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Electroporation : Application in Biology and Medicine

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Electroporation is a phenomenon where a cell membrane is temporarily made permeable exposure to an intense electric field. It is a physical method primarily used for introducing genes into wide array of cell types, both eukaryotic and prokaryotic, including animal cells, plant cells, unicellular organisms such as algae or yeast, parasites and bacteria. Besides genes, electroporation can also be applied to introduce other types of molecules into cells ranging from small ions such as calcium to large molecules such as proteins, enzymes, antibodies and drugs. Recently, it has been used as a clinical tool for targeting and enhancing uptake of chemotherapeutic agents by tumours and also as a non-invasive means to enhance transdermal transport of drugs. This article briefly reviews the technique of electroporation and its major applications in biology and medicine.

Electroporation or electropermeabilisation involves the creation of transient aqueous pathways across lipid bilayers by applying short but high voltage pulses (microseconds to milliseconds)[1,2]. Permeability and electrical conductance of bilayer membranes are rapidly and transiently increased by orders of magnitude. It is a universal phenomenon which occurs in lipid bilayers of non-living systems such as liposomes and red blood cell ghosts as well as the plasma membranes of living cells, either isolated or *in situ*[3]. It also occurs in such events as electric injury, electrocution and cardiac procedures involving electric shocks[4]. The phenomenon of electroporation is known from last four decades and its reported application in molecular biology is only a decade old[5].

The phenomenon of electroporation can be explained as follows. The cell membrane, which envelops the cell, is a thin elastic structure which only 7.5 to 10 nm thick[6]. It is composed of lipid bilayer embedding large globular protein molecules. As we know, the intracellular and extracellular fluids contain numerous ions and can therefore carry currents. Lipid bilayer, however, contains very few charged groups and cannot carry current. Therefore, the lipid bilayers of the plasma membrane are regions of high electrical resistance separating two water compartments-the intracellular fluid and the extracellular fluid-of low resistance[7]. So naturally, when an electrical current (or voltage) is applied across a cell membrane, there is a build up of the charges across the lipid bilayer (i.e the lipid bilayer functions as a capacitor) resulting in induced membrane potential, V_m, at point Z (Fig. 1) which is given by[8];

$$V_m = C.r.E. \cos \theta \qquad (1)$$

where θ is the angle made by point Z relative to the direction of field and r is the radius of the cell C is a constant, the value of which is 1.5 under normal physiological conditions. The induced membrane potential is highest at points were where $\cos \theta = \pm 1$, i.e. at points X and Y in Fig. 1 in line with the direction of field, i.e.

$$V_m = 1.5\ r\ E \qquad (2)$$

When the applied field strength is so high that the induced membrane potential (V_m) exceeds a threshold potential V_t, membrane capacitor can no longer withstand

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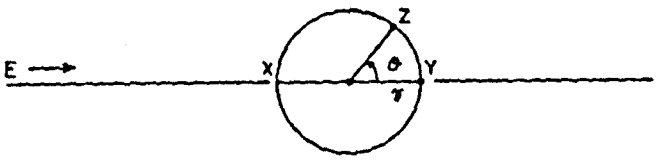

Fig. 1 : Schematic representation of equation 1 (E is the direction of electrical field, X and Y are the two points on the cell membrane in line with direction of field, r, is the radius of the cell, for details see text)

the accumulation of charges and results in an electrical breakdown; at this point the cell membrane can no longer maintain its structural integrity and becomes permeable. This phenomenon is called 'electroporation' or electropermeabilisation. For most cells, the threshold potential is typically on the order of 0.5-1.5 V, which is about 10 times larger than the normal resting potential of an animal cell[1,4]. According to equation 2, the electrical field required for electroporation of living cells (with radius or long axis varying between 2-7 μm) is of the order 10^3-10^4 V/cm. One can take advantage of the relationship between V_m and r to achieve selective permeabilisation of the larger cells in presence of many smaller cells.

