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Revisiting the role of pulsed electric fields in overcoming the barriers to *in vivo* gene electrotransfer

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ABSTRACT

Gene therapies are revolutionizing medicine by providing a way to cure hitherto incurable diseases. The scientific and technological advances have enabled the first gene therapies to become clinically approved. In addition, with the ongoing COVID-19 pandemic, we are witnessing record speeds in the development and distribution of gene-based vaccines. For gene therapy to take effect, the therapeutic nucleic acids (RNA or DNA) need to overcome several barriers before they can execute their function of producing a protein or silencing a defective or overexpressing gene. This includes the barriers of the interstitium, the cell membrane, the cytoplasmic barriers and (in case of DNA) the nuclear envelope. Gene electro-transfer (GET), i.e., transfection by means of pulsed electric fields, is a non-viral technique that can overcome these barriers in a safe and effective manner. GET has reached the clinical stage of investigations where it is currently being evaluated for its therapeutic benefits across a wide variety of indications. In this review, we formalize our current understanding of GET from a biophysical perspective and critically discuss the mechanisms by which electric field can aid in overcoming the barriers. We also identify the gaps in knowledge that are hindering optimization of GET *in vivo*.

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1. Introduction

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Gene therapy is revolutionising the field of medicine by offering potential unprecedented treatments to devastating diseases of various origins, with cancer, inheritable diseases, infectious diseases

by offering ases of varus diseases been approved for cancers such as head and neck squamous cell carcinoma, Acute Lymphoblastic Leukaemia, B-cell Lymphoma and unresectable Metastatic Melanoma, and for inheritable diseases such as Lipoprotein Lipase Deficiency, Adenosine Deaminase Deficiency - Severe Combined Immunodeficiency or ADA-SCID and Retinal Dystrophy [1,3]. Approval of these therapies, especially

and cardio-vascular diseases currently holding the major share of indications [1,2]. Treatments based on (cell and) gene therapy have

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with the advent of Chimeric Antigen Receptor T-Cell (CAR-T cell) therapy which is contingent upon genetic engineering of T-cells, represents a hallmark in the field of medicine since they provide supreme remission rates to untenable cancers [4]. Additionally, gene-based vaccines made of ribonucleic acid (RNA) (BNT1262b2 and mRNA-1273) were the first to receive approval for vaccination against the infectious Corona Virus Disease - 2019 (COVID-19) pandemic at a record breaking speed of less than 12 months [5]. Their safety, potency, low cost, rapid production and scalability following identification of the virion make them superior to previous generation vaccines [6-8]. With gene editing technologies, especially CRISPR/Cas9, gene therapies are no longer limited to adding a specific gene to the target cells but are now also capable of editing entire defective genetic sequences [9]. Although still in the nascent stage, such gene editing technologies have not only expanded the indications falling under the gambit of gene therapies but have also elevated the potential impact of gene therapies in the field of medicine.

For gene therapies to take effect, DNA or RNA (deoxyribonucleic acid or ribonucleic acid) must enter the cell to produce a protein or to silence a defective or overexpressing gene. This entails the nucleic acid to overcome several barriers before it can reach the cytoplasm of the cell (for RNA) or the nucleus of the cell (for DNA) to enable its therapeutic action. These barriers are: the interstitial barriers, the cell membrane barrier, the cytoplasmic barriers and the nuclear envelope. After the pioneering work of Wolff et al. [10], who injected naked DNA and RNA into mouse skeletal muscle in vivo and observed trans-gene (protein) expression, it soon became clear that these barriers severely limit the efficiency of gene therapies mediated by naked DNA and RNA injection. Following DNA or RNA injection into the muscle, only minute amounts can enter the cell. For instance, DNA starts to degrade as soon as 5 min after injection into mouse muscles [11]. Thus, researchers are actively investigating possibilities to devise strategies that can overcome these barriers.

So far, viral and non-viral vectors have been researched for DNA and RNA delivery. Viral vectors possess excellent capability to overcome the barriers and are now being approved for treating diseases. Till date, 13 gene therapies have been approved in vivo based on using viral vectors to overcome the barriers [12]. However, viral vectors have some alarming drawbacks: pre-existing immunity and immune reactions following injection of viral vectors can reduce the effectiveness of the therapy and cause immunotoxicity - precluding the use of particular viruses in certain geographic locations and certain patients [13]. While most of these concerns related to viral vectors are being addressed by modifying and engineering the viral vectors, non-viral vectors are gaining traction as feasible and, in some cases, even superior (at least in terms of safety) alternatives to viral gene delivery [14]. Non-viral vectors that are being developed fall broadly into the categories of chemical vectors (e.g., polymer, lipid-based and various inorganic nano-carriers etc.) and physical vectors (e.g., ballistic, laser, ultrasound, electroporation etc.). Non-viral vectors are in principle devoid of an immune response per se but they lack the high efficiency of viral vectors in vivo [1,3,14,15].

A non-viral method which shows great promise is naked DNA injection followed by the application of pulsed electric field (PEF). DNA transfection, referred to as cellular uptake of DNA and subsequent gene expression, mediated by PEF is known as Gene Electrotransfer (GET). DNA transfection, GET and a few other terms that will be used throughout the review are formally defined in Section 2. Although GET is applicable to both DNA and RNA, we will be restricting the purview of this review, and of GET, to only DNA as it has been the prevalent molecule under investigation in GET literature. GET increases transfection rates of DNA by 100–2000 times and improves reproducibility of transfection compared

to naked DNA injection without the application of PEF [16-20]. Several clinical trials are underway evaluating the efficacy of GET in oncology for treatment of, and vaccination against, cancer, and for vaccination against infectious diseases [21-33]. A GET based DNA vaccine [34,35] is currently under Phase (2/3) investigation for COVID-19 pandemic (NCT04336410 and the INNOVATE trial -NCT04642638). Further, since GET is capable of delivering large genetic payloads, it is considered as a promising technique for CRISPR/Cas9 gene editing applications [36,37]. CRISPR/Cas9 applications mediated by GET greatly amplify the prospect of GET in the field of medicine and therapeutics.

