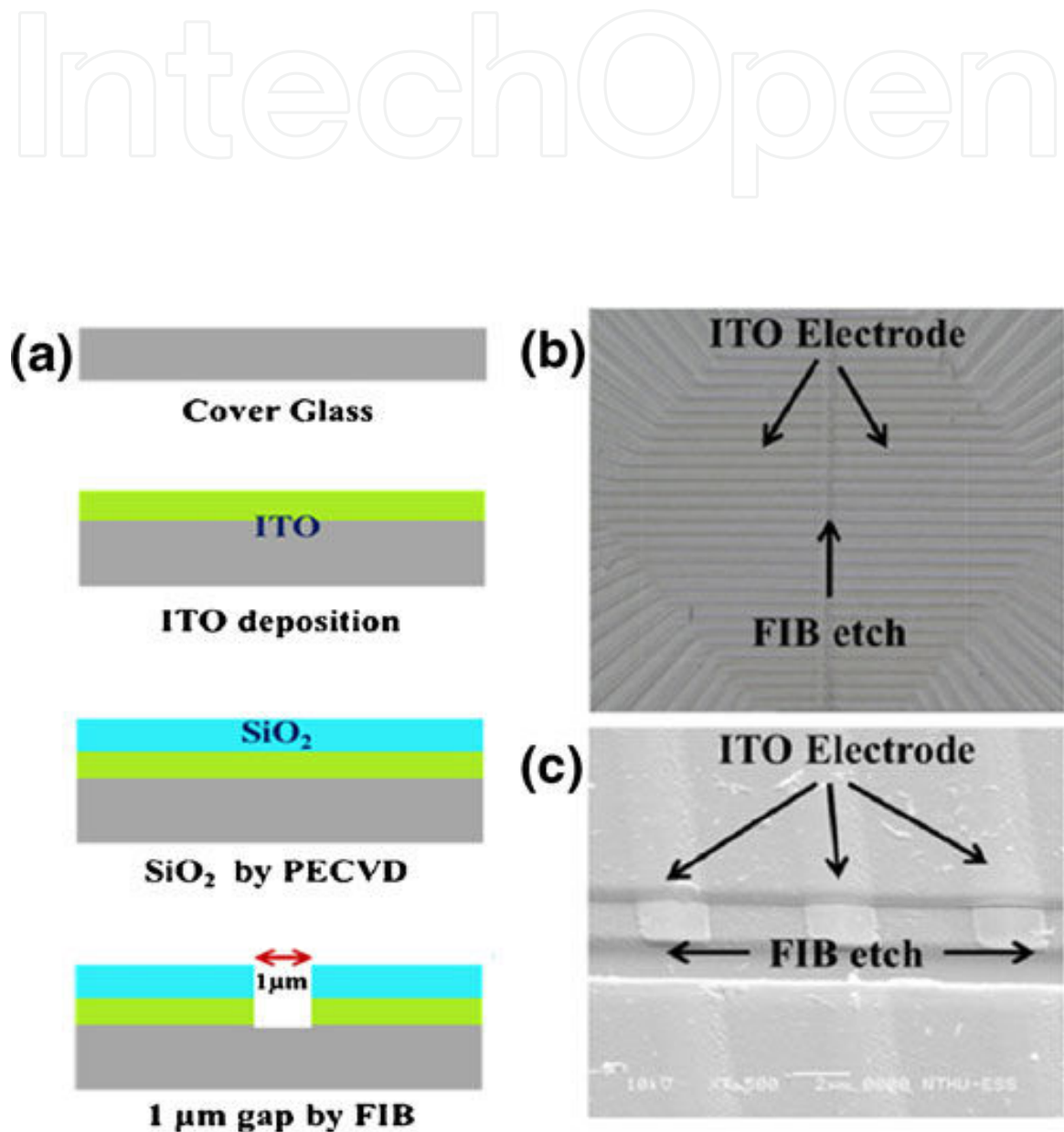


Figure 5. Fabrication process of ITO microelectrode based localized single cell electroporation chip. (a) Fabrication process step (b) Optical microscope image of patterned ITO microelectrodes. (c) SEM image of ITO microelectrodes with micro channel (FIB etch), Permission to reprint obtained from Springer [63].

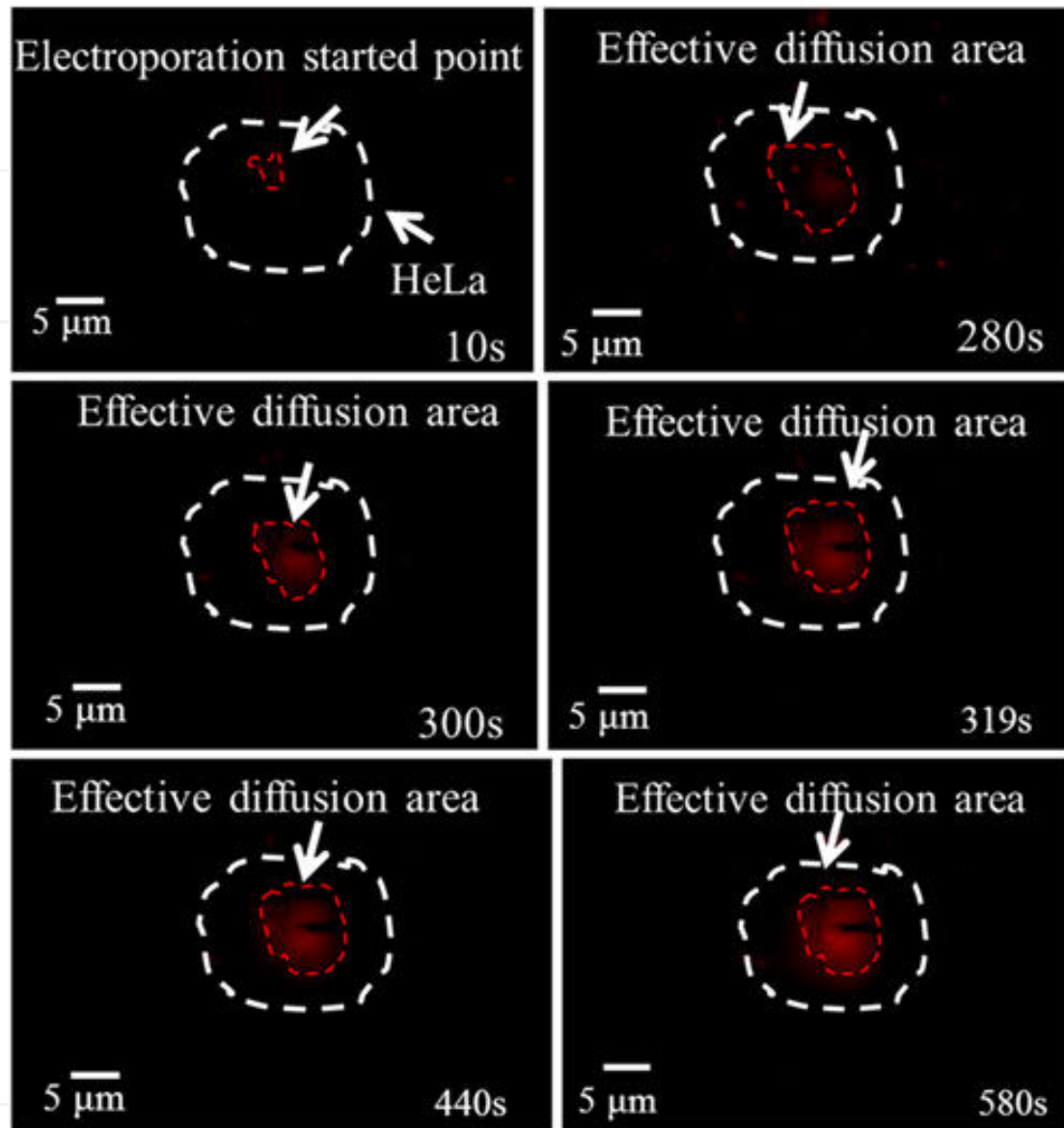


Figure 6. After application of 8Vpp 20 ms pulse, cell survival fluorescence image of HeLa cell at different time scale, Permission to reprint obtained from Springer [63].

According to the results, $0.93 \mu\text{m}$ electroporation regions were achieved successfully with 60% cell viability for 20 microsecond pulse. Fig.6. demonstrates the cell survival fluorescence image of HeLa cell at different time scale during LSCMEP process.

3.2. Pore formation on SCEP

In single cell electroporation technique, electroporation occurs in adherent cell and tissue. However single cell electroporation can be visualized for cell in suspension. In BEP, mostly the cells are in suspension as spheres, in which homogeneous electric field can be applied. But

for single cell electroporation, electric field is in inhomogeneous form, which targets on a particular cell without effecting neighboring cells. Generally cell membrane described in terms of fluid mosaic membrane model [64]. Due to application of an electric electric field, the formation of pores into the cell membrane depends upon field strength with low conductance, which is approximated as electrical capacitors with infinite resistance. The pore as liquid capacitor which converts to the electrical force associated with transmembrane potential U into an expanding pressure within the aqueous pore interior [65-68]. The pore creation energy ΔE can be calculated with pressure balance by removal of planar area πr^2 and creation of a cylindrical pore edge of length $2\pi r$, can be written as

$$\Delta E = 2\pi\gamma r - \pi r^2\Gamma \quad (5)$$

where surface energy approximately $\Gamma = 1 \times 10 \text{ J/m}^2$ and the edge energy approximately $\gamma = 1$ to $6 \times 10^{-11} \text{ J/m}$ [69-71]. Here γ is constant even it is a function of r [70, 72-73]. To expand the pore radius from zero radius to r can be written as

$$\Delta E(r) = 2\pi\gamma r - \pi r^2\Gamma + A/r^4 \quad (6)$$

The first term is energy related stressed pore edge with length $2\pi r$. The second term is energy to remove a circular flat lipid membrane having energy per unit area Γ and the third term is steric repulsion of the lipids with constant A . Fourth term arises when transmembrane potential V_m is nonzero, which is related to,

$$-0.5C_p V_m^2 r^2 \quad (7)$$

The transmembrane potential $\Delta\Psi_E$ in a uniform electric field E at a point M with time t can be written as

$$\Delta\psi_E(t) = \psi_{in} - \psi_{out} = -fg(\lambda)RE \cos\theta(M) \times (1 - \exp(-t/\tau)) \quad (8)$$

where f is the shape of the spheroidal cell [74] and τ is the charging time of the cell membrane, g depends upon the conductivities and R is the radius of the spherical cell. E is the field strength and $\theta(M)$ is the angle between normal to the membrane at the position M and direction of the field [55]. The exponential term can be ignored if the pulse length is longer than a few microseconds. Because induction time $\tau < 1\mu\text{s}$, the value f is generally $3/2$, which is for completely insulating membrane [75].