After a cell membrane has been permeabilised by an applied electrical field, molecules that normally could not penetrate the membrane can now pass through easily. These molecules include ions, metabolites, carbohydrates, proteins and nucleic acids. Some of the molecules that enter the cell during the electropermeabilised state are very large. It has been reported that DNA molecules of sizes up to 150 kb can be taken up by electropermeabilised cells[9]. The uptake of these very large molecules however may involve some complex processes as for small molecules such as small carbohydrates or proteins of low molecular weight, it is evident that the particles can enter the cell by simple diffusion through the permeabilized cell membrane. For charged molecules (such as DNA or charged proteins), the transport may be because of electrophoretic mechanism (electrorepulsion from similarly charged electrode).

If the external electric field is applied only for a brief period of time (microseconds to milliseconds), the bilayer will recover by gradually resealing itself after the external electric field has been turned off[1,2]. The resealing time of artificial lipid-bilayer membranes is reported to be of the order of microseconds, whereas for biological membranes, it ranges between minutes to an hour depending on temperature and species[10]. One can however, delay the resealing process by keeping the cells at O°.

Studies on artificial-lipid bilayer membranes and living cells have provided some insights into the process of electroporation and recovery. From the studies on single lipid-bilayer membranes, it has been suggested that the aqueous pathways (or pores) formed are small (<10 nm), sparse (≤ 0.1% of surface area) and generally short lived (μs to s)[1,2,11]. In living cells however, the pores formed were observed to be much larger (100 nm or more)[12].

Though the creation of transient aqueous pathways is proposed as a mechanism by which electroporation occurs, the exact physical nature of any structural changes remains unresolved. The detail of mechanics of electroporation is discussed elsewhere[13].

Instrumentation:

There are several equipments available for electroporation in first world countries, such as ECM® (Genetronics, Inc., San Diego, CA, U.S.A), Easyject Plus® (Equibio, Seraing, Belgium), Bio-RAD® gene pulser (Bio Rad, Calif, U.S.A.), BTX®, San Diego, CA, U.S.A.) etc. However, a simpler and economical device can be built indigenously[14]. The choice of an electroporation device is governed by four major factors; safety, convenience, reliability and cost. The optimal features for the electroporation device should include voltage density, pulse duration and number of pulses. Currently, three types of electroporation devices are being used. They include, Square wave pulse device, Capacitor based device and Radiofrequency based pulse device[2,15].

In square wave pulse device, a constant voltage output of an external power supply is converted into an approximate square wave pulse of desired duration, whereas in capacitor based device, a capacitor is charged up to a desired voltage and then discharged producing an exponential decay form of the voltage (Fig. 2). By varying the capacitor value, one can manipulate the resulting pulse width. In radiofrequency (RF) based device, a constant voltage is converted into an oscillating electrical field. It is also known as dc shifted radiofrequency (RF) pulse. This device is more effective in electroporating cells of heterogeneous size since under an oscillating electrical field, the induced membrane potential, V_m, is not very sensitive to r. Moreover, this device is more effective in inducing electroporation since under RF pulse structural fatigue is created in the membrane because of an

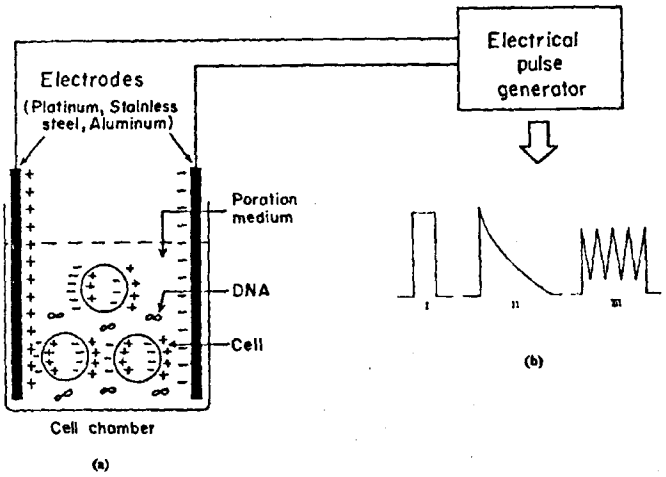

Fig. 2 : (a) Illustrative diagram of electroporation equipment used for introduction of r-DNA (or other molecules) into a cultured cell line. (b) Various types of electrical pulses produced by electrical pulse generator, I-square wave pulse, II-exponential decay pulse and III-oscillating pulse

oscillating motion of the charged membrane lipids and proteins.