Although, several clinical investigations indicate that GET is a safe and an effective clinical technique providing therapeutic benefits, it has taken around 40 years to reach this stage. Over the years, efforts have been made to improve GET. GET of DNA encoding for monoclonal antibodies in large animals such as non-human primates provides a quantitative example to illustrate how optimizations have led to an improvement in the efficiency: dose finding studies in combination with optimizing devices and delivery protocols have led to an increase in the serum antibody levels from a few ng/ml to greater than 30 µg/ml [38]. Some of these optimizations were based on targeting the interstitial barrier using extracellular matrix digesting enzymes and aiding in better distribution of DNA in the interstitium. While GET has taken several strides to reach the clinic, much of the success can be attributed to our increased understanding of how DNA molecules, due to PEF, overcame the barriers they encountered on their way and reached the nucleus. The knowledge of how DNA molecules interact and overcome the barriers during GET is scattered over the literature, which dates as far back as 1982 with the first report of *in vitro* GET [39]. The purpose of this review is to revisit the role of PEF in overcoming the barriers to GET in vivo. Since the cell membrane and intracellular barriers are discussed in detail along with the interstitial barrier, the review is also relevant for understanding GET in vitro. We, thus, critically review existing literature that helps formalize the current understanding of GET and barriers limiting its efficiency. We also identify gaps in current understanding and suggest directions of future research to further enable an enhanced understanding of DNA delivery to cells using GET.

2. Brief overview and current understanding of mechanisms involved in gene electrotransfer

GET is a complex process and to get a good grip on the current understanding of GET it is prudent to explain the process based on *in vitro* systems which are more amenable to investigative rigour even though they are oversimplified compared to processes *in vivo*. According to *in vitro* experiments, GET is a multi-step process which involves (i) interaction of DNA with the cell membrane (Fig. 1 B.1) (ii) translocation through the cell membrane (Fig. 1 B.2) (iii) migration across the cytoplasm (Fig. 1 C.1) (iv) translocation through the nuclear envelope (Fig. 1 D) and (v) gene expression. *In vivo*, an additional step involving the distribution of DNA from the site of injection to enough number of cells in the target tissue needs to be considered. This step entails overcoming the interstitial barriers (Fig. 1 A). The subsequent steps (i-v) are the same both *in vivo* and *in vitro*.

In vitro, the DNA molecules suspended in the solution uniformly surround the cells shortly after addition. Once PEF is applied, DNA molecules (being negatively charged) are electrophoretically pushed from the cathode towards the anode (Fig. 1 A). In the process, they encounter the cells, specifically, the cell membrane on the cathode facing side of the cell. PEF, in addition to electrophoretically pushing the DNA molecules towards the cell membrane, also increases the permeability of the cell membrane via a



Fig. 1. Overview of the gene electrotransfer (GET) process and definitions of the terminologies used. (A) Negatively charged DNA molecules are electrophoretically pushed towards the cell membrane. (B.1–3) Events taking place at the membrane level during GET. (B.1) Formation of DNA-membrane complexes or DNA aggregates at the cell membrane. (B.2) Endocytic translocation of DNA aggregates. (B.3) Direct DNA translocation into the cell without the formation of DNA aggregates. (C.1–3) Events taking place inside the cytoplasm during GET. (C.1) Intra-cellular trafficking of endocytic vesicles (endosomes) containing DNA aggregates. (C.2) Escape of DNA molecules from endocytic vesicles. (C.3) Intracellular trafficking of naked DNA molecules which have gained direct access to the cytoplasm by translocating through the membrane without forming DNA aggregates. (D) DNA transport across the nuclear envelope.

phenomenon termed electropermeabilization (also referred to as electroporation). Experiments have suggested that DNA enters the cells only if the PEF intensity is similar to, or higher than, that required for electropermeabilization [40,41].

Electropermeabilization, or the transient increase of membrane permeability due to PEF, is attributed to formation of hydrophilic pores in the lipid domains of the cell membrane (Fig. 2 A), oxidation of membrane lipids (Fig. 2 B), denaturation of membrane proteins (Fig. 2 C) and/or a combination of these [42]. These mechanisms of electropermeabilization, depicted in Fig. 2, explain a large number of observations related to *trans*-membrane transport of ions and small molecules which is primarily governed by electrophoresis and diffusion [43-49]. The "threshold" PEF intensity leading to electropermeabilization, known as the electropermeabilization threshold, is usually determined as the minimum PEF intensity required for detecting such ions or small molecules (e.g., propidium iodide dye) inside the cells [50].

Only if the PEF intensity is above the electropermeabilization threshold, the entry of DNA molecules into cells can be detected. There are two possible pathways of DNA entry. In the first, and the most widely accepted, pathway the DNA molecules, which are electrophoretically pushed towards the cells, interact with the permeabilized membrane on the cathode facing side of the cell (Fig. 1 B.1-B.2). The DNA interaction with the permeabilized membrane can be visualized in terms of DNA aggregates or DNAmembrane complexes (Fig. 1 B.1). Such trapped (or immobilized) DNA molecules, henceforth referred to as DNA aggregates, are internalized *via* endocytosis and appear inside the cell in the minutes following PEF application [40,51,52].

In the second pathway, which is less accepted, the electrophoretically pushed DNA enters the cell directly by translocating through the permeabilized membrane on the cathode facing side of the cell (Fig. 1 B.3). In this pathway, the DNA molecules, prior to and/or during translocation, might interact with the cell membrane in the form of DNA adsorption on the cell membrane. Using molecular dynamics (MD) simulations, siRNA molecules have shown to translocate through hydrophilic pores by being adsorbed to the lipid bi-layer [53]. DNA interaction with the cell membrane in the form of DNA adsorption is different from DNA aggregation at the permeabilized membrane.