4. Bulk electroporation

4.1. Electric field effect on cell membrane

Biological systems are mainly heterogeneous from electrical point of view [76-77]. When a high electric field pulses is applied across the cell membrane, due to rapid polarization, cell membrane can deform mechanically (e.g., suspended vesicles and cells) and is allowed to redistribute ionic charges due to electrolyte conductivities and distributed capacitance. Initially every bilayer cell membrane structure is dielectric in nature. After application of electric field pulses, membrane conductivity can increase due to structural change of the cell membrane cause the formation of hydrophilic pores from initially formatted hydrophobic pores [78]. Generally the breakdown potential of lipid bilayer is 100-300 mv, which depends upon the lipid compositions [79]. If the pulse electric field (PEF) decreases, then breakdown voltage can increase [80-81].

To consider a cell as a sphere with a small volume of V and current is flow of charges. Both current and charges have relationship between them. If we consider the total current flow through small volume of cell V , then the current must be equal to the net flow of charges with in volume V or equal to the rate of decrease of charge with in volume or net flow of current into volume V must be accompanied by an increase of charge with in volume V . This is the principle of conservation of charge, which can be mathematically expressed as

$$I = \int_S J \cdot ds \quad (9)$$

$$= -\frac{\partial}{\partial t} \int_V \rho dV \quad (10)$$

Using divergence theorem, $\int_S A \cdot n ds = \int_V \nabla \cdot A dV$ then the equation can be written as

$$\int_V \nabla \cdot J dV = -\int_V \frac{\partial \rho}{\partial t} dV \quad (11)$$

where J is the current density and ρ is the volume charge density. Now we can write equation [11] as

$$\int_V \left(\nabla \cdot J + \frac{\partial \rho}{\partial t} \right) dV = 0 \quad (12)$$

Since equation [12] must be true irrespective of the volume, so we can write equation [12] as

$$\nabla \cdot J + \frac{\partial \rho}{\partial t} = 0 \quad (13)$$

This is equation of continuity, which is the principle of conservation of charge where steady current involve $\frac{\partial \rho}{\partial t} = 0$ and if charges are not generated into the cell during application of electric field pulses, then $\nabla \cdot J = 0$. Now electric field is the gradient of electric potential. So Maxwell equation becomes $\Delta^2 \psi = 0$, where Ψ denotes the electrical potential. If the conductivity of cytoplasm and external medium of the cell is higher than the cell membrane conductivity, then $\Delta \Psi$, the field induced transmembrane potential can be written as:

$$\Delta \psi = 1.5 a_{\text{cell}} E_e \cos \theta \quad (14)$$

where a_{cell} is the outer radius of the cell, E_e is the applied electric field strength and θ is angle between field line and normal to the point of interest in the membrane which can be either 0° or 180° [82-85]. Under the ideal experimental conditions like pulse width, electric field, number of pulses, removal of external electric field for resealing of the pore membrane, pulse duration and rearrangement of the membrane protein can be preserved the cell viability. If the membrane is not spherical, then equation [14] may not be right explanation. If we consider that the cell has ellipsoidal structure, then equation [14] will not be applicable. But for any practical purpose this equation can be used to evaluate the field induced transmembrane potential.

4.2. Reversible electroporation

When a strong external electric field applied across cell and tissue, then membrane conductance and permeability can increase significantly due to strong polarization of the cell membrane, as a result membrane can form nano scale defects (called nanopores). But when we switched off the external electric field, membrane can return from its conducting state to its normal state. This phenomenon is called reversible electric breakdown or reversible electroporation [86-87]. The reversible electroporation generally involves reversible electric breakdown (REB), which is generally a temporary high conducting state. This reversible electroporation influences both cell membrane as well as artificial planner bilayer lipid membrane. Reversible electroporation involve with rapid creation of many small pores, where membrane discharge occur before any critical pores can evolve from the small pores. To understand the method of electroporation of bilayer lipid membrane, it is necessary to use the method of voltage clamp [65,71,88] and charge relaxation [80,89] techniques, where for charge relaxation, kinetics of voltage decreases across the membrane after the application of short pulses (20 nsec to 10 μ sec). It was also fact that originally membrane breakdown can occur before the start of membrane discharge. From the charge relaxation method, it used to show

that, when membrane of oxidized cholesterol are rapidly ($\sim 500\text{nsec}$) charged (approximately 1 V), then membrane resistance reversibly decreased by almost nine orders of magnitude [80, 89]. By this way it was first observed that reversible breakdown of planar lipid bilayer membrane and the charged could not be exceeded beyond 1.2 V, even pulse amplitude was increased further. After first electroporation, it was able to recharge again. This same phenomenon was investigated later with azolectin bilayers modified UO_2^{2+} ions and the membranes of lecithin and cholesterol in the presence of alkaloid holoturin A [90-92]. The different types of behavior of planer oxidized cholesterol membrane are shown in table III [78,80,93].

Characteristic electrical behavior	Pulse magnitude
"Reversible electrical breakdown"(REB); membrane discharge to $U=0$	Largest
Incomplete REB(discharge halts at $U>0$)	Smaller
Rupture (mechanical); slow, sigmoidal electrical discharge	Still smaller
Membrane charging without dramatic behavior on U	Smallest

Table 3. Planar bilayer membrane electroporation. Permission to reprint obtained from Elsevier [78, 80, 93].

For voltage clamp method, the time resolution is 5-10 μs to monitor continuously charge at specific conductance of membrane from 10^{-8} to $10^{-1} \Omega^{-1}/\text{cm}^2$. Thus voltage clamp technique and charge relaxation technique are complement to each other [94].

4.3. Irreversible electroporation

In our earlier discussion of reversible electroporation, external electric field can permeabilize the cell membrane temporarily by which, the cell membrane can survive and the process known as "reversible electroporation" whereas, some of strong external electric field can cause the cell membrane to permanently permeabilize (membrane becomes weak effect on conductance), by which the cell can die and the process is refer to as "irreversible electroporation". This irreversible electroporation was observed in early 1754 due to discharge of static electrical generator of the skin [95-96]. The main phenomenon of irreversible breakdown was stochastic quantities by which mean life time of membrane can abruptly decreased with increased of voltage. The pores of the bilayer membrane can be hydrophilic or hydrophobic [65]. For hydrophobic cases, the pores can be formed by hydrocarbon lipid tails. Whereas the inner surface of the pores can be covered by polar tails. The hydrophobic pores which can fill by water are energetically unfavorable [66] and thus should be short-lived. The formation of the pores during reversible electroporation can exist for longer periods of time due to hydrophilic pores. The accumulation of pores during reversible electroporation is due to membrane containing lysolecithin, which can decrease the linear tension of hydrophilic pores [97-98]. The hydrophilic pores can cause the reversible and irreversible breakdown of lipid membrane. Also every electrical field can produce the thermal effect as familiar as Joule effect is disputed, where as certain electric field is undisputed, which can provide irreversible electroporation [95]. Irreversible electroporation can affect only the membrane of living cells and spares of

tissues scaffold. During irreversible electroporation, the membrane survives in two stages as (a) steady state current stage and (b) fluctuating current stage. The phenomena of irreversible electroporation can cause by charge pulse technique [80] in which membrane is charged at $U=0.1$ V (with pulse width 400 ns) and discharged was very slow. The large pulse of the same width, can charge the membrane towards 0.4 V, but after 300-400 μ s, charges can be decreased as a sigmoidal manner up to zero because of membrane rupture [78].

5. Applications

5.1. Bulk electroporation

From the last decade, the application of electroporation has been increasing rapidly. Nowadays, the electroporation technique can be applied in many way to deliver drugs, antibodies, oligonucleotides, proteins, RNA, DNA and plasmid in vivo for clinical, biotechnological and biomedical applications [42, 99-101]. Table 4 described details about gene transfer by electroporation technique with the variation of molecules/gene, targeted cells, different types of electric pulses [7, 102-117].

Ref.	Year	Authors	Recipient cells	Plasmid /gene	Pulsing $CD:E_0:\tau$	Results
7	1982	Neumann et al.	Mouse L tk ⁻ Fibroblast cells	pAGO with tk gene herpes virus(HAT select.)	CD:3x8 KV/CM;5 μ s 20°C, 10 min postincub., HBS (without Mg ²⁺)	Sharp optimum in field strength, incubation after pulse necessary, linear plasmid better than circular, 100col./10 ⁶ cells/ μ g DNA
102	1983	Shivarova et al.	Bacillus cereus protoplasts	pUB110 from B. thuringiensis(kn resistance)	CD: 3x 14 KV/cm; 5 μ sec40% PEG present, 20°C, 10 min incub.	Small objects, high electric field strength necessary, 10-fold increase in stable transformation.
103	1984	Falkner et al.	Mouse lymphoid cell lines	Plasmid with Ig κ gene	CD: 3x8 KV/cm; 5 μ sec 20°C, 10 min incub., DME medium + 20 mM MgCl ₂ (plastic cuvette)	Two to five copies of plasmid per genome integrated in transformed clones.
104	1984	Potter et al.	Mouse B and T lymphocytes and fibroblasts	Mouse and human Ig κ gene	Pulse: ISCO 494 power supply directly discharged through cuvette, no definite pulse parameters given, estimated: 320 V/cm; 17 msec, 0°C, 5 min preincub.,10 min postincub., PBS	Up to 300 transf./10 ⁶ cells, linear > supercoiled, low temperature favorable, few copy number (1-15) integrated, mitotic arrest by colcemid favorable