In molecular biology studies, electroporation of variety of cells is achieved using a cell chamber, which is generally a small plastic cuvette (0.1-1 cm) having two metal electrodes placed against the opposite walls (Fig. 2). The cells to be electroporated are suspended in the cell chamber along with the molecules to be transported (DNA for example) in the cell. A high voltage pulse is applied to the electrodes and thus to the cell-DNA suspension. Fig. 2 depicts the illustrative diagram of electroporation equipment used for introduction of r-DNA (or other molecules) into a cultured cell line. Certain cells may be prone for electrical shock, for such cells low voltage high capacitance (longer pulse duration) devices can be used. In general, 40-60% cell viability is desired in electroporation[15].

There are various factors which may affect the efficiency of electroporation Table 1, the most important being the applied field strength and pulse duration. The applied field strength must be high enough to induce electroporation. Moreover, the electrical field should be applied for a sufficiently longer duration (longer than the relaxation time of the cell, which is typically on order of magnitude of microseconds e.g. 20 μs for red blood cells)[1,2].

TABLE 1 : FACTORS AFFECTING EFFICIENCY OF ELECTROPORATION OF CELLS[1-5, 8-12]

Electrical factors
Electrical field strength
Nature of pulse
Pulse duration
Number of pulses applied
Type of electrode
Cell related factors
Type or cell species
Size
Growth phase of cells
Miscellaneous factors
Temperature
Composition of poration medium
pH and ionic strength of poration medium
Presence of extraneous ions

APPLICATIONS

Gene Transfer:

The most important application of electroporation is the introduction of genes into wide array of cell types both eukaryotic and prokaryotic, including animal cells, plant cells, unicellular organisms such as algae or yeast, parasites and bacteria for variety of purposes[1,2,15]. The first reported application of electroporation for gene transfer was only in the year 1982, when Neumann used electroporation for introducing a plasmid DNA containing the simplex virus thymidine kinase (TK) gene into mouse L cells deficient in the TK gene[5]. Stable transformants were obtained in his experiment. Today, electroporation is used to introduce exogenous DNA into a variety of cells, including fibroblasts, lymphocytes, neuronal cells, endocrine cells, human epithelial cells, primary animal cultures, hepatoma cells, hematopoietic stem cells and mammary carcinoma cells[16-26]. Although there are various other methods of gene transfer such as microinjection, calcium phosphate precipitation or retroviruses, electroporation has several distinctive advantages Table 2.

One common application of electroporation mediated gene transfer is the study of gene regulation. Usually a

reporter gene is inserted into plasmid vector with specific transcription promoter and enhancer sequences. By comparing the efficiency of gene expression under various conditions, such as the availability of certain transcriptional factors, one can gain knowledge about how the gene of interest is regulated. Alternatively, one may mutate the upstream sequences of a certain gene, fuse it with the reporter gene and introduce it into cells by electroporation. By comparing the resulting level of gene expression under various mutation conditions, one can identify the major promoter or enhancer sequences.

In addition, functional genes can be introduced into mammalian cells by stable transfection using electroporation. This can be done by coinserting a plasmid with the gene of interest into the cell genome and then subjecting the cell to a selection medium to which a plasmid confers resistance. Using this method, a large number of different types of DNA have been transfected into a variety of mammalian cells. For example, the complete human parathyroidharmone gene has been permanently transfected and expressed in rat pituitary cell line GH_4 C1 by electroporation with transfection efficiency of 1 per 10^5 to 10^6 cells[18].

