

# Electrotransfer as a Non Viral Method of Gene Delivery

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**Abstract:** Over the last few decades, various vectors have been developed in the field of gene therapy. There still exist a number of important unresolved problems associated with the use of viral as well as non viral vectors. These techniques can suffer from secondary toxicity or low gene transfer efficiency. Therefore an efficient and safe method of DNA delivery still needs to be found for medical applications. DNA electrotransfer is a physical method that consists of the local application of electric pulses after the introduction of DNA into the extra cellular medium. As electrotransfer has proven to be one of the most efficient and simple non viral methods of delivery, it may provide an important alternative technique in the field of gene therapy. The present review focuses on questions related to the mechanism of DNA electrotransfer, i.e. the basic physical processes responsible for the electroporation of lipid membranes. It also addresses the current limitations of the method as applied to DNA transfer, in particular its efficiency in achieving *in vitro* gene expression in cells and also its potential use for *in vivo* gene delivery.

**Keywords:** Gene transfer, gene expression, plasmid DNA, membrane, electric field, electroporation, electroporation, electroporation.

## 1. INTRODUCTION

In the field of gene delivery various different methods have been developed over the years for the delivery of DNA. These methods are usually based on the use of viruses as biological vectors or on the exploitation of chemical and/or physical phenomena. However, after decades of research and development, gene therapy still lacks techniques that permit risk free and successful DNA delivery. This lack of a safe and efficient method presents a significant bottleneck in the quest to develop successful gene therapy.

The use of electric pulses to deliver therapeutic molecules to tissues and organs has seen rapid development over the last few years. The method relies on the transient increase in the permeability of cell membranes, which arises when they are subjected to pulses of an externally applied electric field. This phenomenon is known as *electroporation* and is often referred to as *electroporation* [Neumann E *et al.*, 1989, Weaver, 1995] because one theory of this phenomenon is based on the formation of pore like structures in the cell membrane. It is these pores that are claimed to be responsible for the apparent permeabilisation. Upon the application of the field, hydrophilic molecules, which would otherwise be incapable of entering the cell, can cross the cell membrane and reach the cytosol of the cell. Electroporation can be used to deliver a wide range of therapeutic molecules; these include: anticancer drugs, proteins, and nucleic acids. *In vivo* electrotransfer of plasmid DNA is of particular interest because of its low cost, its practical ease and its safety. Moreover, the method has the capacity to deliver large DNA based macromolecules. The method is not

only promising for the treatment of genetic disorders, but also for the systemic secretion of therapeutic proteins, vaccination being another field of application [Babiuk *et al.*, 2006, Gehl, 2003, Heller and Heller, 2006, Mir *et al.*, 2005].

The successful electrotransfer of plasmid DNA into cells depends on cell membrane permeabilisation and on the way DNA is transported from the plasma membrane towards the nuclear envelope. However, the mechanisms underlying cell membrane permeabilisation and the subsequent DNA transfer have not yet been fully elucidated. A full understanding of the permeabilisation phenomenon is of great importance for the future development of the method, both to improve its efficiency and also to address safety and dosage issues in its *in vivo* applications. Many theoretical models have been proposed to explain the mechanisms of membrane permeabilisation and gene transfer, and to date no clear or comprehensive evidence has been obtained on how DNA interacts with the electroporated cell plasma membrane. Several models have been proposed in the case of mammalian cells. Such models to explain the crossing of the membrane by the plasmid are based on the following physical postulates (or combinations of them): 1) the existence of long-lived "electropores" [Neumann E. *et al.*, 1982, Xie and Tsong, 1993, de Gennes, 1999], 2) a preliminary binding step at the cell surface due to membrane plasmid interaction and then diffusion through the electropores [Xie and Tsong, 1993], 3) electrophoretic forces generated by the external field which push the plasmid through the membrane [Klenchin *et al.*, 1991, Sukharev *et al.*, 1992] and 4) adsorption by sphingosine/DNA interactions, insertion and passage of DNA through a hydrophilic percolated porous zone [Hristova *et al.*, 1997]. However, these models only describe the initial step of DNA transfer across the plasma membrane and do not address the behaviour of the plasmid DNA once it is inside the cell. It is the general consensus that little is known about what is really

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occurring in the cell after electropermeabilisation [Teissie *et al.*, 2005]. In addition, open questions still exist about the actual permeabilisation process and the subsequent behaviour of the membrane both while the field is applied and after it is cut. Also, there is little understanding of how DNA is transported in complex environments, such as those found in cells and tissues. These issues need to be addressed before a complete theory of electrotransfer gene delivery can be developed.

The aim of this chapter is to describe the different aspects of what is known about the mechanism on membrane permeabilisation and associated gene transfer, while at the same time highlighting outstanding questions and difficulties associated with DNA electrotransfer into cells.

## 2. THEORETICAL BACKGROUND OF ELECTROPERMEABILISATION

### 2.1. Basic Physical Considerations

The application of an external electric field to a cell leads to a number of physical effects on it. The major effects are: thermal effects due to the heat generated by the flow of electric current, electrochemical effects and orientational, structural and shape changes induced by electrostatic stresses. This latter category of effects includes the phenomenon of electropermeabilisation, which is ultimately responsible for the passage of molecules across the cell membrane. As well as having a direct effect on the target cell, the electric field will also act on the extracellular medium, in particular the molecules that are transferred into the cell. Electrophoretic forces act on charged molecules, for instance negatively charged models such as DNA are pushed away from the cathode towards the anode. The importance of the electrophoretic forces is dependent on the charge carried by these molecules; for negatively charged DNA they are much more important than for small molecules which carry a smaller electric charge.