Ref.	Year	Authors	Recipient cells	Plasmid /gene	Pulsing CD:E ₀ :τ	Results
105	1985	Prochownik	Mouse M12 myeloma cells (transient expression)	Plasmid carrying CAT gene	Pulse: ISCO power supply (acc. To Potter) 0°C. PBS, Cuvette	Successfully transformation, CAT actively after 48 hr observed
106	1985	Zerbib et al.	Hamster CHO tk cells in suspension or monolayer	pAGO with tk gene from herpes virus	CD: 3x6 KV/cm; τ= 10 μsec (20°C, HBS) Square pulse: 3x1.5 KV/cm; 50 μsec(low ionic strength)	150 transf./10 ⁶ cells/μg DNA, threshold: >4 KV/cm 70 transf./10 ⁶ cells/μg DNA, 4 plasmids/transformed cell in monolayer
107	1986	Weir and Leder	Mouse B and pre-B cell lines	Functionally rearranged VκII gene	Pulse: ISCO power supply(acc.to potter)	Gene successfully introduced both transiently and permanently by electroporation
108	1986	Yancopoulos et al.	Tk- derivative of 38B9 A-MuLV-transformed pre-B cell line	T cell receptor variable region gene segments on special plasmid construct	Pulse: ISCO power supply (acc. To potter) 0°C, PBS	Linearized plasmid successfully transfected
109	1987	Boston et al.	Daucus carota protoplasts (W001C)	pCATTi, pCATZ2(supercoiled)	Pulse: ISCO power supply(acc. To Potter) Preincub. 5 min 45°C + 5 min on ice with PEG; postincub. 10 min at RT; PCM; Cuvette with AL foil electrodes (acc. To Potter)	2.0 KV setting results in 40% intact viable cells and maximum CAT activity; presence of PEG is necessary (no sharp optimum related to concentration); no effect of heat-shock treatment; linear DNA and presence of carrier DNA decreases CAT expression
110	1992	Puchalski et al.	COS-M6 Monkey kidney cells	Glutathione Stransferase (GST) gene.	(University of Wisconsin Medical Electronics, Madison, WI) (4 0C, 1-cm-wide aluminum electrodes, and 1-cm gap)	With lipofection, only 1% of the surviving cells expressed recombinant GST, although 2.540% of the cells that survived transfection formed colonies.
111	1996	Heller et al.	Rat liver tissue	Psv-β-galactosidase. The BamHI-XhoI fragment carrying the Luc coding sequence from pGEM- Luc was	DC generator, (T820,BTX,Inc.;San Diego, CA) and a switch box (195-7460; BTX,Inc.;San Diego, CA). Field strength 1000V/cm, 6 pulses, duration=99 μs	Gene transfer by electroporation in Vivo may avoid anatomical constraints and low transfection efficiency.

Ref.	Year	Authors	Recipient cells	Plasmid /gene	Pulsing CD:E ₀ :τ	Results
				cloned into pRc/ CMV plasmid		
112	2000	Dujardin et al.	Rat keratinocytes	pEGFP-N1 with CMV promoter	Cytopulse PA-4000 (Cyto Pulse Sciences, Inc., Maryland, USA), 10 pulses of 1000V and 100μs duration	A localized expression of GFP was observed for at least 7 days in the epidermis. Skin viability was not compromised by electroporation
113	2004	Yamauchi et al.	Human embryonic kidney cells, HEK293	pEGFP-C1 and pDsRed-C1	ElectroSquare-Porator T820, BTX, San Diego, 100v/cm, 10ms	Efficient to transfer multiple genes, in parallel, into cultured mammalian cells for high-throughput reverse genetics research.
114	2006	Yamaoka et al.	Male Japanese white rabbits (2.5–3.0 kg body wt; Kyudo, Tosu, Saga, Japan)	Plasmid DNA	Electric pulse generator (model CUY 201 BTX) P _{on} =5ms, P _{off} =95 ms, No of pulse 10	Optimal gene transfer efficiency in the in situ jugular veins of rabbits, and transgene expression was observed primarily in endothelial cells.
115	2008	Takei et al.	MKN-1, PC-3, F12	VEGF Si RNA	Square Electro Porator (CUY21; Nepagene).	The delivery efficiency correlated to the electric current. The electric current correlated to the microvascular density and vascular endothelial growth factor (VEGF) expression and exhibited a threshold that guaranteed efficient delivery.
116	2010	Kaufman et al.	A549 cells (ATCC, Manassas, VA,USA) a human lung adenocarcinoma cell line	Plasmid DNA	BTX ECM 830 , Electroporation coupled with a Petri-Pulser PP35–2P electrode (Harvard Apparatus, Holliston, MA, USA) using a single 10 ms 160 V square wave	cyclic stretching of the murine lung using ventilation immediately after endotracheal administration and transthoracic electroporation of plasmid DNA increases exogenous gene expression up to fourfold in mice that were not ventilated after plasmid administrationand transfection by electroporation in vivo

Ref.	Year	Authors	Recipient cells	Plasmid /gene	Pulsing CD:E ₀ :τ	Results
117	2011	Geng et al.	CHO-K1 cells (ATCC)	pEGFP-C1 plasmid(Clontec), cat.no.6084-1	DC power supply (ps350; Stanford research system) with alligator chip leads.	Enable to continuous transfection of cells by flow through electroporation in PDMS fluidic channel with alternating wide and narrow section

Table 4. Modified table of gene delivery by electroporation technique. Permission to reprint obtained from Springer book series [94]

In vivo electroporation is a special kind of interest for all researchers because it is nonviral gene delivery with low cost, safety and ease of realization. Recently nucleic acid based gene transfer has been investigated successfully which could be helpful for more clinical trials in human body [118]. This technique can be applied for food industry [119]. For cancer treatment, electrochemotherapy has emerged and this therapy successfully used for clinical trials [42,99, 120-123]. The different types of application of electroporation has mention below.

5.1.1. Electroporation for DNA transfer

The first reversible electroporation with DNA electrotransfer has been investigated in 1982 [7]. After application of an external electric pulse, cell membrane can permeabilize and DNA will move towards the cell membrane by electrophoretic force and finally it can enter into cytoplasm of the cell. It has been reported that, small molecules can diffuse into the cell before membrane reseals but DNA cannot transfer inside the cell, if DNA is added immediately after the pulse applications [124]. For better DNA electrotransfer, electric field pulses are important. The electroporating pulse can stimulate a vascular lock (i.e., a transient hypoperfusion) as well as affects the blood circulation to the electropulsed tissues, caused by histamine dependent physiological reaction [125]. For better electrotransfer, electric field pulses have three steps which includes,

- Molecules can increase the electrophoretic displacement of the charged molecules due to application of electric filed pulses
- Cell membrane can enhance the permeabilization
- Exposed tissues can stimulate the vascular lock [126].

Moreover to deliver the electric pulse for DNA is electrotransfer, just short or high amplitude pulse (e.g. six pulses, 100μs and 1.4 kV cm⁻¹) required to deliver small molecules [127]. For better electrophoretic effect, longer pulses with low amplitude (e.g. eight pulses, 20ms, 200 kV cm⁻¹) are required to increase the transfection rates [124]. However short, high amplitude pulse can follow the long low amplitude pulse. From these two pulses, high amplitude pulse can permeabilize the cell membrane, then long duration low voltage pulse can play the role to drive the DNA into destabilized membrane of the cell [128]. The transfection threshold values are the same for cell electroporeabilization [39]. The transfection efficiency maintains the following equation as mentioned below

$$\text{Transfection Efficiency} = KNT^{2.3}(1 - E_p/E)f(\text{ADN}) \quad (15)$$

where plasmid concentration $f(\text{AND})$ is complex and high level of plasmid is toxic [129] and K is constant. As results, for DNA electrotransfer, the pulse effect (Field strength, short high amplitude pulse, long low amplitude pulse) are very important and which is the major parameters for efficient gene expression into cell and tissues.

5.1.2. Electrotransfer for clinical developments

The electroporation technique has been used widely for transfection of plasmid in vitro and in vivo. Recently this technique has been used for application of DNA vaccine and gene therapies for clinical trials. Electroporation technology are not only the basis for human studies, but also it influence veterinary medical for animals, which can make the bridge between human and animal studies [130-134]. In this section, different clinical trials with electroporation techniques are mentioned below.

5.1.2.1. DNA vaccine

DNA vaccines have excellent potential as preventive or therapeutic agents against cancers and infectious diseases. For a successful DNA delivery into the cell or tissues, DNA must need to subsequently achieve gene expression of the encoded protein at desired level or for the desired duration of time. In vivo electroporation, which can enhance the delivery efficiency and the cellular uptake of an agent by 1,000 times and it can increase the levels of gene expression (i.e. production of the coded protein) by 100 times or more compared to plasmid DNA delivered without other delivery enhancements. DNA vaccination by electroporation technique has been developed in last several years [134-140]. For DNA vaccination by electroporation, preclinical trials for mouse studies revealed that xenogeneic DNA vaccination with gene encoding tyrosinase family membrane can induced antibody and cytotoxic T cell responses resulted in tumor rejection [141-142]. DNA vaccine, p.DOM-PSMA encoded a domain (DOM) of fragment C of tetanus toxin to induced CD⁴⁺ T cell helps to fuse to a tumor-derived epitope from prostate-specific membrane antigen (PSMA) for use in HLA-A2⁺ patients with recurrent prostate cancer [139]. For this open level phase I/II work, DNA was delivered by intracellular injection followed by electroporation with five patients per dose level. Plasmid DNA vaccination using electroporation able to elicited robust humoral and CD⁸⁺ T-cell immune responses, while limited invasiveness of delivery [140]. DNA delivered method which included phase I clinical trial investigated safety and immunogenicity of xenogenic tyrosinase DNA vaccine, administered intramuscularly with electroporation to patient with stage IIB, IIC,III or IV melanoma (Clinical Trials. Gov ID NCT00471133). Electroporation with xenogeneic tyrosinase DNA vaccine can increase the human response and anti-tumor effects compared to the vaccine alone [143].

5.1.2.2. RNA electroporation

The RNA transfer by electroporation technique has been increases continuously recently. RNA can enter inside the cell alone or be used for transfection of dendritic cells, showing several advantages as a vaccine including feasibility, applicability, safeness, and effectiveness, when it comes to the generation of immune responses. In vitro experiment, dendritic cells (DC) pulsed with whole tumor RNA or RNA encoding specific antigen like TAAs induced the generation of specific positive cytotoxic T lymphocytes (CTLs) into the cell [144]. Electroporated monocyte-derived DCs with whole RNA from LP-1, U266 cell lines and induced specific CTLs that lysed LP-1 and U266 myeloma cells [145]. The RNA delivery into the dendritic cells (DCs) can be achieved by using electroporation of dendritic cells in the presence of RNA [146]. In vivo study was performed by TriMix dendritic cells (DCs) [146]. The transfection of Dendritic cells (DCs) were performed by electroporation technique with mRNA encoding CD40L, CD70, and a constitutively activated TLR4 as enhancing elements. Additionally the cells were electroporated with either Mage-A3, Mage-C2, tyrosinase, or gp100 mRNA. The intra-dermal injections at four different sites of 1.25×10^7 TriMix DCs per antigen were provided to two melanoma patient in four times per week. While antigen-specific CD8 T cell responses was detected in both patients after finished treatment, but no data published for this trials [147].