Before proceeding, we would like to define a few terms that will be used repeatedly through the text and could potentially lead to a confusion if they are not explicitly defined. The event of DNA crossing the cell membrane is a multi-step process which involves interaction of DNA with the permeabilized membrane in the form of DNA aggregates and subsequent internalization of the DNA aggre-



Fig. 2. (A-C) Mechanisms of cell membrane permeabilization due to its exposure to pulsed electric fields (PEF). Image reproduced from [42] with permission. Lipid molecules are depicted in blue and membrane protein is depicted in green. The electric field is represented by a red arrow on the left. The length of the arrow depicts the strength of the electric field and the arrow points in the direction of the electric field. The black arrows in between membrane states depict the transition between the states and the length of the arrow depicts the transition rates. Longer arrows depict faster transition rates and shorter arrows depict field. (B) Chemical modification (e.g. lipid peroxidation) of lipids, specifically their tails, leading to their deformation resulting in increased permeability. (C) Denaturation of membrane proteins (e.g., voltage gated on-channels) in the presence of an electric field, making them non-selectively permeable. Both (B) and (C), and/or their combination, can be responsible for prolonged permeability observed in cells which is of *O*(10–15) mins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

gates into the cell via endocytosis. We will refer to the combination of these steps i.e. DNA aggregate formation and subsequent internalization via endocytosis, including any intermediate steps, as DNA transport across the cell membrane. For instance, processes B.1 and B.2 in Fig. 1 represent a DNA transport event. We will exclusively refer to DNA translocation as an event in which DNA only crosses the permeabilized cell membrane and reaches the cytoplasm. For instance, endocytosis of aggregated DNA, i.e. Fig. 1 B.2, is a translocation event. In addition, DNA translocating through the permeabilized membrane without the formation of DNA aggregates and directly reaching the cytoplasm, i.e. Fig. 1 B.3, is also a translocation event. Further, we consider DNA transfection (or transfection) to imply (and be inclusive of) the complete sequence of events - DNA translocation across the cell membrane, DNA transport through the cytoplasm, DNA transport across the nuclear envelope and gene expression. GET is referred to as DNA transfection mediated by PEF. See bottom half of Fig. 1 for elucidation. Thus, GET efficiency implies DNA transfection efficiency in which DNA transfection is mediated by PEF. Lastly, throughout the text, we have used the notation of O(n) [units]' to quantify various parameters in appropriate units. The 'O(n) [units]' simply implies that the value of the parameter is approximately n in the given [units]. E.g., "...electrophoretic migration of O(1) µm.." implies that the electrophoretic migrations is approximately 1 µm. Rather than concerning with precise values, we have used this notation to provide approximate values or order of magnitude estimates, which are often sufficient to illustrate our point.

Along with electropermeabilization, electrophoresis is also believed to be necessary for GET. So much so that these two processes need to take place simultaneously. If DNA molecules are added after the application of PEF, DNA transfection is not observed even though the membrane is permeable to small molecules [41,54]. Role of electrophoresis is further evident from the fact that DNA aggregates are formed only on the cathode facing side of the cells [40]. Moreover, DNA molecules are electrophoretically added to the existing DNA aggregates in subsequent pulses [51], and DNA transfection is a vectorial process that depends on the direction of PEF [54]. Recent experiments have suggested that small DNA molecules of size 15–25 base pairs (bp) and siRNA molecules have direct access to the cytoplasm (without forming DNA or RNA aggregates) and they enter the cell from the permeabilized membrane on the cathode facing side of cell, indicating an electrophoretic DNA and RNA translocation across permeabilized membrane [55,56].

Overall, PEF is thought to play a dual role in GET. One role is to permeabilise the cell membrane. The other role is to electrophoretically push the DNA molecules and bring them close to the permeabilized membrane, allowing DNA molecules to either form DNA aggregates at the permeabilized membrane (Fig. 1 B.1) which later appear inside the cytoplasm *via* endocytosis (Fig. 1 B.2) or translocate through the permeabilized membrane directly into the cytoplasm (Fig. 1 B.3). While both, membrane permeabilization and electrophoresis, appear to be necessary for GET, the reason for this necessity as well as their precise role in GET still remains elusive.

The cell membrane has a residual negative charge on the outer surface [57]. For negatively charged DNA to interact with the permeabilized membrane and form DNA aggregates, DNA must overcome an electrostatic barrier. The role of electrophoresis could thus be to, directly or indirectly, overcome the electrostatic barrier, enabling the interaction of DNA with the permeabilized membrane and enabling the formation of DNA aggregates (Fig. 1 B.1). In case of DNA molecules that have direct access to the cytoplasm, electrophoresis could help drive the translocation of DNA molecule through the permeabilized membrane during PEF (Fig. 1 B.3) [58]. Another possible role of electrophoresis (specific to *in vivo*) could be to transport DNA in the interstitial space through the dense network of the Extra-Cellular Matrix (ECM) fibres. For instance, diffusion is negligible compared to electrophoresis in the ECM, and the DNA molecules primarily rely on electrophoresis as the dominant mode of transport [59-61]. Thus, another role of electrophoresis could be to overcome the interstitial barriers by transporting DNA in the tissue and improving the interstitial distribution of DNA molecules.

The pathways by which the DNA is translocated across the cell membrane are understood only to a limited extent. Direct translocation of DNA in its native configuration through hydrophilic pores formed in the permeabilized membrane (Fig. 2 A) could explain the internalization of DNA molecules that have direct access to the cytoplasm (Fig. 1 B.3) [55,62]. However, research has mainly focussed on investigating DNA aggregate formation. As a result, there is now increasing evidence that DNA aggregates are translocated *via* endocytic pathways [63-65]. Understanding the mecha-

nism(s) of DNA translocation are crucial for successful GET since the intra-cellular fate of the DNA molecules depends on whether they have direct access to the cytoplasm or they are endocytosed inside vesicles.

Once internalized, irrespective of the translocation pathway, the DNA molecules are presented with yet another barrier - the cytoplasm, which primarily comprises of the dense cytoskeleton network. DNA aggregates that have been endocytosed are encapsulated in endocytic vesicles (endosomes - Fig. 1 B.2) and are protected against degradation by intra-cellular nuclease(s). DNA molecules inside endosomes rely on endosomal trafficking mediated by the actin and the microtubule network and their associated molecular motors (myosin and dynein) to reach the nucleus (or its vicinity) (Fig. 1 C.1) [65,66]. However, the endosomal membrane presents an extra barrier since the DNA molecules must escape from the endosome (Fig. 1 C.2) in order to cross the nuclear envelope and get expressed.