Most theories of electropermeabilisation are based on a continuum description of the system and do not start at a molecular level. The bulk of progress in descriptions at the molecular level has been made via numerical simulations. The analytic theories that exist are based on descriptions in terms of local effective electrical and mechanical properties that cannot be easily extrapolated to a molecular description. At the simplest level, the system can be treated as being composed of three components, the region outside the cell between the two plates, the cell membrane and the interior of the cell. The electrical properties of these regions are described by their dielectric constant  $\epsilon$  and their conductivity  $\lambda$ . The mechanical properties of the membrane are described by bulk elastic constants, such as the rigidity and the bulk stretching modulus. These bulk mechanical constants express how the membrane bends and is stretched by the application of external forces or stresses, and can be used to predict shape changes due to the applied field. In order to investigate the possibility of pores being formed, which are presumably responsible for the permeabilisation, one needs to know the surface tension of the membrane  $\Gamma$  as well as the line tension which would be associated with the edges of the pore  $\gamma$ . The surface tension is the (free) energy per unit area of the membrane and the line tension is the energy per unit length of the

edges of a pore. The line tension is positive (so it will cost free energy) as can be seen from the following heuristic physical argument. If we cut a hole in a membrane that is in equilibrium, at the edges the hydrophobic core of the membrane will be exposed to water and this will lead to an increase in the free energy of the system. However the edges will heal in such a way that the hydrophilic lipid heads will occupy the edge in order to hide the hydrophobic core. This mechanical rearrangement at the pore edge will cost mechanical energy, for instance bending energy. Hence, despite the reduction that the mechanical rearrangement achieves, the overall energy change due to pore formation will be positive. Note that if the line tension were not positive the membrane would not be stable and would become full of holes without any external influence. However, because of the thermal energy of the system, fluctuations can occur, allowing the transient formation of pores. If the membrane is impermeable in the absence of an external influence, this means that the pores that might be formed due to thermal fluctuations are too small or too short lived to permeabilise the membrane. Theories of electroporation are based on the idea that an external perturbation (via an applied electric field or a mechanically applied stress, via a micro-pipette for example) energetically favours the formation of a pore and thus reduces the energy barrier (to the order of the thermal energy available to the system) that must be overcome to form a pore.

The simplest theories of electropermeabilisation ignore the mechanical properties of the cell and the shape deformation that can be induced by electrostatic stress. In the simplest approximation, the cell is taken to be spherical. In the steady state, the electric potential  $\psi$  in the system obeys Laplace's equation:

$$\nabla \cdot (\lambda \nabla \psi(r, \theta)) = 0 \quad (1)$$

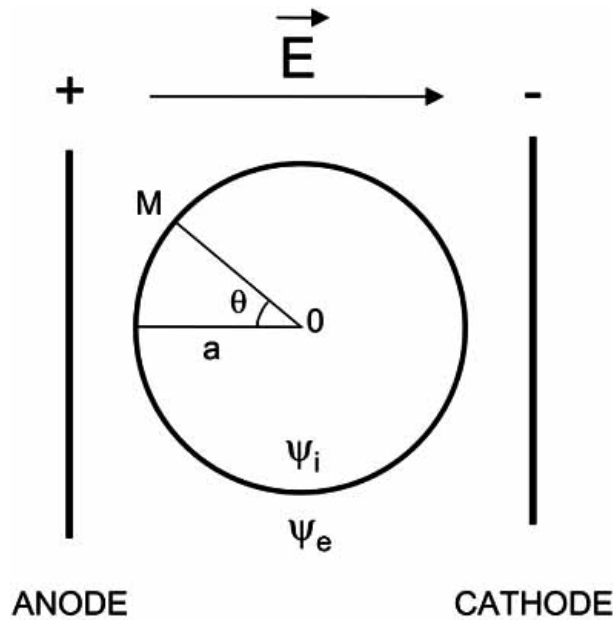
In what follows, we will use the following notation:  $r$  is the distance from the centre of the cell and  $\theta$  is the angle with the normal to the electrodes, which is the direction of the applied electric field far from the cell.

The equation (1) is difficult to solve because of the spatial variation of the conductivity  $\lambda$  and has been studied in various approximations for many years [Bernhardt and Pauly, 1973, Cartee and Plonsey, 1992, Neumann *et al.*, 1989, Schwan, 1957, Zimmermann *et al.*, 1974]. In the limit of a thin membrane, it can be shown that the potential drop across the membrane is given by [Cartee and Plonsey, 1992]:

$$\Delta\psi = \frac{3\lambda_i \lambda_e R_m a E \cos\theta}{a(\lambda_i + 2\lambda_e) + 2\lambda_i \lambda_e R_m} \quad (2)$$

where  $R_m$  is the membrane resistance and  $a$  is the cell radius. The indices  $i$  and  $e$  refer the interior and exterior of the cell and  $E$  is the magnitude of the applied electric field (see Fig. 1). In the limit where the membrane conductivity is much smaller than that of the exterior and interior, this equation simplifies to the Schwan equation [Schwan, 1957]:

$$\Delta\psi = \frac{3aE \cos\theta}{2} \quad (3)$$



**Fig. (1).** Physical principle of electroporation. At a given point  $M$ , the value of the potential depend on  $\psi_i$  intracellular potential,  $\psi_e$  extracellular potential,  $a$  the cell radius and  $\theta$  the angle between the normal to the cell surface and the electric field.