5.1.2.3. HIV vaccine

Since early 1980s, for causative agent of acquired immundeficiency syndrome (AIDS), an effective vaccine has been continuously tried to find to recover AIDS. Nowadays, the HIV vaccine is introduced by electroporation technique. In vivo experiment on mice, electroporation technique can amplify cellular and humoral immune response to a HIV type 1 EnvDNA vaccine, capable of tenfold reduction in vaccine dose and resulting in an increased recruitment of inflammatory cells [148]. The plasmid HIV vaccine, ADVAX env/gag+ADVAX pol/nef-tat (ADVAX), ongoing to examine in phase I trials for uninfected adults (Clinical Trials.gov Identifier: NCT00249106) combination with electroporation as a potential protective vaccine against HIV (Clinical Trials.gov Identifier:NCT00545987). Now more recent study was going on for safety and immunogenicity of an IM injection of two dose of ADVAX using Electroporation TriGrid Delivery Systems (Inchor Medical Systems, Clinical Tials.gov Identifier: NCT00545987)[143].

5.1.2.4. Cancer treatment

Electroporation technique for cancertreatment (Electrochemotherapy) have been increasing rapidly after first reported of clinical use of electroporation [122]. Electrochemotherapy can combine electroporation and chemotherapeutic agents [149-150]. The treatment of cutaneous and subcutaneous tumors has reached for clinical trials using bleomycin or cisplatin by antitumor electrochemotherapy process [120,151-157]. For localized therapy to avoid systematic drug delivery, bleomycin can be injected directly into the tumors by using electrochemotherapy process. Bleomycin is hydrophilic in nature, which can be internalized in limited amounts only in normal condition [158]. The use of bleomycin for electroporation process can directly enter into cytosol and its cytotoxicity can be increased up to

300-5000 fold [159-161]. Different types of cancer can be treated by electroporation technique. The prostate cancer is one of the most common cancer, which is increasing day to day. For this cancer prostate specific antigen (PSA), targeted to the prostate cancer cell for immunotherapeutic approach. The phase I clinical trials with PSA DNA vaccine for human prostate cancer is safe and which can include cellular and humoral immune responses against PSA protein [162-163]. The PSA-DNA vaccine has been investigated by electroporation technique [164-165]. Electroporation treated with CD^{4+} , CD^{8+} cells and antibodies were detected in patient successfully with safe and tolerated mode. Electrochemotherapy has also been investigated for treatment of human colorectal cell line and liver tumours [166-167]. The local treatment of electrochemotherapy (ECT) with master cell tumours of Dog has been experimented in where size of the tumors was 5.2 cm^3 and 2.9 cm^3 treated by surgery and ECT. The ECT treatment was easy, effective and safe local treatment for master cell tumors of Dogs [168]. Recently, electrochemotherapy has been developed in more advancement for treatment of internal tumors using surgical procedures, endoscopic routes or percutaneous approaches to gain access to the treatment area [169-170].

5.1.3. Skin electroporation

Molecule or DNA vaccine can transport across targeted tissue of human skin is of great interest for transdermal drug delivery and non-invasive chemical sensing. Skin has capability to produce therapeutic molecules, which not only acts as a systematically or locally, but it can create immunological response, when antigen presenting cells will be targeted. The skin containing antigen presenting cells like dendritic cells, langerhans cells, and mononuclear cells. The gene delivery through the skin electroporation is feasible, efficient and comparable to other tissues [171]. The first skin electroporation study was observed in Newborn mice which transfected with a plasmid coding for a neomycin resistance gene [172]. The transfection efficiency can depend upon the age of the skin, where the higher transfection efficiency can be achieve for younger mice compare to the older mice [173]. Skin electroporation, only clinical study has been reported belonging to metastatic melanoma [118]. To date, the skin electroporation has been studied broadly for animal infectious diseases. For most cases Hapatitis B has been investigated for animals through skin electroporation [137,174-176]. Also experiments have been performed vaccine against HIV, smallpox, malaria [177-179].

5.2. Single cell electroporation

By using single cell electroporation technique, it is possible to deliver the molecules such as drugs, DNA, RNA, peptide, nucleic acid into the cell membrane in vivo and vitro for single cell analysis. The plasmid delivery inside the cell membrane with high efficiency in adherent cells and tissues has been studied in vitro [180-184] and in vivo [52,183-186]. Fig.7. show the different applications of single cell electroporation, where membrane can permeabilized to transport protein, small and large molecules inside the single cell.

When two single cells are closed to each other, then cell fusion can occur. Due to high electric field strength, which exceeds the critical value of cell membrane, irreversible electroporation can occur, resulting in cell membrane rupture and finally cell death. This electroporation

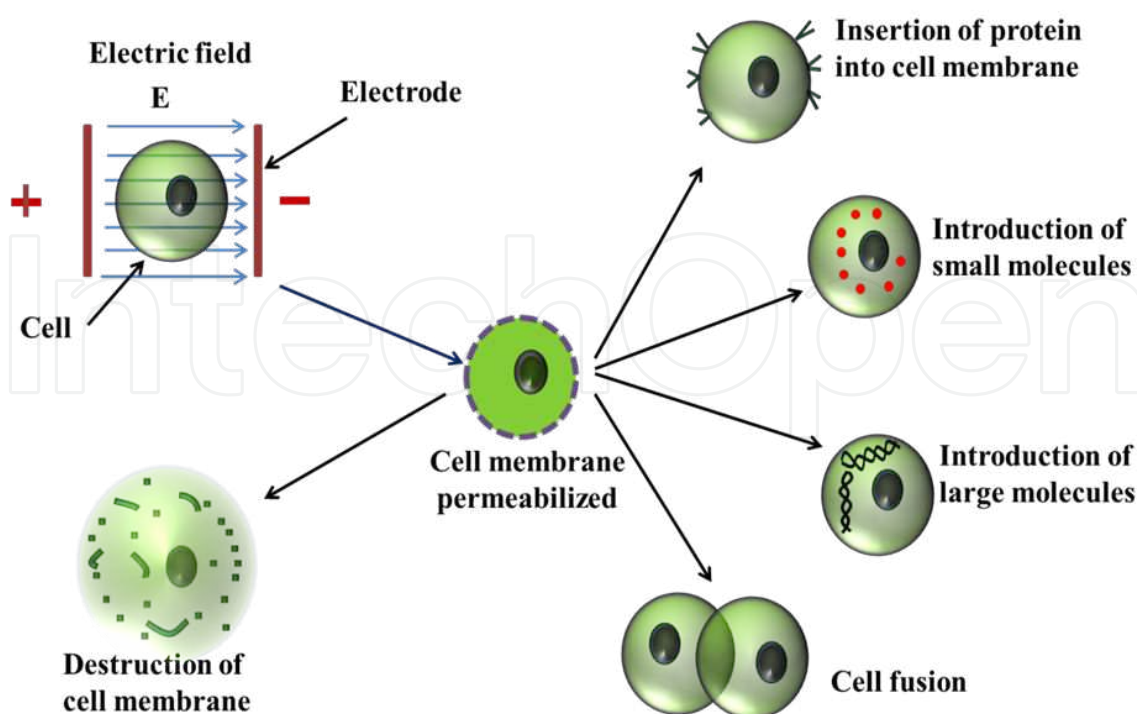


Figure 7. Different application of single cell electroporation. When external applied electric field reaches to the threshold values of the cell membrane, then cell membrane can permeabilized to deliver protein, small and large molecules inside the cell. If two single cells are close to each other, then cell fusion can occur. To apply an intense electric field, which exceeds certain critical value, irreversible electroporation can occur resulting cell membrane rupture and finally cell death. Figure has redrawn from reference. Figure has redrawn with reprint permission obtained from Springer [187].

successfully investigated cell to cell intracellular biochemical variation from millions of cells. However this technique needs a lot of research in the future for more improvement because this technology is in underdeveloped stage. For intracellular targeting, single cell electroporation based systems can be developed for genomic characterization, where a tagged antisense oligonucleotide is introduced to block expression and proteins can be profile by tagged markers [188]. To reduce the electrode size in nanoscale label, selective manipulation of single organelles within a cell can be possible. Thus the localized single cell membrane electroporation (LSCMEP) concept has come in frontier research in last several years [61-63]. This technique can control spatial-temporal process successfully and its have ability to monitor the transfection results in real time situation. To reduce the electrode size in nanoscale label, effective electroporation region should be reduce. As results transfection efficiency should be increase with high cell viability. Florescent markers with single cell electroporation permits direct visualization of cell morphology, cell growth, and intracellular events over timescales ranging from seconds to days. Fluorescent dye or plasmid DNA can enter the neurons with the intact brain of albino *Xenopus* tadpoles [189]. Individual neurons can be electroporated by this technique in vivo and in vitro including mature and fully differentiated neurons. The transfection of neurons into brain slices and in intact brains of living animals is possible to use

this technique. The neuron transfection achievable up to 1 mm dip into a tissue and electrophysiological recording of individual neuron was possible by use of SCEP [190]

6. Conclusions

This chapter described the detailed concepts about bulk electroporation (BEP) as well as single cell electroporation (SCEP) techniques. In both electroporation technique different types of exogenous molecules such as DNA, RNA, proteins, anticancer drugs, ions, oligonucleotides can be transported into the cell cytosol in vivo or in vitro. For bulk electroporation, the clinical development of DNA based vaccine and immunotherapeutic delivery is progressing. As a nonviral gene transfer, this technique is important for clinical gene transfer regarding efficacy and safety issue compared to other gene transfer techniques. The new technique such as single cell electroporation (SCEP) makes the possibility to judge cell to cell variations with their organelles and intracellular biochemical effect. The development of SCEP technique at clinical level and for biomedical application needs more research in the future. In SCEP, there still lacks the are lack of understanding of theory and molecular delivery inside the cell. But this technique can initiate new root of research, such as single cell biophysics and drug delivery inside single cell. To reduce the electrode gap at nanoscale level, it is possible to do localized single cell membrane electroporation (LSCMEP) by which selective specific single cell organelles can be manipulated with higher transfection rate and high cell viability.