TABLE 2 : METHODS OF GENE TRANSFER AND ADVANTAGES OFFERED BY ELECTROPORATION[1,15,27,28]

Method	Disadvantage	Advantage offered by electroporation*
Microinjection	Technically tedious and only one cell can be injected at a time.	Simple and technically easy, can be considered as effective mass micro-injection technique (thousands or millions of cells can be injected at a time)
Calcium Phosphate Precipitation	1. Insertion of multiple copies of recombinant gene; number of copies uncontrollable	Insertion of number of copies is controllable by manipulating electroporation conditions
	2. Chances of chemical hazard (e.g. mutation)	Physical method, no chance of chemical hazard.
	3. Prior treatment of DNA with $CaCl_2$ necessary	No prior treatment of DNA needed.
Retroviral Method	1. Insertion of r-DNA into random site on genome (non HR) and could lead to mutagenesis or tumorogenic potential.	r-DNA can be introduced into desired site of genome by linkage with proper flanking sequences (gene targeting or HR possible)
	2. To be infected by retroviruses, cells must express appropriate surface proteins/receptors.	Physical method; not sensitive to surface properties of cell
	3. Applicable only to dividing cells	Applicable to wide array of cell types
	4. Manufacture/Quality Control/Quality Assurance of retroviral vectors costly and difficult.	Direct gene transfer into desired cell eliminating the need for viral material treatment.

*Selective biochemical loading (DNA or other bio molecules) of one size cell in the presence of many smaller cells is possible only in electroporation by manipulating electroporation conditions.

HR : homologous recombination; non HR : non homologous recombination.

Not only can electroporation be used for introducing genes into cells *in vitro* but also for direct gene transfer *in vivo*. Aihara and colleagues at the Osaka University, Japan, used electroporation for *in vivo* gene transfer in ti-bialis anterior muscle of mice[29]. They injected the plasmid DNA (expressing interleukin-5, as vector) into the muscle followed by electroporation of the injected site with a pair of inserted needle electrodes. Five days later, the serum IL-5 levels were assayed. Mice that did not receive electroporation had serum levels of 0.2 ng/ml. Electroporation enhanced the levels of over 20 ng/ml. Histochemical analysis of muscles injected with a lacZ expression plasmid showed that *in vivo* electroporation increased both the number of muscle fibers taking up plasmid DNA and the copy number of plasmids introduced into the cells indicating the efficiency of *in vivo* electroporation. Similar *in vivo* protein and gene transfer has also been reported in murine melanoma by a direct injection of either the protein or the plasmid in the tumour, followed by electroporation[30].

Besides introduction of genes into cells to produce permanently transfected cell lines, a similar method called electrofusion (fusion of cells under the influence of high voltage pulses) can be used to prepare heterokaryons, hybridoma, hybrid embryos etc[4]. Even the much-talked sheep, Dolly, the first mammal to be cloned in history, has been produced by the same method of electrofusion of donor cell with oocyte[31]. It has been reported that transgenic fish can be produced by electroporating desired foreign DNA into unfertilised or newly fertilised eggs using many different fish species[32].

Electroporation has also been used to introduce genes into plant cells as well; protoplasts of a number of plants including carrot, maize and tobacco leaf mesophile cells have been transfected[15]. It has been reported that electroporation can be used for the generation of transgenic cereal plants such as rice and sorghum. Electroporation of unicellular organisms, bacteria and fungi has also been reported[15].

Electroinjection of other molecules:

Besides genes, electroporation can also be used to introduce other molecules into living cells. Earlier, it has been used to introduce small ions such as calcium and small molecules such as ATP into cells[8,15]. Subsequently, it has been used for introducing fluoroscent-labeled dextrans and molecular probes such as Calcium Green-1[1,4]. It can also be used for introduction of large molecules such as antibodies[15]. For example, using electroporation monoclonal antibodies have been introduced into HeLa cells and murine lymphoma cells. In addition, various cellular metabolites/antimetabolites have been electroporated into cells studying the various aspects of cellular metabolism[15], identification of cell signaling molecules[33], cell growth and differentiation[34], apoptosis[35] and in understanding the mechanism of various enzymes and enzyme inhibitors on proliferative cell growth[36].