The time dependent generalization of the above equations is :

$$\Delta\psi(t) = \Delta\psi(1 - e^{-t/\tau}) \quad (4)$$

where  $\tau$  is called the membrane polarisation time, or the membrane charging time, and  $t$  is the time elapsed after the (constant) pulse is applied. Equation (3) shows that, for a given external electrical field, the induced transmembrane potential will be maximal at the poles of the cells facing the electrodes ( $\theta = 0$  or  $\pi$ ). However it should be zero at the equator of the cell ( $\theta = \pm \pi/2$ ). We also see that the potential difference is bigger for bigger cells. In order to achieve the full steady state potential drop across the membrane, one must apply the field for a time longer than  $\tau$ . If  $\tau$  is sufficiently short, the steady state membrane potential difference can be quickly attained before heating effects, due to the flow of current, become important. Experimentally determined values for the membrane polarisation time typically lie in the ns range [Beebe *et al.*, 2003, Frey *et al.*, 2006].

When an external electric field is applied, in the simplest approximation, the induced potential is added to the native or rest potential of the cell. The latter varies with the cellular type but is generally found to be between  $-70$  and  $-100$  mV. When the sum of native and induced transmembrane potential reaches a critical value :

$$\Delta\psi_{nat} + \Delta\psi_{ind} = \Delta\psi_m \geq \Delta\psi_c \quad (5)$$

The lipid membrane becomes permeable [Hibino *et al.*, 1991, Kotnik and Miklavcic, 2000]. This critical value has been evaluated to be of the order of 200-300 mV and appears to be rather insensitive to the cell type [Gabriel and Teissie, 1997, Teissie and Rols, 1993]. We shall see later that the existing theories imply that the degree of permeabilisation

depends on the absolute value of the potential drop across the cell  $|\Delta\psi|$ . The presence of a rest potential implies that there is a difference between the regions where the angle to the field is  $\theta$  and that where it is  $-\theta$  and this asymmetry has been observed experimentally [Gabriel and Teissie, 1997].

## 2.2. The Effect of the Electric Field on a Membrane

Having established that one of the major effects of applying an electric field across a cell is to induce a potential drop across its membrane, we will now explore the physical consequences for the membrane. Systems that are subjected to extremely large electric fields can under go dielectric breakdown where the electric field disrupts their molecular structure. Dielectric breakdown of the cell membrane due to an induced electric field was first observed in the early 1970s [Crowley, 1973, Kinoshita and Tsong, 1977, Neumann E. and Rosenheck, 1972]. Varying the different parameters of the induced electric field across the membrane (value, duration of the pulse, delay between each pulse) can lead to different effects: a slight increase in membrane conductivity, incomplete or totally reversible electrical breakdown, or destruction of the cell membrane [Benz *et al.*, 1979, Ho and Mittal, 1996].

As mentioned previously, in the presence of electric fields lipid bilayers are deformed because of the electric stress, this stress can be calculated using the Maxwell stress tensor and the subsequent induced deformation is determined by the mechanical properties of the membrane. This effect of deformation has been studied theoretically on lipid vesicles, both for alternating fields [Hyuga *et al.*, 1993, Kumorrow and Helfrich, 1991] and for square-wave pulses [Hyuga *et al.*, 1991a, Hyuga *et al.*, 1991b]. The basic theory of dielectric breakdown [Crowley, 1973] is based on a flat membrane geometry and thus ignores possibly important geometric effects. It is based on a model of an elastic capacitor that can be compressed. The drop in electric potential across the membrane causes a compressive force and it can be shown that beyond a critical potential drop that an equilibrium is no longer possible between the elastic and electrostatic forces. This instability is interpreted as the signal of a dielectric breakdown. This theory has a number of problems, firstly it does not actually describe the physics of what happens after the breakdown and secondly it predicts a variation in the capacitance of the membrane as the applied field is changed; this variation in capacitance is not observed experimentally.

What is, however, clear is that the membrane will respond to the applied electric field in order to reduce the electrostatic energy of the system and establish an equilibrium between mechanical and electric forces. Compression and deformation are two possible responses, however the phenomenon of permeabilisation suggests another possibility, the formation of pores. Experiments show that membranes subjected to an electric field can allow the passage of charged molecules. The change in the electrostatic energy of an ion going between water (with a high dielectric constant) into the membrane (which has a low dielectric constant) is extremely large and thus unlikely at room temperature. A way of getting around this problem is to assume that pores can form which are water filled, the presence of water in the pores reduces the energy barrier that the ion would need to

cross [Parsegian, 1969]. In addition, the formation of pores can reduce the electrostatic energy of the system [Weaver, 1995]. As discussed above, if a pore is formed, there will be positive contribution to the energy of the system coming from its line tension. The existence of spontaneous pore formation due solely to thermal fluctuation inside the membrane (without any external electric field) has been observed [Karatekin *et al.*, 2003, Sandre *et al.*, 1999]. The energy cost to form an hydrophilic pore in a planar lipidic membrane suspended in a frame is given by :

$$W(r_p) = 2\pi\gamma r_p - \Gamma\pi r_p^2 \quad (6)$$

where  $r_p$  is the radius of the pore. We have assumed that the pore is circular and therefore the first term is the energy cost due to the line tension (proportional to the pore circumference) and the second term comes from the diminution of the surface area. This picture is only valid for the suspended planar membrane and the situation for a cell or vesicle is considerably more subtle and complicated. However, for our purposes, the planar example will suffice to give the basic idea of the physics involved.

The creation of a pore relies on the interplay between the line tension and the surface tension of the lipid bilayer as can be seen in Eq. (6). For small  $r$ , we see that the pore energy  $W$  is positive and thus an energy barrier has to be overcome in order to create a pore. If the radius of the pore becomes too big, equation (6) describes an unstable system which is thought to signify membrane rupture [Weaver, 1995]. The basic idea behind the theory of electroporation (and here we very much paraphrase the full theoretical knowledge on this subject) is that the presence of the applied electric field (of magnitude  $E$ ) effectively increases the surface tension of the system and, in the simplest of approximations, this effective surface tension is given as a function of  $E$  by :

$$\Gamma(E) = \Gamma(0) + AE^2 \quad (7)$$

where  $A$  is a positive constant depending on the physical properties of the membrane.