Acknowledgements

The authors greatly appreciate the financial support from National Science Council (NSC) of Taiwan ROC through National Nanotechnology and Nanoscience Program under Contract no. NSC- 98-2120-M-007-003 and NSC 99-2120-M-007-009.

Author details

Tuhin Subhra Santra¹, Pen-Cheng Wang² and Fang Gang Tseng^{1,2,3}

1 Institute of NanoEngineering and Microsystems (NEMS), National Tsing Hua University, Hsinchu, Taiwan

2 Department of Engineering and System Science (ESS), National Tsing Hua University, Hsinchu, Taiwan

3 Division of Mechanics, Research Center for Applied Sciences, Academia Sinica, Taipei, Taiwan

References

- [1] U.Zimmermann, G.Pilwat, F.Friemann, Dielectric breakdown of cell membranes, *Biophys.J.* 1974;14: 881-899.
- [2] H.A.Pohl, J.S.Crane, Dielectrophoresis of cells, *Biophys.J* 1971;11: 711-727.
- [3] A.J.H.Sale, W.A.Hamilton, Effect of high electric fields on microorganisms II. lysis of erythrocytes and protoplasts, *Biochem. Biophys. Acta* 1968;163: 37-43.
- [4] J.M.Crowley, Electrical breakdown of bimolecular lipid membranes as an electromechanical instability, *Biophys.J.* 1973;13: 711-714.
- [5] H.G.L.Coster, A quantitative analysis of the voltage-current relationship of fixed charge membranes and the associated property of "punch through", *Biophys.J.* 1965;5: 669-586.
- [6] A.J.H. Sale, W.A.Hamilton, Effects of high electric fields on microorganisms I. killing of bacteria and yeasts, *Biochem. Biophys. Acta* 1967;148: 781-788.
- [7] E.Neumann, M.S-Ridder, Y.Wang, P.H.Hofschneider, Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO.J.* 1982;1: 841-845.
- [8] J.C.Weaver, Electroporation of cell and tissues, *IEEE transactions on plasma science* 2000; 28(1): 24-33.
- [9] U.Pliquett, J.C.Weaver, Electroporation of human skin: Simultaneous measurement of changes in the transport of two fluorescent molecules and in the passive electrical properties, *Bioelectrochemistry and Bioenergetics* 1996;39(1): 1-12
- [10] K.K.Ewert, A.Ahmed, N.F.Bouxsein, H.M.Evans, C.R.Safinya, Non-viral gene delivery with cationic liposome-DNA complexes, *Methods Mol. Biol.* 2008;433:159-170.
- [11] D.H.Fuller, P.Loudon, C.Schmaljohn, Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases, *Methods* 2006;40:86-97
- [12] S.Ohta, K.Suzuki, Y.Ogino, S.Miyagawa, A.Murashima, D.Matsumaru, G.Yamada, Gene transduction by sonoporation. *Dev Growth Differ* 200; 50(6):517-520.
- [13] J.A.O'Brien, S.C.R.Lummis, Biolistic transfection of neuronal cultures using a hand-held gene gun, *Nature Protoc.* 2006;1:977-981.
- [14] M.R.Capecci, High efficiency transformation by direct microinjection of DNA into cultured mammalian cells, *Cell* 1980; 22: 479-488.
- [15] A.Naga, M.Gertsenstein, K.Vintersten, R.Behringer, *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Laboratory, 2003)
- [16] D.J.Wells, Gene therapy progress and prospects: electroporation and other physical methods, *Gene Ther.* 2004; 11:1363-1369.

- [17] G.L.Prasanna, T.Panda, Electroporation: basic principles, practical considerations and application in molecular biology, *Bioprocess Engineering* 1997;16: 261-264.
- [18] E.M.Miller, J.A.Nickoloff, *Escherichia coli* electrotransformation. In: J.A.Nickoloff, editor. *electroporation protocols for microorganisms*. Totowa, New Jersey: Humana Press. 1995; 105-114.
- [19] H.L.Withers, Direct plasmid transfer between bacterial species and electrocuring. In: J.A.Nickoloff, editor. *Electroporation protocols for microorganisms*. Totowa, New Jersey: Humana Press. 1995; 47-54.
- [20] T.Tryfona, M.T.Bustard, Enhancement of biomolecule transport by electroporation: A review of theory and practical application to transformation of *Corynebacterium*. *Bio-technol.and Bioengg* 2006;93(3): 413-423.
- [21] J.A.Nickoloff (edited), *Methods in molecular Biology, Animal cell electroporation and electrofusion protocols*, Humana press Inc. Totowa, NJ, Vol 48(1995) ISBN-10 / ASIN: 089603304X, ISBN-13 / EAN: 9780896033047.
- [22] J.Teissie, N.Eynard, B.Gabriel, M.Rols, Electroporabilization of cell membranes. *Adv Drug Deliv Rev* 1999;35: 3-19.
- [23] M.Pavlin, N.Pavselj, D.Miklavcic, Dependence of induced transmembrane potential on cell density, arrangements, and cell position inside the cell system. *IEEE Trans Biomed Eng* 2002;49: 605-612.
- [24] E.T.Jordan, M.Collins, J.Terefe, L.Ugozzoli, T.Rubio, J. *Biomolecular Techniques* 2008;19: 328-334.
- [25] J.A.Sunders, C.H.Lin, B.H.Hou, J.Cheng, N.Tsengwa, J. JLin, C.R.Smith, M.S.McIntosh, S.V.Wert, *Molecular biotechnology* 1995;3: 181-190.
- [26] K.Vafai (Editor), *Porous media, Applications in biological systems and biotechnology*, CRC press, Taylor and Francis group, Boca Raton, London, New York. 2011
- [27] R.Lanza, R.Langer, J.P.Vacanti, *Principle of tissue engineering*, second edition, academic press, London UK, 2002.
- [28] O.C.Tuncay, D.Ho, M.K.Barker, Oxygentension regulates osteoblast function. *American Journal of orthodontics and dentofacial orthopedics* 1994;105: 457-463.
- [29] T.Arnett, D.Gibbons, J.Utting, I.Orriss, A.Hoebertz, M.Rosendaal, S.Mejhji, Hypoxia is a major stimulator of osteoclast formation and bone resorption, *Journal of Cellular Physiology* 2003;196: 2-8.
- [30] M.Muraji, W.Tatebe, H.Berg, The influence of extracellular alkali and alkaline earth ions on electroporabilization of *Saccharomyces cerevisiae*. *Bioelectrochem Bioenergetics* 1998;46: 293-295.

- [31] W.Y.Gu, W.M.Lai, V.C.Mow, A mixture theory for charged hydrated soft tissues containing multi-electrolytes: passive transport and swelling behavior, *Journal of Biomechanical Engineering* 1998;120: 169-180.
- [32] A.Maton, J.Hopkins, S.Johnson, D.LaHart, M.Q.Warner, J.D.Wright, *Cells buildings block of the life*, Prentice Hall, Upper Saddle River, NJ, 1997.
- [33] J.M.Huyghe, J.D.Janssen, Quadriphasic mechanics of swelling incompressible porous media, *International Journal of Engineering Science* 1997;35: 793-802.
- [34] T.Ohshima, M.Sato, Bacterial sterilization and intracellular protein release by pulsed electric field. *Adv. Biochem Eng Biotechnol* 2004;90: 113-133.
- [35] [35]K.Kinosita, T.Y.Tsong, Formation and resealing of pores of controlled sizes in human erythrocyte membrane, *Nature* 1977b;286: 438-441.
- [36] B.J.Wards, D.M.Collins, Electroporation at elevated temperatures substantially improved transformation efficiency of slow growing mycobacteria, *FEMS Microbiol Lett* 1996;145: 101-105.
- [37] M.P.Rols, C.Delteil, G.Serin,, J.Teissie, Temperature effects on electrotransfection of mammalian cells, *Nucleic Acids Res* 1994;22: 540.
- [38] S.Fielder, R.Wirth, Transformation of bacteria with plasmid DNA by electroporation, *Analyt Biochem* 1988;170: 38-44.
- [39] H.Wolf, M.P.Rols, E.Boldt, E.Neumann, J.Teissie, Control by pulse parameters of electric field-mediated gene transfer in mammalian cells, *Biophys J* 1994;66(2): 524-531.
- [40] D.J.Winterbourne, S.Thomas, J.Hermon-Taylor, I.Hussain, A.P.Johnstone, Electric shock-mediated transfection of cells. Characterization and optimization of electrical parameters, *Biochem J* 1988;251: 135-160.
- [41] E.Neumann, S.Kakorin, I.Tsoneva, B.Nikolova, T.Tomov, Calcium –mediated DNA adsorption to yeast cells and kinetics of cell transformation by electroporation, *Biophys J* 1996;71: 868-877.
- [42] J.Gehl, Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research, *Acta Physiol Scand* 2003;177: 437-447.
- [43] E.Neumann, B.Rosenheck, Permibility induced by electric impulsions in vesicular membranes, *J.Membr.Biol* 1972;10; 279-290.
- [44] K.H.Schoenbach, S.J.Beebe, E.S.Buescher, Intracellular effect of ultrashort electrical pulses, *Bioelectromagnetics* 2001;22: 440-448.
- [45] B.Gabriel, J.Teissie, Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electroporemeabilized cell membrane, *Biophys J* 1997;73: 2630-2637.