DNA molecules that gain direct access to the cytoplasm (Fig. 1 B.3), however, have to rely on hindered diffusion to reach the nucleus (Fig. 1 C.3). The diffusion of molecules inside the cytoplasm is size dependent and is hindered by the actin network [67,68]. Large DNA molecules, such as the plasmid DNA (pDNA) of around 5 kbp, have extremely low diffusion coefficients and are practically immobile [68]. They are, thus, highly susceptible to degradation by the intracellular nuclease(s) [69,70]. However, experiments have shown that naked DNA molecules are able to complex with intra-cellular proteins that may aid in their trafficking inside the cytoplasm [71].

The final (physical) barrier to GET is the nuclear envelope. DNA molecules in the *peri*-nuclear space that are not trapped in endosomes (endocytic vesicles), need to cross the nuclear envelope to reach the nucleus for transcription. The nuclear envelope is temporarily disrupted during cell division and synchronising GET (or gene transfer in general) with the mitotic phase of cells has shown to increase DNA transfection efficiency [72,73]. DNA molecules can also enter the nucleus of non-dividing, slow-dividing and terminally-differentiated cells using specific gene sequences in the DNA molecule that are able to bind to proteins in the cytoplasm that facilitate the entry of DNA molecules into the nucleus [71]. However, transfection has also been obtained with DNA molecules lacking these specific gene sequences [74].

Nanosecond PEF have shown to permeabilize membranes of intra-cellular organelles, vesicles and vacuoles [75-77]. However, results on applying nanosecond PEF after conventional PEF to improve GET efficiency by disrupting or permeabilizing the nuclear membrane have been inconclusive [78-81].

Various mechanisms of nuclear import have also been proposed for DNA molecules trapped within endosomes. For instance, endosomes containing DNA aggregates could fuse with the endoplasmic reticulum, transferring their (DNA) load to the endoplasmic reticulum. DNA molecules could then utilize the network between reticulum and nuclear membrane to enter into the nucleus [82]. Alternatively, or additionally, nuclear envelope associated endosomes could transfer DNA to the nucleus by fusing with the nuclear envelope [83].

3. Interstitial barriers

The interstitial space, or the interstitium, constitutes the environment surrounding the cells. Apart from cell-cell junctions, the interstitial space comprises of a network of macromolecules known as the Extra-Cellular Matrix (ECM). The major components of the ECM are polysaccharides - glycosaminoglycans (such as hyaluronan, chondroitin sulphate, dermatan sulphate, heparan sulphate) and fibrous proteins (such as collagen, elastin, fibronectin, laminin) [84]. Physical impediments of cell-cell junctions, ECM and cell-ECM junctions, along with specific interaction of DNA with these components (e.g., electrostatic interactions), significantly limit mobility and distribution of DNA in target tissues and prevent DNA molecules to come in contact with large number of cells. In addition, DNA is highly susceptible to degradation by extracellular nuclease(s). Nucleases are present in the intra-cellular as well as in the extra-cellular (i.e. the interstitial) space [85]. While the precise function of nucleases is still debated, they are expected to regulate the extra-cellular concentration of DNA through the action of DNA cleavage [86]. Nevertheless, they pose a great threat to the functionality of DNA introduced into tissues for therapeutic purposes. Some studies have reported that DNA starts to degrade as soon as 5 mins after injection into mouse muscles [11], whereas others have reported half-life of 120 mins in the tumour interstitium [87]. Hindered distribution of DNA due to structural components of the interstitium and degradation of DNA by the nucleases present in the interstitium make it a potentially limiting barrier to GET in vivo.

Scale-up studies have shown that higher levels of connective tissue/ECM in muscles of larger/older animals correspond to lower levels of DNA transfection compared to smaller/younger animals [88-91]. This suggests that ECM is a limiting barrier to GET. Enzymes that can digest certain components of the ECM have, thus, been used to increase the efficiency of GET. For instance, hyaluronidase, an enzyme digesting hyaluronan, has been used to increase GET efficiency [92]. In another study, tumours with different levels of ECM were treated with ECM digesting enzymes - hyaluronidase and collagenase, and the transfection efficiency was compared for each type of tumour. Tumours with different levels of ECM responded differently to GET post the enzymatic treatment. It was observed that tumours with high levels of ECM responded better to GET post enzymatic treatment compared to tumours with low levels of ECM [93].

Several studies mimicking an in vivo environment have also demonstrated that cell-cell junctions and components of the ECM indeed limit the distribution of DNA. contributing to diminished GET efficiency. For instance, experiments on 3D spheroid models mimicking an in vivo environment revealed that cells only on the outer layer of the spheroid interacted with the DNA molecules even though the cells deep inside the spheroid were permeabilized as was evident by the uptake of small propidium iodide dye [94]. In another study, less than 1% of the cells in the spheroid could be transfected even though a transfection efficiency greater than 20% was achieved for cells in suspension under similar electroporation (PEF) conditions [95]. The low efficiency of GET can predominantly be attributed to the lack of DNA distribution inside the spheroid due to a dense cell arrangement with corresponding cell-cell junctions and possibly some ECM that can be deposited from cells within the spheroid [94]. In addition, non-uniform distribution of electric field and a reduced induced transmembrane voltage due to dense packing of cells inside a spheroid could also be a reason for limited electropermeabilization and concomitant reduced DNA transfection [95-97]. Reduced uptake of another molecule (Calcein) via PEF by cells in a spheroid was attributed to diminished distribution of Calcein due to dense packing of cells, reduced electric field inside the spheroid and lower induced transmembrane voltage for cells inside the spheroid [98]. Other components of the ECM such as collagen fibres also hinder diffusion and electrophoresis of DNA in the interstitium as determined by experiments in reconstructed tissues in vitro [99] and in ex-vivo models [60]

The ECM, thus, severely limits the distribution of molecules including DNA in the tissues. While PEF overcomes the cell membrane barrier by permeabilizing the cell membrane and allowing the entry of DNA into cells, PEF also provides the necessary push



Fig. 3. Distribution of DNA *in vitro* and *in vivo*. (A) Distribution of DNA molecules *in vitro* under sub-optimal/low and optimal/high DNA concentrations. The DNA molecules are in the suspension and homogenously distribute throughout the suspension. (B) Distribution of DNA molecules *in vivo*. Due to interstitial barriers, the injected DNA molecules are heterogeneously distributed such that regions of sub-optimal/low and optimal/high DNA concentrations exist in the target tissue. The average cell-DNA/DNA-DNA distance is given in (A) and (B) for high/optimal and low/sub-optimal DNA concentrations. The distances have been calculated according to calculations in Appendix A. (C) Electrophoretic migration of DNA molecules (5900 bp) as a function of accumulated time for different electric field intensities. The calculations of electrophoretic migration by the DNA molecule are presented in Appendix B.

to DNA *via* electrophoresis and possibly overcomes, at least partially, the interstitial barrier as well.