The presence of the field thus increases the surface tension and consequently lowers the energy barrier associated with pore formation. One should bear in mind that the above argument is based on the physics of a flat membrane. Experiments on cells and vesicles suggest a critical field strength for permeabilisation, this could be explained by the fact that the membrane can tolerate a certain amount of electrostatic stress by shape deformation or the smoothing of membrane undulations [Kakorin *et al.*, 2005] before the surface tension is significantly increased. A major problem with this picture is that it is, like the theory of dielectric breakdown, based on bulk thermodynamic quantities and a molecular interpretation is not available. This certainly poses conceptual problems for the first stage of pore formation as equation (6) does not make much sense when  $r_p$  is the order of the size of a lipid head group.

### 2.3. Formation of Pores in Lipid Membranes as Seen by Molecular Modelling

Recently, computer simulations at the nano length scale have become possible. However the complexity and, more

importantly, the size of the systems that can be simulated are still rather limited. These simulations have shed the light on the first steps of electropore formation. It has been shown that, under a high electric field, pore formation can be induced in bilayers on a nanosecond time scale [Tieleman *et al.*, 2003]. It has been found that, for a large enough system, multiples pores with sizes up to 10 nm formed independently [Tieleman, 2004]. These simulations suggest that pore formation occurs in two steps:

- penetration of water molecules in a single file line or wire into the hydrophobic core of the bilayer, apparently favoured by local defects in the lipid headgroup region.
- growth in the length of these wires and their expansion to form water filled pores stabilized by lipid head groups that migrate from the membrane-water interface to the middle of the bilayer.

A recent molecular dynamics study on a small patch (64 lipids in each leaf of the bilayer) of a bilayer has shown translocation of DOPS from one layer to the other by electrophoresis along the electropore created during the pulse [Vernier *et al.*, 2006]. Nevertheless, it is important to notice that electropores in simulations are obtained under field values much larger and time exposure much smaller than those experimentally required to induce reversible membrane permeabilisation [Tarek, 2005].

### 2.4. Experiments on Electric Field Effects on Model Lipid Membranes

Experiments on the electrodeformation of lipidic vesicles have been performed on small unilamellar vesicles (SUVs) [Kakorin and Neumann, 2002, Neumann *et al.*, 1998] and on large vesicles [Teissié and Tsong, 1981] using indirect observations (changes in turbidity and absorbance, electrical conductance monitoring, radioactive loading and leakage monitoring). Most of these studies on SUVs have indicated the formation of aqueous pores allowing molecules to cross the membrane. Over the last few years, studies have been performed using giant unilamellar vesicle (GUVs) whose sizes are closer to those of cell plasma membranes. Moreover GUVs have sizes that allow them to be visualized by optical microscopy. Using pure DOPC GUVs, Tekle *et al.*, [Tekle *et al.*, 2001] observed pores of  $\sim 3 \mu\text{m}$  lasting for  $\sim 150$  ms after the end of the field pulse (700  $\mu\text{s}$ ). They also observed a loss of lipids associated with pore formation. Recently, experiments have been conducted using ultra-fast microscopy (1 image every 33  $\mu\text{s}$ ) in order to directly visualised the formation of pores [Riske and Dimova, 2005]. They observed the existence of macropores (radius  $\sim 1 \mu\text{m}$  in vesicles of 10  $\mu\text{m}$  radius) lasting 10 ms after the end of the field application (300  $\mu\text{s}$  duration) showing that membrane integrity recovery takes longer than pore creation. Finally, it has recently been shown that the presence of cholesterol in the membrane of SUVs reduces the degree of electroporation [Kakorin *et al.*, 2005]. The addition of cholesterol was shown to alter the mechanical properties of the membrane, increasing both the membrane rigidity and the membrane stretching modulus. This clearly demonstrates the way in which the phenomenon of electroporation for cells and vesicles is intrinsically dependent on their mechanical properties.

## 2.5. Effect of Electric Field on Cell Membranes

All the results exposed in section 2.4 strongly suggest that even in a "simple" (without proteins) lipid bilayer, lipid composition, surface tension and solvent composition will strongly affect the process of permeabilisation. It is thus very difficult to extrapolate these results to the case of a cell membrane.

The cell plasma membrane is a mosaic and should be considered as a highly complex molecular system with different lateral organisation at different length-scales. This lateral organisation (micro and macro-domain) is also in direct interaction with the interior of the cell. From a purely physical point of view, embedding proteins inside a lipid membrane, or "attaching" the membrane to fixed proteins (such as actine or tubuline networks), will dramatically change the local values of  $\gamma$  and  $\Gamma$  and thus will alter the energy necessary to create a pore and also the mean size and the mean lifetime of these pores.

Up to now, large pores, arising from primary pores, have only been detected in cells pulsed under hypo osmotic conditions [Chang and Reese, 1990] suggesting, in this case, a very high value of  $\Gamma$  for the red blood cell membrane which favours the creation of pores.

Few experimental results on the molecular changes accompanying membrane electropermeabilisation have been published.  $^{31}\text{P}$  NMR studies, performed both on model membranes and on mammalian cells, point to a reorganisation of the polar head group region of the phospholipids, leading to a weakening of the hydration layer [Lopez *et al.*, 1988, Stulen, 1981]. Transbilayer reorientation of phospholipid probes has been observed in the human erythrocyte membrane, suggesting an increase in the flip-flop of phospholipids as a direct consequence of electropermeabilisation [Haest *et al.*, 1997].