Cancer Chemotherapy : Electrochemotherapy of Cancer:

Electrochemotherapy (ECT) is a new technique wherein high-voltage electric pulses delivered into a neoplasm transiently increase cell membrane permeability to large molecules, including cytotoxic agents[37]. ECT has been studied in experimental animals by direct injection of the cytotoxic drug (or immunomodulator) into tumour followed by the application of electrical pulses with surface electrodes in contact with the skin.

There are several reports of successful ECT treatment both *in vitro* and *in vivo* in experimental animals as well as in human subjects. In one such study conducted in rats bearing squamous cell carcinoma and a hepatocellular carcinoma on tongue, tumours were no longer visible from the third day after ECT treatment[38]. The histological examination revealed complete regression, whereas tumours that received either high voltage impulses alone or bleomycin alone showed no detectable changes.

In another phase I/II study, scientists at Medical Center, Chicago proved that electroporation can offer promising possibilities in the local treatment of head and neck cancer[39]. When they combined the electroporation with extremely low dose intralesional bleomycin in head and neck cancer patients and assessed tumour responses, they could detect 50% complete responders and 30% partial responders with only 20% non -responders, indicating the potential of ECT in treatment of surface tumours.

Electroporation also appears to offer great potential for the target oriented drug delivery. For example, it has been demonstrated that combined bleomycin treatment with EP offered a potentially beneficial way to minimise the amount of antiproliferative agent used and localised

the effect of anticellular proliferation resulting in potentially safer and more effective glaucoma filtering surgery of eye[40].

Transdermal drug delivery:

In case of transdermal drug delivery, the skin's outermost layer, the stratum corneum (SC), is the main barrier. If this barrier is destroyed, for example by mechanically abrading the stratum corneum, then the protection vanishes and skin becomes permeable to most of the molecules. In order to accomplish the latter without significant damage, the idea of using 'high voltage pulses' across the skin is to physically open the stratum corneum on a microscopic scale, to temporarily transport the drug and allow natural recovery processes to re-establish the barrier. Although, the stratum corneum is much more complicated than a single phospholipid bilayer (it is a multilammellar structure containing corneocytes) it seems possible to create new aqueous pathways within this structure[41-42]. Extensive research on skin electroporation has indicated that the multilammelar lipid bilayers of human stratum corneum could electroporate at voltages on the order of 50-100 V.

Electroporation has shown to enhance *in vitro* transdermal transport of compounds into or across skin ranging in size from small ions (e.g. Na^+, Cl^-)[43,44] to moderate sized molecules (e.g. calcein[45], sulforhodamine[46], metoprolol[47], fentanyl[48,49], methylene blue[50]) to macromolecules e.g. LHRH[51], heparin[52], oligonucleotidses[53]) to latex microspheres of micron dimensions[54]. Moreover, the electrical measurement studies (impedance/capacitance) has indicated the reversible nature of skin permeability following electroporation, which means the original skin resistance and impermeability can be restored after a given time interval[44].

In brief, the above observations support the idea that electrical measurements and electrical control of charged molecules are promising approaches either for topical drug therapy or non invasive systemic drug delivery once the safety aspects of this process is proved in human subjects.

Encapsulation of drugs in cellular carriers:

Electroporation can be used to encapsulate drugs, enzymes, vaccines and genetic materials in wide array of cells viz.; erythrocytes, lymphocytes, platelets and other somatic cells[10,55]. The encapsulated cells can be used as drug delivery systems to alter the *in vivo* distribution of drugs. The advantages of using these cellular carriers are self-evident. There is little chance of adverse side effects or immunogenic potential due to the carrier material, particularly if the cells used as carriers are derived from the patient to be treated. Moreover, by electroporation, cells that still retain haemoglobin (in case of erythrocytes) or other cellular constituents (enzymes for example) can be obtained, which is unlikely with the other methods of cellular drug entrapment.