To further investigate the role of electrophoresis, a strategy consisting of high voltage (HV) "short" pulses along with low voltage (LV) "long" pulses were employed *in vivo* [100,101]. This strategy was first proposed for *in vitro* experiments [62,102], and the purpose was two-fold – (i) to decouple the process of electropermeabilization from electrophoresis and (ii) to enhance the electrophoresis. The HV pulses were above the electropermeabilization threshold with an electric field amplitude of O(100)V/cm and with a pulse duration of O(100) µs. Since these pulses were of shorter duration, the HV pulses did not provide enough electrophoresis compared to LV pulses. The LV pulses were below the electropermeabilization threshold with an electric field amplitude of O(10) V/cm, but with a longer duration of O(100) ms to enhance the electrophoresis of DNA.

Increase in GET efficiency was observed for the combination of HV + LV pulses compared to using HV or LV pulses alone [100,101]. Since the LV pulse alone did not result in significant electropermeabilization [100,101], even though some transfection was observed for the LV pulse alone [100], the increase in GET efficiency was attributed to direct effect of LV pulses on DNA assuming that LV pulses are contributing to electrophoresis. Enhanced GET efficiency was obtained if DNA was added either before the HV pulse or in between the HV and the LV pulses of the HV + LV protocol, indicating the crucial role of electrophoresis in enhancing the efficiency of GET [101].

The strategy consisting of HV pulses for permeabilization and LV pulses for electrophoresis was employed leading to a higher efficiency of GET *in vivo* in further studies [103-105]. Long duration pulses are currently being employed in clinical settings. For instance, CELLECTRA® devices by Inovio Pharmaceuticals employ electric current-controlled (0.2 A for intra-dermal and 0.5 A for intra-muscular) long-duration pulses of 52 ms for its GET based DNA vaccination programs [29,32,34,35,106]. Interestingly, exponentially decaying pulses that have been successfully used in early studies of GET have a leading high peak, corresponding to HV component, and a long tail, corresponding to the LV component [102]. In light of these observations regarding long duration pulses of pulses of a statement of the pulses of pulses.

52 ms, it is also important to note that short duration pulses of $100 \,\mu$ s (at $1.3-1.5 \,kV/cm$) are also being successfully used for GET in clinical settings [24-27,33].

The results on improved GET efficiency in vivo as a result of employing HV + LV pulses (or enhancing electrophoresis with separate LV pulses for electrophoresis) were, however, not observed in subsequent in vitro studies. At optimal DNA concentrations for in vitro, HV + LV pulses did not lead to higher transfection efficiency compared to HV pulses alone; however, for sub-optimal DNA concentrations, HV + LV resulted in an increased transfection efficiency compared to HV alone [107,108]. In this case, optimal concentration was defined as concentration beyond which transfection efficiency did not increase with an increase in DNA concentration, and transfection efficiency was defined as the percentage of transfected cells. Experiments at these sub-optimal and optimal DNA concentrations in vitro revealed the role of HV + LV pulses and of electrophoresis (Fig. 3 A). Due to hindered distribution of DNA in target tissues, regions of suboptimal DNA concentrations are expected to always exist in vivo (Fig. 3 B). Employing HV + LV pulses in vivo is thus expected to enhance GET efficiency due to the presence of these regions of sub-optimal DNA concentrations.

Although HV + LV pulse experiments *in vitro* and *in vivo* provide a possible mechanism of how LV pulses, through electrophoresis, enhance GET efficiency *in vivo*, it is worthwhile to look deeper into the role of electrophoresis in this enhancement. As argued by Bureau *et al.* [100], electrophoresis could enhance efficiency of GET by (i) improving DNA diffusion (distribution) in tissues (ii) by improving the contact between DNA molecules and the cell membrane (iii) by allowing DNA molecules to interact with and/ or "insert" into the permeabilized cell membrane, i.e., DNA aggregation (Fig. 1 B.1) and (iv) aiding direct DNA translocation through the permeabilized membrane (Fig. 1 B.3). Possibilities (iii) and (iv) require the membrane to be permeabilized, whereas this is not necessary for (i) and (ii).

Bureau et al. further ruled out the possibilities (i) and (ii) by arguing that the application of LV pulses before HV pulses or LV pulses alone did not lead to an enhancement in GET efficiency, implying that a permeabilized state of the membrane is necessary to observe the effect of LV electrophoresis [100]. Usually, LV pulses



Fig. 4. Distribution of intramuscularly injected DNA. (A-E) Tibialis anterior muscle of mice; images from [113] Copyright 2000. The American Association of Immunologists, Inc. (A) Brightfield image of tibialis anterior muscle. White arrow marks the site injection. (B) Fluorescent image of the whole tibialis anterior muscle with DNA (labelled) in red, 5 mins after injection. White arrow marks the site of injection. (C) Fluorescent image of the lateral view of tibialis anterior muscle with DNA (labelled) in red, 5 mins after injection. White arrow marks the point of injection and white arrowhead points to accumulated DNA along the myotendinous junction. (D) Fluorescent image of vibratome transverse section (150 µm) of the tibialis anterior muscle with DNA (labelled) in red, 5 mins after injection. White arrowheads mark DNA in between muscle fibres/cells and white arrows mark DNA (labelled) in red, 5 mins after injection. White arrowheads mark DNA in between muscle fibres/cells and white arrows mark DNA (labelled) in red, 5 mins after injection. White arrowheads mark DNA in between muscle fibres/cells (E) Vibratome longitudinal section of the tibialis anterior muscle with DNA (labelled) in red, 5 mins after injection. DNA is located between muscle fibres/cells. (F-H) Tibial cranial muscle of mice with DNA (radioactively labelled) in black; images from [11]. (F) Transverse section of the tibial cranial muscle, 5 mins after injection. (G) Higher magnification of a transverse section of the tibial cranial muscle, 5 after injection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are applied after HV pulses to increase the efficiency of GET. However, applying LV pulse before the HV pulse has been shown to marginally (although not statistically significantly) enhance DNA transfection compared to HV pulses alone in certain *in vitro* experiments [108,109]. Lack of significant enhancement in GET efficiency when LV pulses are applied before HV pulses does provide some evidence against possibility (ii), further studies might, however, be required to completely rule out this possibility.