Finally, in most cases, pores have never been observed in living cells submitted to external electric field, showing that the standard theory of electroporation is far from being experimentally proven [Teissie *et al.*, 2005].

As a final cautionary note, even if the term electroporation is commonly used among biologists, one should strictly use the term of electropermeabilisation, which describes the observed phenomenon and does not presuppose its physical origin.

## 3. EFFECT OF EXTERNAL ELECTRIC FIELD ON DNA UPTAKE

### 3.1. DNA Interaction with Model Lipid Membranes

Despite the fact that the pioneering work on plasmid DNA electrotransfer in cells was initiated more than 20 years ago [Neumann E. *et al.*, 1982], many of the mechanisms underlying DNA electrotransfer remain to be elucidated. Since membrane lipids are mainly zwitterionic or negatively charged, it seems unlikely that a direct electrostatic interaction is sufficient to induce the initial interaction between the membrane and the DNA. The electrostatic affinity for membrane-DNA interaction can be increased by the introduction into the system of divalent cations [Francescu *et al.*, 2006] or positively charged sphingosides [Angelova *et al.*, 1999].

However, interactions that have been observed between DNA and model lipid bilayers suggest that other mechanisms may play a role in membrane-DNA interaction. For example, DNA-induced endocytosis has been observed, in the absence of any electric field, when DNA is injected using a micro pipette next to the surface of a giant unilamellar vesicle [Angelova *et al.*, 1999, Angelova and Tsoneva, 1999]. A possible mechanism for this DNA/lipid membrane type of interaction is DNA encapsulation within an inverted micelle included in the lipid membrane. High molecular mass DNA can be efficiently taken up by large unilamellar vesicles exposed to a short pulse of electric field (0.1-1 ms) with an intensity as high as 12.5 kV/cm and there is evidence that this DNA take up is the result of the electrostimulated formation of endosome-like vesicles rather than via field-induced membrane pores [Chernomordik *et al.*, 1990].

Other experiments suggest that the electrotransfer of DNA through lipid bilayers could be mediated by transient complexes between the DNA and the lipids in the pore edges of elongated, electropercolated hydrophilic pore zones [Hristova *et al.*, 1997]. Moreover, the association of DNA with a lipid bilayer greatly facilitates the transport of small ions. This suggests the existence of a locally conductive DNA/lipid interaction zone where parts of the DNA strand may be transiently inserted into the bilayer and with, probably, the rest of the DNA protruding out from the outer surface of the bilayer.

Direct DNA crossing through electropores in the lipid bilayer has also been suggested. De Gennes has theoretically shown the possibility that DNA, once inserted in an electropore, can pass through the lipid bilayer, in a time of the order of a second [de Gennes, 1999], due to the presence of a thermodynamic gradient. This passage is aided by the presence of an electric field as the DNA is subjected to an electrophoretic force which pulls it through the permeabilised zones to the inside of membrane while leaving the bilayer structure basically intact at the end of the process [Spassova *et al.*, 1994].

Finally, a recent result has shown that adsorption of DNA at the surface of anionic vesicles in the presence of added divalent cations decrease the rigidity of the membrane and it is argued that this facilitates electropore formation. This is thus an example of a cooperative effect that can be exploited to optimise the uptake of DNA [Francescu *et al.*, 2005].

### 3.2. DNA Interaction with the Cell Membrane

In the case of cells, the process involved in electrically mediated DNA uptake are surely more complex than purely an interaction of DNA with the plasma membrane. In addition DNA uptake is much more complex than the simple and rapid transfer of small hydrophilic molecules into the cytosol. Small soluble molecules can freely cross the permeabilised membrane for a time much longer than the time during which the electric pulse is applied, i.e. the membrane remains permeable to these molecules for a significant time after the field is cut. However DNA transfer requires that the DNA is present during the application of the electric field pulses and involves complex steps, presumably occurring over relatively large time scales. These steps include the initial interaction with the electropermeabilised membrane, the

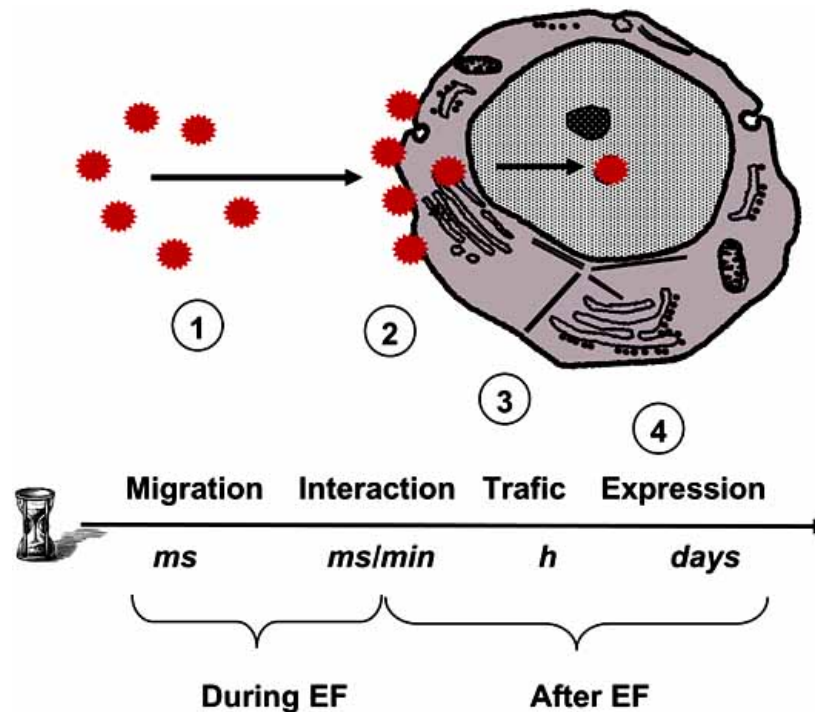
crossing of the membrane, transport within the cell and finally gene expression.