Erythrocytes loaded with various enzymes are particularly attractive to replace a missing or deficient enzyme in a metabolic disorder or to degrade toxic compounds accumulated in the blood due to a disease or the environment. Model experiments *in vitro* with erythrocyte ghosts in which urease had been electrically entrapped (8 KV/cm, 40 μs) demonstrated that these loaded cells could split urea added to the external solution into ammonia and CO_2 over a period of 24 h at 37° (It should be noted that membrane has no limitation on urea and ammonia diffusion)[56]. Leakage of urease from the ghost cells was not observed without lysing the cells a second time indicating the integrity of cells after electroporation.

It has also been reported that electroporation of erythrocytes permits free exchange of the native haemoglobin with the exogenous haemoglobin in the surrounding medium. This method can be used to introduce natural or genetically engineered haemoglobins with altered oxygen binding characteristics into erythrocytes and use them in treatment of tissue hypoxia from a variety of causes. Fisher *et al.* used electroporation to exchange a fish root effect haemoglobin into rat RBCs[57]. The Resulting RBC's exhibited oxygen transport characteristics, unloading at high pressure at acidic pH indicating their potential to treat tissue hypoxia.

Erythrocytes have also been electroencapsulated with methotrexate[58], sucrose[59] and cynocobalamine[60]. It has been observed from *in vivo* biokinetic study of the drug loaded erythrocytes that the rate of elimination of drug was considerably reduced as compared to that of the free drug, indicating that drug delivery by electroporation can provide a sustained release of drug.

Similar to erythrocytes, blood platelets can also be electroencapsulated with drugs and can be targeted to a vessel wall injury site, primarily because of their natural

haemostatic properties[55]. This is particularly important during certain invasive cardiological procedures such as angioplasty or thrombolysis procedures, where the vessels might get damaged with subsequent accumulation of platelets and formation of thrombus or restenosis. The local delivery of anti-platelet drug to the vessel wall might reduce the incidence of such occlusive events. Several researchers have investigated the effects of autologous platelets electroloaded with anti-platelet drug, iloprost on platelet aggregation and adhesion to fibrillar collagen and injured arteries both *in vitro* and *in vivo* in rats, rabbits and pigs[55,61,62]. The electroloaded platelets substantially reduced platelet deposition at the lesion site as compared with control platelets indicating their potential for site specific delivery to the injured vessels.

Eosinophils have also been encapsulated with exogenous DNA by electroporation technique[63]. These altered eosinophils can be used as an important tool in the immunoregulatory and pathological processes.

Conclusions and Future Perspectives:

With the commencement of Human Genome Project and the possibility of identifying the majority of genes responsible for genetic defects, the advent of the molecular genetic medicine or the gene therapy seems to be inevitable. Present approaches in gene therapy strongly relies on the use of retroviral vectors for transfecting the cells with genes of interest. One of the major concerns with retroviral vectors is the possibility that a replication-competent retroviruses (RCR) could arise during the manufacturing process. Furthermore, as every mammalian cell contains endogenous retroviruses, additional viral sequences could be incorporated into the RCR, perhaps producing a pathogenic virus. There are various other potential problems with retroviral vectors that limit their application in gene transfection Table 2. Electroporation being relatively 'clean' method (no bio/chemical hazard) would be a much safer choice for future use in gene therapy. Also, in cell biology, electroporation has poised to become a novel method to study the various aspects of cell related to metabolism and growth. By electroporating the molecule of interest, one can assess its importance in cell biology. From drug delivery point of view, electroporation can be valuable tool either for non-invasively administering the drug molecules or for enhancing their efficacy. Further research in this direction is, however needed to establish the safety and efficacy of this technology in human subjects.

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