- [46] J. A. Lundqvist, F. Sahlin, M. A. Aberg, A. Strimberg, P. S. Eriksson and O. Orwar, Altering the biochemical state of individual cultured cells and organelles with ultra-microelectrodes, *Proc. Natl. Acad. Sci* 1998; 95: 10356–10360.
- [47] E. Takle, R. D. Astumian, P. B. Chock, Electro-permeabilization of cell membranes: effect of the resting membrane potential, *Biochem Biophys Res Commun* 1990; 172: 282–287.
- [48] F. J. Chang, T. S. Reese, Change in membrane structure induced by electroporation as revealed by rapid-freezing electron microscopy, *Biophys J* 1990; 58: 1–12.
- [49] S. Y. Ho, G. S. Mittal, Electroporation of cell membrane: A review. *Crit Rev Biotechnol* 1996; 16(4): 349–362.
- [50] J. Teissie, M. Golzio, M. P. Rols, Mechanisms of cell membrane electroporation: A minireview of our present (lack of ?) knowledge, *Biochimica et Biophysica Acta* 2005; 1724: 270–280.
- [51] D. Ovcharenko, R. Jarvis, S. Hunicke-Smith, K. Kelnar, D. Brown, High throughput RNAi screening in vitro: From cell lines to primary cells, *RNA* 2005; 11: 985–993.
- [52] J. E. Bestman, R. C. Ewald, S.-L. Chiu, H. T. Cline, In vivo single cell electroporation for transfer of DNA and macromolecules, *Nature Protocols* 2006; 1(3): 1268–1272.
- [53] M. Khine, A. Lau, C. I. Zanetti, J. Seo, L. P. Lee, A single cell electroporation chip, *Lab on a chip* 2005; 5: 38–43.
- [54] D. C. Chang, B. M. Chassy, J. A. Saunders, A. E. Sowers, *Guide to Electroporation and Electrofusion*, Academic Press, Inc., San Diego, 1992.
- [55] M. Wang, O. Orwar, J. Olofsson, S. G. Weber, Single cell electroporation, *Anal Bioanal Chem* 2010; 397: 3235–3248.
- [56] F. Ryttsen, C. Farre, C. Brennan, S. G. Weber, K. Nolkranz, K. Jardmark, D. T. Chiu, O. Orwar, Characterization of single cell electroporation by using Patch-Clamp and Fluorescence microscopy, *Biophysical Journal* 2000; 79: 1993–2001.
- [57] K. Nolkranz, C. Farre, A. Brederlau, R. I. D. Karlsson, C. Brennan, P. S. Eriksson, S. G. Weber, M. Sandberg, O. Orwar, Electroporation of single cells and tissues with an electrolyte-filled capillary, *Anal Chem* 2001; 73: 4469–4477.
- [58] K. Haas, W. C. Sin, A. Javaherian, Z. Li and H. T. Cline, Single cell electroporation for gene transfer in Vivo, *Neuron* 2001; 29: 583–591.
- [59] Y. Huang and B. Rubinsky, Microfabricated electroporation chip for single cell membrane permeabilization, *Sens. Actuators A* 2001; 89: 242–249.
- [60] K. Nolkranz, C. Farre, K. J. Hurtig, P. Rylander and O. Orwar, Functional screening of intracellular proteins in single cells and in patterned cell arrays using electroporation, *Anal. Chem* 2002; 74: 4300–4305.

- [61] D.Nawarathna, K.Unal, H.K.Wickramasinghe, Localized electroporation and molecular delivery into single living cells by atomic force microscopy, *Appl. Phys. Lett* 2008;93: 15311-15313.
- [62] P.E.Boukany, A.Morss, W-C Liao, B.Henslee, H.Jung, X.Zhang, B.Yu, X.Wang, Y.Wu, L.Li, K.Gao, X.Hu, X.Zhao, O.Hemminger, W.Lu, G.P.Lafyatis, L.J.Lee, *Nature Nanotechnology* 2011;6: 747-754.
- [63] S-C. Chen, T.S.Santra, C-J Chang, T-J Chen, P-C Wang, F-G Tseng, *Biomedical Microdevices*, DOI 10.1007/s10544-012-9660-9.
- [64] S.J.Singer, G.L.Nicolson, The fluid mosaic model of the structure of cell membranes, *Science* 1972;175: 720-731.
- [65] I.G.Abidor, V.B.Arakelyan, L.V.Chernomordik, Y.A.Chizmadzhev, V.F.Pastushenko, M.R.Tarasevich, Electric breakdown of bilayer lipid membrane.I. The main experimental facts and their qualitative discussions, *Bioelectrochem.Bioenerg* 1979;6: 37-52.
- [66] J.C.Weaver,R.A.Mintzer, Decreased bilayer stability due to transmembrane potentials, *Phys. Lett* 1981;86A: 57-59.
- [67] I.P.Sugar, The effects of external fields on the structure of lipid bilayers, *J.Physiol. Paris* 1981;77: 103-1042.
- [68] K.T.Powell, J.C.Weaver, Transient aqueous pores in bilayer membranes: A statistical theory, *Bioelectrochem. Bioenerg* 1986;15: 211-227.
- [69] J.D.Litster, Stability of lipid bilayers and red blood cell membranes, *Phy. Lett* 1975;53A: 193-194.
- [70] A.G.Petrov, M.D.Mitov, A.I.Derzhanski,, Edge energy and pore stability in bilayer lipid membranes , in :*Advances in Liquid crystal research and applications* (L. Bata, ed.), Pergamon Press, Elmsford, NY 1980;695-737.
- [71] L.V.Chernomordik, S.I.Sukharev, I.G.Abidor, Y.A.Chizmadzhev, Breakdown of lipid bilayer membranes in an electric field, *Biochim. Biophys. Acta* 1983;736: 202-213.
- [72] J.Panyam, V.Labhasetwar, Dyanamics of endocytosis and exocytosis of poly (D,L-Lactide-co-Glycolide) nanoparticles in vascular smoothmuscle cells. *Pharmaceutical Research* 2003;20(2): 212-219.
- [73] I.R.Miller, Structural and energetic aspects of change transport in lipid bylayers and biological membranes, in: *Topics in Bioelectrochemistry and Bioenergetics*(G. Milazzo, ed.), Wiley, New York 1981; 161-224.
- [74] J.Gimsa, D.Wachner, Analytical description of the transmembrane voltage induced on arbitrarily oriented ellipsoidal and cylindrical cells *Biophysical J* 2001;81: 1888–1896.

- [75] T.Kotnik, D.Miklavcic, Analytical description of transmembrane voltage induced by electric fields on spheroidal cells, *Biophysics. J* 2000;79: 670-679.
- [76] C.Polk, E.Postow, Eds., *CRC Handbook of biological effects of electromagnetic fields*, 2nd ed. Boca Ratom, FL:CRC, 1996.
- [77] E.A.Gift, J.C.Weaver, Observation of extreamly heterogeneouselectroporative upta-
kewhich changes with electric field pulse amplitude in *saccharomyces cerevisiae*, *Bi-
ochem. Biophys.Acta* 1995;1234: 52-56.
- [78] J.C.Weaver, Yu.A.Chizmadzhev, Theory of electroporation: A review, *Bioelectro-
chem. Bioenerget* 1996;41: 135-160.
- [79] H.T.Tien, *Bilayer lipid membrane (BLM)*, Dekker, New York 1974.
- [80] R.Benz, F.Beckers, U.Zimmermann, Reversibale electrical breakdown of lipid bilayer
membranes: A charge pulse relaxation study, *J.Membr.Biol* 1979;48: 181-204.
- [81] R.Benz, U.Zimmermann, Relaxation studies on cell membranes and lipid bilayers in
the high electric field range, *Bioelectrochem.Bioenerg* 1980b;7: 723-739.
- [82] T.Y.Tsong, T.T.Tsong, E.Kingsley, R.Siliciano, Relaxation phenomena in human er-
ythrocyte suspensions, *Biophys J* 1976;16: 1091-1104.
- [83] P.Linder, E.Neumann, K.Rosenheck, Kineticsof permeability changes induced by
electric impulses in chromaffin granules *J.Membr.Biol* 1977;32: 231-254.
- [84] K.S.Cole, *Membranes, Ions and Impulses*, University of California Press, Berkeley,
1972
- [85] H.Pauly, W.P.Schwan, Dielectric properties and ion mobility in erythrocytes, *Bio-
phys.J* 1966;6: 621-639.
- [86] R.Stampfli, Reversible electrical breakdown of the excitable membrane of a Ranvier
node, *Ann. Acad. Bras. Cien* 1958;30: 57-63.
- [87] U.Zimmermann, Electric field-mediated fusion and related electrical phenomena, *Bi-
ochim.Biophys. Acta* 1982;694: 222-227.
- [88] L.V.Chernomordik, S.I.Sukharev, S.V.Popov, V.F.Pastushenko, A.V.Sokirko, I.G.Abi-
dor, Y.A.Chizmadzhev, The electrical breakdown of cell and lipid membranes: The
similarity of phenomenologies, *Biochim, Biophys. Acta* 1987;902: 360-373.
- [89] R.Benz,U.Zimmermann, The resealing process of lipid bilars after reversible electrical
breakdown, *Biochim. Biophys. Acta* 1981;640: 169-178.
- [90] I.G.Abidor, S.I.Sukharev, L.V.Chernomordik, Y.A.Chizmadzhev, The reversible elec-
trical breakdown of bilayer lipid membranes modified by UO_2^{2+} ions, *Bioelectro-
chem.Bioenerg* 1982;9: 141-148.

- [91] L.V.Chernomordik, S.I.Sukharev, I.G.Abidor, Y.A.Chizmadzhev, The study of the BLM reversible electrical breakdown mechanism in the presence of UO_2^{2+} ions, *Bioelectrochem.Bioenerg* 1982;9: 149-155.
- [92] [92]S.I.Sukharev,L.V.Chernomordik, I.G.Abidor, Reversible electrical breakdown of holoturinmodified bilayer lipid membranes, *Biofizika* 1983;28: 423-426.
- [93] A.Barnett, J.C.Weaver, Electroporation:a unified, quantitative theory of reversible electrical breakdown and reupture, *Bioelectrochem. Bioenerg* 1991;25: 163-182.
- [94] A.E.Sowers, C.A.Jordan, "Electroporation and electrofusion in cell biology" Edited by E.Neumann, Planam press, New York 1989; 308-315.
- [95] [95]B.Rubinsky, Irreversible electroporation in medicine, *Technol. In Cancer Research Treatment*, ISSN 1533-0346, 2007;6: 255-259.
- [96] J.A.Nollet, *Researches sur les causes particulieres des phenomenes electriques*, Paris: Chez H.L. Guerin &L.F.Delatour 1754.
- [97] R.W.Glaser, A.Wagner, E.Donath, Volume and ionic composition changes in erythrocytes after electric breakdown: Simulation and experiment, *Bioelectrochem. Bioenerg* 1986;16: 455-470.
- [98] L.V.Chernomordik, M.M.Kozlov, G.B.Melikyan, I.G.Adibor, V.S.Markin, A.Y.Chizmadzhev, The shape of lipid molecules and monolayer membrane fusion, *Biochim, Biophys. Acta* 1985;812: 643-655.
- [99] A.Gothelf, L.M.Mir, J.Gehl, Electrochemotherapy: Results of cancer treatment using enhanced delivery of bleomycin by electroporation, *Cancer Treat. Rev* 2003;29: 371-387.
- [100] S.Orlowski, L.M.Mir, Cell electroporabilization: a new tool for biochemical and pharmacological studies, *Biochim. Biophys. Acta* 1993;1154: 51-63.
- [101] M.Golzio, M.P.Rols, J.Teissie, In vitro and vivo electric field-mediated permeabilization, gene transfer, and expression, *Methods* 2004;33: 126-135.
- [102] N.Shivarova, W.Forster, H-E.Jacob, R.Grigorova, Microbiological implications of electric field effects. VII. Stimulation of plasmid transformation of *Bacillus cereus* protoplasts by electric field pulses, *Z.Allg. Mikrobiol* 1983;23: 595-599.
- [103] F.G.Falkner, E.Neumann, H.G.Zachau, Tissue specificity of the initiation of immunoglobulin κ gene transcription, *Hoppe-Seyler's Z physiol. Chem* 1984;365: 1331-1343.
- [104] H.Potter, L.Weir, P.Leder, Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation, *Proc. Natl. Acad. Sci. USA* 1984;81: 7161-7165