Maximum DNA migration observed in tumour interstitium *ex vivo* [60] and *in vivo* [61] was around 0.37 μ m and 0.23 μ m, respectively, for a 50 ms pulse duration. It should be noted that a pulse duration of 50 ms is representative of pulse durations used in LV GET protocols. Even with the application of 10 such pulses, only a microscopic distribution of $O(1) \mu$ m is achieved. Thus, electrophoresis (by LV pulses) is not sufficient to improve DNA distribution in target tissues, effectively ruling out possibility (i). It is

likely that the distribution of DNA observed over macroscopic distances is due to the convection forces through injection [110-112].

It then appears that a permeabilized membrane is indeed necessary to observe the effect of electrophoresis provided by the LV pulses, and role of electrophoresis is to enhance the local concentration around the permeabilized membrane so that more DNA molecules can interact with the permeabilized membrane forming DNA aggregates, i.e., possibility (iii) (Fig. 1 B.1) or DNA molecules can directly translocate through the permeabilized membrane, i.e., possibility (iv) (Fig. 1 B.3).

According to our estimates, the DNA-cell distance is O(0.5-1) µm *in vitro* and O(0.1-0.5) µm *in vivo*, in regions with high/optimal DNA concentration (see Fig. 3 A and B, and refer to Appendix A for calculations). Thus, for high/optimal DNA concentrations which can be easily achieved *in vitro* and almost impossible to achieve *in vivo*, there already are DNA molecules in close proximity to

the cell. In this case, HV pulses alone are sufficient in bringing enough DNA molecules close to the permeabilized membrane for the purpose of DNA aggregation and/or direct DNA translocation. The DNA electrophoretic migration (electrophoresis) provided by HV pulses is $O(0.1-1) \mu m$ (Fig. 3 C, Appendix B for calculations) which is similar to the cell-DNA distance in regions with high/optimal DNA concentrations. Therefore, LV pulses add little to nothing in their contribution to bring enough DNA molecules close to the cell *via* electrophoretic migration (electrophoresis).

On the contrary, we estimate the DNA-cell distance to be $O(1-50) \mu m$ *in vitro* and $O(1-10) \mu m$ *in vivo* at low/sub-optimal DNA concentrations (Fig. 3 A and B, Appendix A for calculations). As a result, the number of DNA molecules close enough to make contact

Table 1

Values of hydraulic conductivities for different types of tissues and tumors. From [114].

	Tissue Type	Hydraulic Conductivity (K $^{'}$) [cm $^{2}/mm$ Hg s \times 10^{-8}]
Normal Tissue	Rat abdominal muscle	15-78
	Rat dermis	5.33
	Mouse tail skin	70–150
	Subcutaneous plane	0.6-0.85
	Subcutaneous slice	6
	Aortic media and intima	0.4-2.0
Tumors	MCaIV tumor	248
	LS174T tumor	45
	U87 tumor	65, 7000
	HSTS26T tumor	9.2
	Rat fibrosarcoma	1.36–1360
	B16.F10 murine tumor	4100-11000
	4 T1 murine tumor	950-2300
	Hepatoma	0.8-4.1, 28

with the cell membrane is low. Electrophoretic migration (electrophoresis) provided by the LV pulse is $O(1-100) \ \mu m$ (Fig. 3 C, Appendix B for calculations). In this case, LV pulse aids in electrophoretically migrating DNA molecules from far away to the cells, bringing enough DNA molecules close to the permeabilized membrane for the purpose of DNA aggregation and/or direct DNA translocation.

As mentioned above, the experimental electrophoretic migration observed for 50 ms long pulses (i.e., in the LV pulse regime) in tumour interstitium *ex vivo* was $O(0.1-1) \mu m$ [60,61]. Therefore, electrophoretic migration of $O(1-100) \mu m$ by LV pulses based on Fig. 3 C and calculations presented in Appendix B should be taken as an upper limit to the electrophoretic migration.

Overall, both HV and LV pulses are crucial for GET *in vivo*, whereby LV pulses, and the associated electrophoresis, appear to be especially critical for enhancing the efficiency of GET *in vivo*. *In vivo*, the interstitium limits the distribution of DNA molecules, providing a heterogenous distribution of DNA in the target tissue (Fig. 3 B). As a result, regions of low/sub-optimal DNA concentrations exist in the target tissue. LV pulses are able to offset the low efficiency of GET which results from the existence of these regions of low/sub-optimal DNA concentrations [107,108]. LV pulses accomplish this by electrophoretically bringing more DNA molecules close to the permeabilized membrane for DNA-aggregation and/or direct DNA translocation.

The question that then arises is - how are DNA molecules distributed over macroscopic distances in the target tissue? Further, is the distribution inhomogeneous leading to zones of sub-optimal DNA concentrations?

For intra-muscular injections in the tibialis anterior muscles of mice, DNA was distributed in the entire muscle 5 min after injection as shown in Fig. 4 A-C [113]. The white arrows mark the point of injection whereas the white arrowhead indicate the accumula-



Fig. 5. Influence of convective forces from injection of fluids on their macroscopic distribution in target tissues. (A-B) Injection of insulin in (pig) adipose tissue; images from [110]. (A) Histologically stained cross section of the sub-cutaneous tissue (pig adipose) with injected insulin shown in red. Injection fluid volume was 100µl and the scale bar corresponds to 1mm implying distribution over macroscopic distances. (B) X-ray computed tomographic scan of a similar sub-cutaneous injection process. The injection channel is visible along with the back-flow of the fluid to the skin surface. (C-D) Injection of Urografin fluid into adipose tissue; images first published in Journal of Mechanics and Material Structures in Vol. 6 (2011), No 1, published by Mathematical Sciences Publishers [111]. (C) A composite of X-ray image of 500µl of 150 Urografin fluid (opaque dye) injected into porcine adipose tissue. (D) Cross-section from a 3D reconstruction of 720 X-ray images of the dye-injection in (C). (E-G) Injection of dye (blue) into pig adipose tissue; images from [112]. (E) Images after single 100µl dye injection into adipose tissue and squeezing the site in-between electrodes. (F) Sagittal plane of the dye injection site after dissection to show the distribution of dye within the tissue. The dye is primarily found between the collagenous septa diving adipose lobes. (G) Distribution of the references to color in this figure legend, the reader is referred to the web version of this article.)