Single-cell microscopy and fluorescent plasmids can be used to monitor these different steps of electrotransfection [Golzio *et al.*, 2002a]. DNA molecules, which we recall are negatively charged, migrate electrophoretically when submitted to the electric field. Under electric fields which are too small to permeabilise the membrane, the DNA simply flows around the membrane in the direction of the anode. However, beyond a critical field value, above which cell permeabilisation occurs, the DNA interacts with the plasma membrane. This interaction only occurs at the pole of the cell opposite the cathode and this demonstrates the importance of electrophoretic forces in the initial phase of the DNA/membrane interaction. We recall that the electric field drives the DNA in the direction of the anode. When the membrane is not permeabilised, the electric field lines follow the outer profile of the cell and the DNA flows in the direction of the field lines around the cell to the anode. When the membrane is permeabilised, the field lines can enter it. The DNA can thus be trapped in the region opposite the cathode where it is effectively pushed up against the membrane by the electrophoretic force and the interaction with the permeabilised membrane prevents it from flowing around the cell. At the pole of the cell opposite the anode, even if this face is permeabilised, the electrophoretic force on the DNA forces it away from this pole and towards the anode. This is contrast with small molecules which are observed to enter both poles [Golzio *et al.*, 2002b]. The difference is due to the fact that small molecules diffuse faster and also carry less net charge than the DNA. The fact that small molecules carry less

charge means that the electrophoretic forces to which they are subjected is much smaller; their transport is thus dominated by diffusion. When the DNA-membrane interaction occurs, one observes the formation of *microdomains* whose dimensions lie between 0.1 and 0.5  $\mu\text{m}$ . Also seen are clusters or aggregates of DNA which grow during the application of the field. However once the field is cut the growth of these clusters stops. These observations are consistent with the hypothesis that plasmids interact with the electropermeabilised part of the cell surface, which allows DNA transfer, but that their accumulation in that region is driven by electrophoretic forces [Phez *et al.*, 2005b, Rols, 2006].

We can thus conclude that electrotransfection is a multi step process (Fig. 2):

- step 1: the negatively charged DNA migrates electrophoretically towards the plasma membrane on the cathode side where it accumulates.
- step 2: for electric field values above a threshold, the plasma membrane is permeabilised allowing the accumulated plasmid DNA to be inserted into it. This interaction, which is observed for several minutes, lasts much longer than the duration of the electric field pulse.
- step 3: translocation of the plasmid from the plasma membrane to the cytoplasm and its subsequent passage towards the nuclear envelope taking place with a kinetics ranging from minutes to hours.
- step 4: when plasmid has reached the nuclei, gene expression can take place and this can be detected up to several weeks later.



**Fig. (2).** The different steps involved in electrotransfection. 1: during the application of the electric field, DNA molecules migrate towards the electropermeabilised plasma membrane where 2) they interact with the membrane and can become inserted. This interaction proceeds for a few ms to a few min. 3: after the electric field application, transport of DNA in the cytoplasm takes place with a kinetics ranging from min to hours, finally leading to 4) gene expression.

Electrically induced DNA uptake by cells can be considered as a vectorial process with the same direction as DNA electrophoresis in an external electric field [Klenchin *et al.*, 1991, Sukharev *et al.*, 1992]. The polarity of the electric field has therefore a direct effect on transfection. Adherent cells facing the cathode exhibit much higher transfection yield as well as gene expression than cells facing the anode [Muller *et al.*, 2003]. This dependence of the transfection efficiency on the direction of the field might be due to the involvement of the electrophoretic force in the translocation of the negatively charged DNA molecule. While cell permeabilization is only slightly affected by reversing the polarity of the electric pulses or by changing the orientation of pulses, transfection level increases are observed. These last effects are due to an increase in the cell membrane area where DNA interacts [Faurie *et al.*, 2004]. *In vivo*, the effect of electrophoresis is not completely understood. A lot of studies show the benefit of the combination of short high-voltage and long low-voltage pulses allowing to evidence the necessity of association of cell electropermeabilization and convenient electrophoretic transport of DNA toward and/or across the permeabilized membrane within the tissue [Satkusas *et al.*, 2005]. But a recent publication indicates that electrophoresis could not play an important role in electro-gene transfer [Liu *et al.*, 2006].

New lines of research are now necessary to characterise the membranes domains observed during electrotransfer. Intriguingly their size is of the same order as the so called raft domains that have been the subject of much recent activity and debate. It has been suggested that these raft domains serve as platforms for various cellular events, such as signalling and membrane trafficking [Brown and London, 1998, Brown and London, 2000]. One can thus pose the question as to whether these rafts are also involved in DNA electro-transfer [Phez *et al.*, 2005a].

### 3.3. DNA Expression

Once the first stage of gene electrotransfection, i.e. migration of the plasmid DNA towards the electropermeabilised plasma membrane and its interaction with it, becomes well understood we will be able to give guidelines to improve this first step in gene electrotransfer protocols. However, successful expression of the plasmid depends on its subsequent migration into the cell. Therefore, the intracellular diffusional properties of plasmid DNA, as well as its metabolic instability and nuclear translocation, represent other cell limiting factors that must be taken into account [Lechardeur and Lukacs, 2002, Lechardeur and Lukacs, 2006].