- [105] E.V.Prochownik, Relationship between an enhancer element in the human antithrombin III gene and an immunoglobulin light-chain gene enhancer, *Nature* 1985;316: 845-848.
- [106] D.Zerbib, F.Amalric, J.Teissie, Electric field-mediated transformation: Isolation and characterization of a TK⁺ subclone, *Biochem. Biophys. Res. Commun* 1985;129: 611-618.
- [107] L.Weir, P.Leder, Structure and expression of a human subgroup II immunoglobulin κ gene, *Nucleic Acids Res* 1986;14: 3957-3970.
- [108] G.D.Yancopoulos, T.K.Blackwell, H.Suh, L.Hood, F.W.Alt, Introduced T cell receptor variable region gene segments recombine in pre-B cells: Evidence that B and T cells use a common recombinase, *Cell* 1986;44: 251-259.
- [109] R.S.Boston, M.R.Becwar, R.D.Ryad, P.B.Goldsbrough, B.A.Larkins, T.K.Hodges, Expression from heterologous promoters in electroporated carrot protoplasts, *Plant physiol* 1987;83: 742-746.
- [110] R.B.Puchalski, W.E.Fahl, Gene transfer by electroporation, lipofection, and DEAE-Dextran transfection: compatibility with cell sorting by flow cytometry, *Cytometry* 1992;13: 23-30.
- [111] R.Heller, M.Jaroszeski, A.Atkin, D.Moradpour, R.Gilbert, J.Wands, C.Nicolau, In vivo gene electroinjection and expression in rat liver, *FEBS Letters* 1996;389: 225-228.
- [112] N.Dujardin, P.V.D.Smissen, V.Preat, Topical gene transfer into rat skin using electroporation, *Pharmaceutical Research* 2000;18(1): 61-66.
- [113] F.Yamauchi, K.Kato, H.Iwata, Spatially and temporally controlled gene transfer by electroporation into adherent cells on plasmid DNA -loaded electrodes, *Nucleic Acids Research* 2004;32(22): e187
- [114] T.Yamaoka, Y.Yonemitsu, K.Komori, H.Baba, T.Matsumoto, T.Onohara, Y.Maehara, Ex vivo electroporation as a potent new strategy for nonviral gene transfer into autologous vein grafts, *Am J Physiol Heart Circ Physiol* 2005;289: H1865-H1872.
- [115] Y.Takei, T.Nemoto, P.Mu, T.Fujishima, T.Ishimoto, Y.Hayakawa, Y.Yuzawa, S.Matsuo, T.Muramatsu, K.Kadomatsu, In vivo silencing of a molecular target by short interfering RNA electroporation: tumor vascularization correlates to delivery efficiency, *Mol. Cancer Ther* 2008;7: 211-221.
- [116] C.D.Kaufman, R.C.Geiger, D.A.Dean, Electroporation and mechanical ventilation-mediated gene transfer to the lung, *Gene Therapy* 2010;17: 1098-1104.
- [117] T.Geng, Y.Zhan, J.Wang, C.Lu, Transfection of cells using flow through electroporation based on constant voltage, *Nature protocols* 2011;6(8): 1192-1208.

- [118] A.I.Daud,R.C.DeConti, S.Andrews, P.Urbas, A.I.Riker, V.K.Sondak,, Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma, *Journal of Clinical Oncology* 2008;26: 5896-5903.
- [119] J.Teissie, N.Eynard, M.C.Vernhes, A.Benichou, V.Ganeva, B.Galutzov, P.A.Cabanes, Recent biotechnological developements of electropulsation.A prospective review, *Bioelectrochemistry* 2002;55: 107-112.
- [120] L.M.Mir, M.Belehradek, C.Domenge, Electrochemotherapy, a new antitumor treatment:first clinical trial. *CR Acad. Sci. III* 1991;313: 613-618.
- [121] L.M.Mir, S.Orlowski, J.Jr.Belehradek,C.Paoletti, Electrochemotherapy potentiation of antitumour effect of bleomycin by local electric pulses, *Eur. J. Cancer* 1991;27: 68-72.
- [122] M.Belehradek, C.Domenge, B.Luboiniski, S.Orlowski,J.Jr.Belehradek,L.M.Mir, Electrochemotherapy, a new antitumor treatment, First clinical phase I-II trial, *Cancer* 1993;72: 3694-3700.
- [123] L.M.Mir, L.F.Glass, J.Teissie, Effetive treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy, *Br.J.Cancer* 1998;77: 2336-2342.
- [124] [124]L.M.Mir, M.F.Bureau, J.Gehl, High efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl Acad Sci. USA* 1999;96: 4262-4267.
- [125] J.Gehl, T.Skovsgaard, L.M.Mir, Vascular reactions to in vivo electroporation:Characterization and consequences for drug and gene delivery, *Biochimica et Biophysica Acta* 2002;1569: 51-58.
- [126] L.M.Mir, Nucleic acids electrotransfer based gene therapy(electrogenetherapy):past, current and future, *Mol Biotechnol* 2009;43: 167-176.
- [127] R.Haller, M.Jarozeski, A.Atkin, In vivo gene electroinjection and expression in rat liver, *FEBS Lett* 1996;389: 225-228.
- [128] M.F.Bureau, J.Gehl, V.Deleuze, L.M.Mir, D.Scherman, Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer, *Biochem Biophys Acta* 2000;1474: 353-359.
- [129] MP.Rols, D.Coulet, J.Tessie, Highly efficient transfection of mammalian cells by electric field pulses, Application to large volumes of cell culture by using a flow system. *Eur. J. Biochem* 1992;206: 115-121.
- [130] K.Dhama, M.Mahendran, P.K.Gupta, A.Rai,, DNA vaccines and their applications in veterinary practice: current prospectives, *Vet Res Commun* 2008;32: 341-356.
- [131] R.Person, A.M.Bodles-Brakhop, M.A.Pope, P.A.Brown, A.S.Khan, R.Draghia-Akli, Growth hormone-releasing hormone plasmid treatment by electroporation decreases offspring mortality over three pregnancies, *Mol Ther* 2008;16: 1891-1897.

- [132] [132]P.A.Brown,A.Bodles-Brakhop, R.Draghia-Akli, Plasmid growth hormone relea-singhormone therapy in healthy and laminitis-afflicted horses-evaluation and pilot study, *J.Gene Med* 2008;10: 564-574.
- [133] P.A.Brown, A.M.Bodles-Brakhop, R.Draghia-Akli, Effects of plasmid growth hor-mone releasing hormone treatment during heat streass, *DNA Cell Biol* 2008;27: 629-635.
- [134] A.M.Bodles-Brakhop, P.A.Brown, M.A.Pope, R.Draghia-Akli, Double blinded, place-bo-controlled plasmid GHRH trial for cancer-associated anemia in dogs, *Mol Ther* 2008;16: 862-870.
- [135] J.Glasspool-Malone, S.Somiari, J.J.Drabick, R.W.Malone, Efficient nonviral cutaneous transfection, *Mol. Ther* 2000;2: 140-146.
- [136] S.Somiari, J.Glasspool-Malone, J.J.Drabick, Theory and in vivo application of electro-porative gene delivery, *Mol Ther* 2000;2: 178-187.
- [137] J.J.Drabick, J.Glasspool-Malone, A.King, R.W.Malone, Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by in vivo electroporpermabilization, *Mol Ther* 2001;3: 249-255.
- [138] F.Liu, L.Huang, A syringe electrode device for simultaneous injection of DNA and electrotransfer, *Mol Ther* 2002;5: 323-328.
- [139] L.Low, A.Mander, K.McCann, D.Dearnaley, T.Tjelle, I.Mathiesen, F.Stevenson, C.H.Ottensmeier, *Human Gene Therapy* 2009;20: 1269-1278.
- [140] A.Brave, S.Nystrom, A-K.Roos, S.E.Applequist, Plasmid DNA vaccination using skin electroporation promotes polyfunctional CD4 T-cell responces,*Immunology and Cell Biology* 2011;89: 492-496.
- [141] L.W.Weber, W.B.Bowne, J.D.Wolchok, R.Srinivasan, J.Qin, Y.Moroi, Tumor immuni-ty and autoimmunity induced by immunization with homologous DNA, *J.Clin In-vest* 1998;102: 1258-1264.
- [142] W.B.Browne, R.Srinivasan, J.D.Wolchok, W.G.Hawkins, N.E.Blachere, R.Dyall, Cou-pling and uncoupling of tumor immunity and autoimmunity, *J.Exp Med* 1999;190: 1717-1722.
- [143] A.M.Bodles-Brakhop, R.Heller, R.D-Akli, Electroporation for the delivery of DNA based vaccines and immunotherapeutics: current clinical developments, *Molecular Therapy* 2009;17: 585-592.
- [144] D.Boczkowski, S.K.Nair, D.Snyder, E.Gilboa, Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo, *J. Ex. Med* 1996;184: 465-472.
- [145] C. Milazzo, V. L. Reichardt, M. R. Muller, F. Grunebach, and P. Brossart, Induction of myeloma-specific cytotoxic T cells using dendritic cells transfected with tumor-de-rived RNA, *Blood* 2003;101: 977-982.