Interstitial fluid volume and ECM composition for different types of tissues and tumors. From [11	17].	•
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	Tissue Type	Interstitial Fluid Volume (ml/g wet weight)	Collagen (mg/g wet weight)	Glycosaminoglycans (mg/g wet weight)	Hyaluron (mg/g wet weight)
Normal Tissue	Skin	0.40-0.45	170-190	3.7-4.2	0.5-1.6
	Muscle	0.07-0.12	10-13	2.2	0.09-0.13
	Lung	0.24	5-35	6.1	0.07-0.13
Tumors	Mammary carcinoma (murine) MCa1V		1.7 ± 0.4		0.22 ± 0.02
	Host: Mouse				
	Colon adenocarcinoma (human) (LS174T)		1.8 ± 0.7		0.10 ± 0.04
	Host: Mouse				
	Glioblastoma (human) (U87)		9 ± 4		0.10 ± 0.04
	Host: Mouse				
	Soft tissue sarcoma (human) (HSTS)		6 ± 1		0.22 ± 0.03
	Host: Mouse				
	Mammary carcinoma (DMBA induced)	0.39 ± 0.02	4.6 ± 0.7		1.9 ± 0.3
	Host: Rat				
	Ovarian carcinoma (OVCAR-3)	0.60 ± 0.03	7.7 ± 0.5		0.08 ± 0.04
	Host: Mouse				
	Ovarian carcinoma (SKOV-3)	0.53 ± 0.11	9 ± 3		0.20 ± 0.02
	Host: Mouse				

tion of DNA (labelled in red) along the myotendinous junction of the tibialis anterior muscle. Closer inspection of the transverse and longitudinal sections of the muscles revealed local distribution of DNA (labelled in red) within the tissue, 5 mins after injection. DNA was distributed in between the muscle cells as observed in transverse (Fig. 4 D) and longitudinal (Fig. 4 E) sections. For the transverse section, the white arrowheads mark the accumulation of DNA in the space between the cells, whereas the white arrows mark the DNA inside cells at the point of injection. Observations on a whole transverse sections of tibial cranial muscles of mice also showed a macroscopic distribution of radio-labelled DNA 5 mins after injection (Fig. 4 F) [11]. Higher magnifications of the transverse (Fig. 4 G) and longitudinal (Fig. 4 H) sections, 3 h and 5 mins after injection, respectively, reveal that the DNA is located in the inter-fibrillar space (i.e., space in between the muscle cells). One can infer similar patterns of DNA distribution in the inter-fibrillar space or the interstitium, after injection, from transverse sections shown in (Fig. 4 D and G) and longitudinal sections shown in (Fig. 4 E and H).

As mentioned earlier, the distribution over macroscopic distances in vivo is likely due to the convection forces while injecting the bolus of DNA solution into the tissue. Injection studies have shown that sub-ml (100 µl) bolus injections into the subcutaneous region led to a "depot" which spans approx. 1 cm in length (Fig. 5 A and B) [110]. The "depot" can be described as a region (or a volume space) within which the injected bolus/dye can be found. In another study, 0.5 ml (500 mm³) bolus occupied a volume of 2300 mm³ once injected into the adipose tissue, implying a macroscopic distribution (Fig. 5 C and D) [111]. Similar observations were made in dye injection studies in which adipose tissue was considered as a target for DNA vaccination using GET (Fig. 5 E-G) [112]. Distribution of fluid in target tissues also depends on the type of tissue (muscle, fat/adipose and skin/dermis) as each of these have different resistances and permeabilities to the injected fluid.

To obtain information of how the tissue type influences the distribution of injected fluid, one can consider the Darcy's equation which describes the flow through porous media (tissue/tumors) as: $\bar{v} = -(K/\mu)\nabla p = -K'\nabla p$, where \bar{v} is bulk-averaged velocity, p is pressure, μ is viscosity, K is specific permeability and K' is hydraulic conductivity [114]. Different types of tissues have different hydraulic conductivities and will influence the convection-based distribution of injected (DNA) solution. Hydraulic conductivities of common types of tissues used as a target during GET is given in Table 1 [114].

There are, however, a few points to be kept in mind while using hydraulic conductivities to interpret convection-based flow. Firstly, hydraulic conductivity of soft porous media such as biological tissues and tumors was found to vary with infusion pressure; with the variations being attributed to pressure-induced deformation of tissues [115,116]. Secondly, hydraulic conductivity of tissues is largely dependent on fractional void volume of the interstitium, the composition of ECM components and the geometry of the ECM [114,117]. For instance, hydraulic conductivity and mechanical (deformative) properties of various tumors were found to be correlated to the constituents of the ECM, specifically the collagen [118]. Various tissues, which are targets for GET, can have widely varying compositions of the ECM constituents and, thus, very different hydraulic conductivities, as evident from Table 1. Composition of commonly used target tissues during GET and a few tumors is shown in Table 2 [117]. Note that one must be careful in correlating the hydraulic conductivity to the composition of the ECM as this approach might be oversimplistic [114]. Interestingly, the increase in GET efficiency correlated with the amount of ECM in tumors when ECM digesting enzymes - collagenase and hyaluronidase – were used [93].

Structural anisotropy in the target tissue can also influence DNA distribution in the interstitium. It was shown that the structural anisotropy in cerebellum, collagen gels and tumor models can lead to anisotropy in diffusion of small and large molecules in the interstitium [119,120]. In addition, drug solution was shown to preferentially permeate along the direction of alignment (i.e. longitudinal) of the muscle tissue and have a higher hydraulic conductivity along that direction compared to the transverse direction [121].

It should be noted that injected volume often exceeds the fluid (holding) capacity of the target tissue leading to swelling or postinjection re-adjustments. For instance, intra-dermal injections lead to the formation of blebs under the skin [122-126].