The cytoplasm is composed of a network of microfilament and microtubule systems, along with a variety of sub-cellular organelles present in the cytosol. The mesh-like structure of the cytoskeleton, the presence of organelles and the high protein concentration means that there is substantial molecular crowding in the cytoplasm which hinders the diffusion of plasmid DNA. The in cell mobility of small molecules (i.e. less than 500-750 kDa) is only 3-4 fold slower than in water, but this reduction is much more significant for larger molecules [Seksek *et al.*, 1997]. The mobility of plasmid DNA is extremely small in the cytoplasm of microin-

jected myotubes [Dowty *et al.*, 1995]. However, successful *in vivo* DNA expression can be obtained by applying electric fields. These apparently contradictory results might be reconciled by the possibility of a disassembly of the cytoskeleton network that may occur during electropermeabilisation [Rols and Teissie, 1992], and is compatible with the idea that the cytoplasm constitutes an important diffusional barrier to gene transfer. In the conditions induced during electropermeabilisation, the time a plasmid DNA takes to reach the nuclei is significantly longer than the time needed for a small molecule [Golzio *et al.*, 2002a]. Therefore, plasmid DNA present in the cytosol after being electrotransferred can be lost before reaching the nucleus, for example because of cell division. Stability of plasmid DNA has been quantitatively assessed by microinjection. In such experiments, 50% of the DNA is shown to be eliminated in 12 hours from HeLa and COS cells and in 4 hours from myotubes [Lechardeur *et al.*, 1999]. Cytosolic elimination of plasmid DNA can not be attributed solely to cell division, since its decrease is also observed in non-dividing cells. In the case of muscles, radio-labelled plasmid is observed to progressively leave muscles and is degraded as soon as 5 min after plasmid injection. A major part of plasmid DNA is rapidly cleared and degraded and the electrotransferable pool of plasmid DNA represents only a very small fraction of the amount initially injected and belongs to another compartment where it is protected from endogenous DNases [Bureau *et al.*, 2004].

Finally, after the cytoskeleton, the nuclear envelope represents the last, but by no means the least important, obstacle to the expression of the plasmid DNA. The inefficient nuclear uptake of plasmid DNA from the cytoplasm was first identified more than 20 years ago. While molecules smaller than 40 kDa can diffuse through the nuclear pore complexes, larger molecules require a specific targeting signal, the nuclear localization sequence, in order to cross. The relatively large size of plasmid DNA (2-10 MDa) makes it unlikely that the nuclear entry occurs by passive diffusion. Dividing cells are highly transfectable compared to quiescent ones, suggesting that DNA enters the nucleus upon the disassembly of the nuclear envelope during mitosis. Synchronizing the electrotransfer protocol with mitosis has been shown to improve gene delivery [Golzio *et al.*, 2002b, Schwachtgen *et al.*, 1994, Takahashi *et al.*, 1991], supporting the hypothesis that the melting of the nuclear membrane facilitates the direct access of plasmid DNA to the nucleus.

### 3.4. New Developments

Clear limits of efficient gene expression using electric pulses are due to cytoplasmic crowding and transfer through the nuclear envelope. A key challenge for electro-mediated gene therapy is to pinpoint the rate limiting steps in this complex process and to find strategies to overcome these obstacles. As mentioned above, the dense latticework of the cytoskeleton impedes free diffusion of DNA. However, since transfections do work, there must be mechanisms by which DNA circumvents cytoplasmic obstacles. One possibility is that plasmids become cargo on cytoskeletal motors, much as viruses do, and move to the nucleus in a directed fashion. Electrotransferred plasmid DNA, containing specific sequences could then use the microtubule network and its as-

sociated motor proteins to move through the cytoplasm to the nucleus [Vaughan and Dean, 2006].

Another alternative, coming from new technologies, concerns nanosecond pulsed electric fields. Recent studies indicate that very short (10-300 ns) but high pulses (up to 300 kV/cm) induce effects, beyond classical electroporation, that primarily affect intracellular structures and functions. As the pulse duration is decreased, below the plasma membrane charging time constant, plasma membrane effects decrease and intracellular effects predominate [Beebe *et al.*, 2003, Schoenbach *et al.*, 2001]. A possible idea, to improve transfection success, is to perform classical membrane permeabilisation allowing plasmid DNA electrotransfer, and then after, when DNA has reached the nuclear envelope, to specifically permeabilise it using these short strong nanopulses. Thus, when used in conjunction with classical electroporation, nanopulses could be used to increase gene expression yields. In this way, it becomes possible not only to simply electroporate cells to transfer DNA into the cytosol, but to fully electromanipulate cells by allowing DNA to be efficiently transferred into the nuclei.