- [146] A. Bonehill, A. M. T. Van Nuffel, J. Corthals, Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients, *Clin. Can. Res* 2009;15: 3366–3375.
- [147] A.Bringmann, S.A.Erika Held, A.Heine, P.Brossart, RNA vaccine in cancer treatment, *Journal of Biomedicine and Biotechnology* 2010;2010: 1-12.
- [148] J.Liu, R.Kjeken, I.Mathiesen, D.H.Barouch, Recruitment of antigen-presenting cells to the site of inoculation and augmentation of human immunodeficiency virus type 1 DNA vaccine immunogenicity by in vivo electroporation, *J.Virol* 2008;82: 5643-5649.
- [149] S.B.Dev, Killing cancer cells with a combination of pulsed electric fields and chemotherapeutic agents, *Cancer Watch* 1994;3: 12-14.
- [150] S.B.Dev, G.A.Hofmann, Electrochemotherapy-A novel method of cancer treatment, *Cancer Treatment Rev* 1994;20: 105-115.
- [151] Z.Rudolf, B.Stabuc, M.Cemazar, D.Miklavcic, L.Vodovnik, G.Sersa, Electrochemotherapy with bleomycin: The first clinical experience in malignant melanoma patients, *Radiol Oncol* 1995;29: 229-235.
- [152] [152]V.M.Munoz, G.P.Ortega, Electrochemotherapy for treatment of skin and soft tissue tumours.Update and definition of its role in multimodal therapy. *Clin Transl Oncol* 2011;13: 18-24 .
- [153] T.Hampton, Electric Pulses Help With Chemotherapy, May Open New Paths for Other Agents, *JAMA* 2011;305: 549-551.
- [154] A.Testori,G.Tosti, C.Martinoli, G.Spadola, F.Cataldo, F.Verrecchia, F.Baldini, M.Moscini, J.Soteldo, I.Tedeschi, C.Passoni, C.Pari, A.di. Pietro, P.F.Ferrucci, Electrochemotherapy for cutaneous and subcutaneous tumor lesions: a novel therapeutic approach, *Dermatol Ther* 2010;23: 651-661.
- [155] M.G.Moller, S.Salwa, D.M.Soden, G.C.O’Sullivan, Electrochemotherapy as an adjunct or alternative to other treatments for unresectable or in-transit melanoma. *Expert Rev Anticancer Ther* 2009;9: 1611-1630.
- [156] G.Sersa, D.Miklavcic, M.Cemazar, Z.Rudolf, G.Pucihar, M.Snoj, Electrochemotherapy in treatment of tumours. *Eur J Surg Oncol* 2008;34: 232-240.
- [157] J.O.Larkin, C.G.Collins, S.Aarons, M.Tangney, M.Whehan, S.O’Reily, O.Breathnach, D.M.Soden, G.C.O’Sullivan, Electrochemotherapy - Aspects of preclinical development and early clinical experience. *Ann Surg* 2007;245: 469-479.
- [158] L.M.Mir, O.Tounekti, S.Orlowski, Bleomycin revival of an old drug, *Gen Pharmacol* 1996;27: 745-748.
- [159] S.Orloswski, J.Jr.Belehradek, C.Paoletti, L.M.Mir, Transient electroporation of cells in culture. Increase of the cytotoxicity of anticancer drugs, *Biochem Pharmacol* 1988;37: 4727-4733.

- [160] J.Gehl, T.Skovsgaard, L.M.Mir, Enhancement of cytotoxicity by electroporabilization: an improved method for screening drugs, *Anticancer Drugs* 1998;9: 319-325.
- [161] M.J.Jaroszeski, V.Dang, C.Pottinger, J.Hickey, R.Gilbert, R.Haller, Toxicity of anti-cancer agents mediated by electroporation in vitro, *Anticancer Drugs* 2000a;11: 201-208.
- [162] M.Pavlenko, A.K.Roos, A.Lundqvist, A.Palmborg, A.M.Miller, V.Ozenci, A phase I trial of DNA vaccination with a plasmid expressing prostate specific antigen in patients with hormone-refractory prostate cancer, *Br. J Cancer* 2004;91: 688-694.
- [163] A.M.Miller, V.Ozenci, R.Kiessling, P.Pisa, Immune monitoring in a phase 1 trial of a PSA DNA vaccine in patients with hormone-refractory prostate cancer, *J.immunother* 2005;28: 389-395.
- [164] A.Kroos, A.King, P.Pisa, DNA vaccination for prostate cancer, *Methods. Mol. Biol* 2008;423: 463-472
- [165] A.K.Roos, S.Moreno, C.Leder, M.Pavlenko, A.King, P.Pisa, Enhancement of cellular immune response to a prostate cancer DNA vaccine by intradermal electroporation, *Mol Ther* 2006;13: 320-327.
- [166] M.Kambe, D.Arita, H.Kikuchi, T.Funato, F.Tezuka, M.Gamo, Y.Murakawa, R.Kanamaru, Enhancing the effect of anticancer drugs against the colorectal cancer cell line with electroporation, *Tohoku, J.Exp.Med* 1996;180: 161-171.
- [167] M.J.Jaroszeski, R.A.Gilbert, R.Heller, In vivo antitumor effects of electrochemotherapy in a hepatoma model, *Biochim. Biophys. Acta* 1997;1334: 15-18.
- [168] V.Kodre, M.Cemazar, J.Pecar, G.Sersa, A.Cor, N.Tozon, Electrochemotherapy compared to surgery for treatment of canine mast cell tumors, *In Vivo* 2009;23(1): 55-62.
- [169] D.Miklavcic, M.Snoj, A.Zupanic, B.Kos, M.Cemazar, M.Kropivnik, M.Bracko, T.Pecnik, E.Gadzijev, G.Sersa, Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. *BioMed Eng OnLine* 2010;9: 1-12.
- [170] D.M.Soden, J.Olarkin, C.G.Collins, M.Tangney, S.Aarons, J.Piggott, A.Morrissey, C.Dunne, G.C.O'Sullivan, Successful application of targeted electrochemotherapy using novel flexible electrodes and low dose bleomycin to solid tumours. *Cancer Lett* 2006;232: 300-310.
- [171] O.Thanaketpaisarn, M.Nishikawa, F.Yamashita, M.Hishida, Tissue-specific characteristics of *in vivo* electric gene transfer by tissue and intravenous injection of plasmid DNA. *Pharm Res* 2005;22(6): 883-891.
- [172] A.V.Titimirov, S.Sukharev, E.Kistanova, *In vivo* electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. *Biochim Biophys Acta* 1991;1088(1): 131-134.

- [173] S.Chesnoy, L.Huang, Enhanced cutaneous gene delivery following intradermal injection of naked DNA in a high ionic strength solution. *Mol Ther* 2002;5(1): 57–62.
- [174] S.Babiuk, M.E.Baca-Estrada, M.Foldvari, L.Baizer, R.Stout, M.Storms, Needle-free topical electroporation improves gene expression from plasmids administered in porcine skin. *Mol Ther* 2003;8(6): 992–998.
- [175] B.M.Medi, S.Hoselton, R.B.Marepalli, J.Singh, Skin targeted DNA vaccine delivery using electroporation in rabbits. I: efficacy. *Int J Pharm* 2005;294(1-2): 53–63.
- [176] L.Zhang, G.Widera, D.Rabussay, Enhancement of the effectiveness of electroporation-augmented cutaneous DNA vaccination by a particulate adjuvant. *Bioelectrochemistry* 2004;63(1-2): 369–373.
- [177] L.A.Hirao, L.Wu, A.S.Khan, A.Satishchandra, R.Draghia-Akli, D.B.Weiner, Intradermal/subcutaneous immunization by electroporation improves plasmid vaccine delivery and potency in pigs and rhesus macaques. *Vaccine* 2008;26(3): 440–448.
- [178] J.W.Hooper, J.W.Golden, A.M.Ferro, A.D.King, Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. *Vaccine* 2007;25(10): 1814–1823.
- [179] C.Dobano, G.Widera, D.Rabussay, D.L.Doolan, Enhancement of antibody and cellular immune responses to malaria DNA vaccines by *in vivo* electroporation. *Vaccine* 2007;25(36): 6635–6645.
- [180] M.Wang, O.Orwar, S.G.Weber, Single cell transfection by electroporation using an electrolyte/plasmid-filled capillary, *Anal Chem* 2009;81: 4060–4067.
- [181] J.Rathenberg, T.Nevian, V.Witzemann, High-efficiency transfection of individual neurons using modified electrophysiology techniques *J. Neurosci Methods* 2003;126: 91–98.
- [182] Y.Saheki, S.T.Li, M.Matsushita, Y.M.Wu, W.H.Cai, F.Y.Wei, Y.F.Lu, A.Moriwaki, K.Tomizawi, H.Matsui, A new approach to inhibiting astrocytic IP₃-induced intracellular calcium increase in an astrocyte-neuron co-culture system, *Brain Research* 2005;1055(1-2): 196–201.
- [183] K.Hass, K.Jensen, W.C.Sin, L.Foa, H.T.Cline, Targeted electroporation in *Xenopus* tadpoles in vivo from single cell to the entire brain, *Differentiation* 2002;70: 148–154.
- [184] S.M.Schanuel, K.A.Bell, S.C.Henderson, A.R.McQuiston, Heterologous expression of the invertebrate FMRFamide-gated sodium channel as a mechanism to selectively activate mammalian neurons, *Neuroscience* 2008;155(2): 374–386.
- [185] K.Hass, W.C.Sin, A.Gavaherian, Z.Li, H.T.Cline, Single cell electroporation for gene transfer in vivo, *Neuron* 2001;29: 583–591.
- [186] B.Judkewitz, M.Rizzi, K.Kitamura, M.Hausser, Targeted single cell electroporation of mammalian neurons in Vivo, *Nature Protocols* 2009;4: 862–869.

- [187] M.Kanduser, D.Miklavcic, Electrotechnologies for extraction from food plants and Biomaterials, E.Vorobiew, N.Lebovka(eds), DOI: 10.1007/978-0-387-79374-0-1, Springer Science + Business Media, LLC 2008.
- [188] J.Olofsson, K.Nolkrantz, F.Ryttzen, B.A.Lambies, S.G.Weber, O.Orwar, Single cell electroporation, Current Opinion in Biotechnology 2003;14: 29-34.
- [189] D.S.Hewapathirane, K.Haas, Single cell electroporation in vivo within the intact developing brain, J.Vis.Exp.17,e705(2008) DOI : 10.3791/705 (2008).
- [190] D.Karra, R.Dahm, The Journal of Euroscience 2010;30(18): 6171-6177.