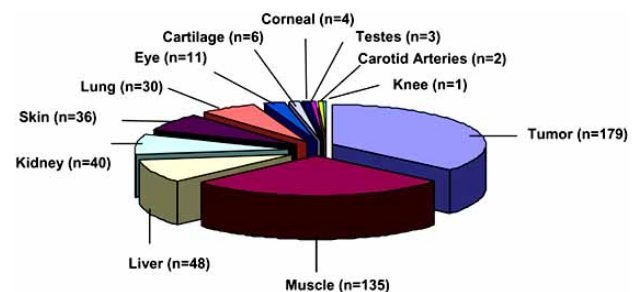
#### 4. *IN VIVO* GENE ELECTRO DELIVERY

Intensive research has gone into the development of safe and efficient methods for therapeutic delivery. In this context, electrotransfer opens interesting perspectives in medicine. The use of electric pulses to safely deliver therapeutic molecules was first developed in oncology. The concept of electrochemotherapy emerged more than 10 years ago [Behrdek *et al.*, 1993, Mir *et al.*, 1991a, Mir *et al.*, 1991b]. Electric pulses, directly applied to tumours following drug injection, enable highly cytotoxic molecules to gain direct access to the cytosol. This process has been successfully applied in clinical trials with bleomycin and cisplatin [Gehl, 2003, Gothelf *et al.*, 2003, Mir *et al.*, 1998, Sersa *et al.*, 1998]. Repetitive treatments can be performed with a 2-week interval in the case of malignant tumours in humans [Mir *et al.*, 1998] and in the case large animal tumours such as cat sarcoma [Mir *et al.*, 1997] and horse sarcoid [Rols *et al.*, 2002]. To date, clinical protocols with electrochemotherapy have been encouraging, with objective responses and effective symptomatic palliation in a variety of cancers [Tozon *et al.*, 2005, Spugnini and Porrello, 2003]. Most of the treated nodules respond, with objective responses in 60–100% of cases. Electrochemotherapy represents proof that electric fields can be safely and efficiently applied to tissues for medical purposes. Because of current technological limitations, clinical experience with electrochemotherapy has been confined to cutaneous and subcutaneous malignancies. However, electrode systems are under development for intraluminal delivery by endoscopes, laparoscopic systems or intravascular catheters [Soden *et al.*, 2006].

As well as drugs, electrotransfer can be used to efficiently deliver a wide range of potentially therapeutic agents including proteins, oligonucleotides, RNA and DNA [Golzio *et al.*, 2004, Golzio *et al.*, 2005]. Its main advantages in the field of gene delivery are that, because only DNA is used, electrotransfer can safely be repeatedly administered and only DNA is electrotransferred into tissues. Moreover, the tissues that are treated are simply selected by placement of

the electrodes. The method is also simpler and more economical than viral approaches, especially for clinical trials. It allows the elimination of the risks and limitations associated with the use of viruses, for example insertional mutagenesis, immunological responses and limitations due to the size of the coding sequence, which are encountered respectively in retroviruses, adenoviruses and adeno associated viruses. It can be performed both *in vitro*, *ex vivo* and *in vivo*. In addition to its potential use in gene therapy, *in vivo* DNA electrotransfer is also, because of its simplicity, a powerful laboratory tool to study *in vivo* gene expression and function in a given tissue [Scherman *et al.*, 2002]. This, together with the capacity to deliver very large DNA constructs, greatly expands the research and clinical applications of *in vivo* DNA electrotransfer [Heller *et al.*, 1996, Nishi *et al.*, 1996, Gehl *et al.*, 1999, Miklavcic *et al.*, 2000, Prud'homme *et al.*, 2006].

Therefore, in the field of gene therapy, electrotransfer represents an increasingly attractive non-viral method, and the exponential increase in number of publications on the subject over the last 10 years testifies to this. Electrotransfer is applicable to all easily accessible organs, but also to internal organs when combined with surgery as shown in Fig. 3. Gene expression can be transient or long term depending on the tissue in question. One of the most widely targeted tissues for DNA transfer is skeletal muscle [Aihara and Miyazaki, 1998, Mir *et al.*, 1999, Rizzuto *et al.*, 1999]. This strategy is promising for the systemic secretion of therapeutic proteins [Scherman *et al.*, 2002], increasing by more than 100-fold the production and secretion obtained by the injection of naked DNA. Vaccination and oncology gene therapy are also major fields of application of electrotransfer [Bloquel *et al.*, 2004]. An increasing number of published studies and reviews have established its potential for therapeutic applications [Bloquel *et al.*, 2004, Cemazar *et al.*, 2004, Cemazar *et al.*, 2006, Dujardin and Preat, 2004, Gehl, 2003, Heller and Heller, 2006, Mir *et al.*, 2005, Scherman *et al.*, 2002, Prud'homme *et al.*, 2006].



**Fig. (3).** Target tissues for gene therapy by electro gene delivery. This graph has been obtained by using Pub Med as a bibliographic resource using electroporation and gene therapy as key words; n represents the number of publications for each organ or tissue type.

#### CONCLUSION

Further studies are still necessary to understand the cascade of events triggered by electroporation at the tissue level. Here, factors arising from tissue organisation and the consequent inhomogeneity of the electric field strength and intercellular distribution of DNA [Pavselj and

Preat, 2005] need to be taken into account. Also, some of the fundamentals of electropermeabilisation still need to be understood in terms of molecular events at the plasma membrane. Clinical trials will however provide the ultimate indication of viability of *in vivo* electrogene transfer. The first clinical trial was initiated on patients with subcutaneous metastases with melanoma in late 2004 at the H. Lee Moffitt Cancer Center and Research Institute (Tampa, Florida, USA). The objective of this trial is the determination of the toxicity and maximum tolerated dose of electrotransferred plasmid coding for the human IL-12 cDNA [Heller and Heller, 2006]. Veterinary clinics may also benefit from electrogenotherapy; similar clinical trial being performed on mast cell tumors in dogs at the Veterinary School of the University of Ljubljana (Slovenia) in collaboration with the institute of Oncology (Ljubljana, Slovenia) [Tozon *et al.*, 2006] as well as in sarcoïd in horses at the Veterinary School of the Université Paul Sabatier (Toulouse, France) in collaboration with the Institute of Pharmacology and Structural Biology (Toulouse, France) of the CNRS and the Claudius Regaud Cancer Center (Toulouse, France) [Tamzali *et al.*, 2006]. The objectives of this last trial are to improve standard electrochemotherapy in horses [Rols *et al.*, 2002] by exploiting electrogenotherapy to stimulate the immune system with local IL-12 expression, an approach called electroimmunogenotherapy. A first indication of the true potential of electropermeabilisation for gene therapy as a safe and efficient non viral method will be given upon the publication of the results of these clinical trials.

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