In case of intra-muscular injections, 50 μ l of DNA injection (exceeding muscle capacity) caused swelling of the anterior epimysial sheath of tibialis anterior muscle of mice [113]. Soon after, the swelling subsided and redistributed the fluid throughout the muscle. Reducing the volume of the fluid during injection to 5 μ l did not lead to swelling of epimysial sheath while still dispersing the DNA throughout the muscle, although to a lesser extent. Interestingly, in the absence of PEF, less DNA uptake (at the site of injection) and less overall transgene expression was observed for the 5 μ l injection compared to 50 μ l injection. The higher transgene expression for the 50 μ l injection was attributed, although specula-

tively, to the swelling of the muscle and the hydrostatic pressure resulting from the excessive fluid volume compared to the fluid (holding) capacity of the muscle, which in turn induced the uptake of DNA by muscle cells.

According to the authors [113], this could potentially explain why naked DNA transfection efficiency is higher for smaller animals compared to larger animals. Dupuis *et al.* [113] argued, based on their experimental observations, that the ratio of the injected fluid volume to the fluid (holding) capacity of the muscle/tissue is higher for smaller animals, owing to the small size of the muscle/tissue. This leads to muscle/tissue swelling and additional (hydrostatic) pressure or mechanical forces being generated that can induce DNA uptake. Mechanically squeezing the cells in a microfluidic environment has led to an enhanced DNA transfection efficiency using GET [127]. On the contrary, muscles/tissues of larger animals have enough capacity to accommodate the incoming injected fluid. This generates less pressure and low (or not enough) mechanical forces in the environment which possibly results in reduced uptake of DNA by muscle cells.

Therefore, while convection forces due to fluid injection help/ aid in macroscopic distribution of DNA in target tissues, other factors associated with injection procedure should be considered while evaluating and investigating GET. One such factor is tissue swelling as a result of injection volume exceeding the fluid holding capacity of the tissues and its associated impact/hydrostatic pressure on cells within the tissues and near the site of injection, leading to DNA uptake or DNA transfection.

Another method to improve macroscopic distribution of DNA is to use injections at multiple sites, as has been observed for GET in rat skeletal muscle [128]. However, the improvement in transfection efficiency due to multiple injections is not always consistent between animals. No variation in DNA transfection efficiency was observed for mice when multiple injections were used compared to a single injection, keeping the total DNA dose constant [129].

While it appears that DNA is distributed over macroscopic distances through convection by injection, studies have also revealed that distribution of DNA is inhomogeneous in the interstitium [130]. From Fig. 4 D and E, it can be observed that the intensity of fluorescently labelled DNA (in red) is unevenly distributed, implying inhomogeneous concentration of DNA in the target tissue/muscle. This is further evident from Fig. 4 G and H where distribution of radioactively labelled DNA (in black) is inhomogeneous in the interstitial space between muscle fibres.

The reason for this inhomogeneity is perhaps the dense environment of the interstitium. The interstitium prevents DNA concentration to be homogenous in the target tissue and allows for zones of low/sub-optimal DNA concentration to exist *in vivo*. As discussed previously, existence of these zones then allows LV electrophoresis, in the HV + LV protocol, to enhance the GET efficiency by pushing DNA molecules over microscopic distances and accumulating enough DNA molecules close to the permeabilized membrane of cells in the low concentration zones, thereby locally increasing the concentration near the permeabilized membrane. In zones where DNA is present at high/optimal concentrations, HV pulses alone are sufficient since enough DNA molecules are already present near the permeabilized membrane of cells.

Eventually, DNA being distributed (or present) over macroscopic distances in the tissue, due to convection by injection, enables DNA transfection into cells at locations where, in addition to DNA, sufficient electric field is present to allow membrane permeabilization.

As mentioned, membrane permeabilization is necessary for DNA transfection, which implies that sufficient electric field intensity which permeabilizes the cell membrane must be present at locations where DNA molecules are present (whether at optimal or sub-optimal concentrations). A broader implication of this (necessity) is that only those cells which are within the electric field distribution emanating from the electrodes are possible targets for GET. This defines a limited geometric area (or volume) constrained by electrode configurations (and distances) within which cells can be transfected through GET [125]. Increasing the target area (or volume) by increasing the distance between electrodes along with sustaining a sufficient electric field for permeabilization is a severe limitation as this requires increasing the voltages to levels that are clinically not viable or are unsafe [131]. In case of viral vector mediated delivery, a wider area can be target based on injection alone as long as appropriate membrane receptors are present on the cells which can accept the viral/chemical vectors [132-135].

Another implication of the requirement of sufficient electric field for permeabilization is that all cells that fall within target area (or within the electrodes) might not get transfected. Electric field suffers the same fate of spatial inhomogeneity within the target area, as does DNA distribution, while going from *in vitro* to *in vivo*. Due to inhomogeneous distribution, there might be pockets well within the target area where electric field is not sufficient. The inhomogeneity arises due to multiple reasons which are discussed below.

Firstly, different tissues have vastly different electrical conductivities [91,136]. Electrical conductivities of different types of tissues are shown in Table 3. If the electric field is applied *trans*cutaneously i.e. the electrodes are in contact with the skin, electric field distribution emanating from the electrodes would be highly heterogenous due to different electrical conductivities of the underlying tissues – skin, adipose, muscle and/or tumor [137,138]. As a result, the electric field in underlying muscle or tumor is less compared to the overlaying skin due to the low electrical conductivity of skin (when an averaged value of all skin layers was considered) [137,139] or of *stratum corneum* [138]. This might result in insufficient electric field within the muscle or tumor for permeabilization and, as a result, for GET.

Secondly, within the same type of tissue, the electric field can be highly heterogenous, as shown numerically for muscles [137]. skin [140-142] and tumors [138], and experimentally for tumors [143]. Skin itself is a heterogenous tissue with different layers (s*tratum corneum* and the lower skin layers – epidermis and dermis) having different conductivities (Table 3). The inhomogeneity in electric field distribution within the same type of tissue, and even within the same layer of the tissue, arises due to conductivity changes resulting from permeabilization of cells within the tissue/layer. These effects have been modelled numerically [137,141,142,144]. Local conductivity changes and the resulting spatial heterogeneity in electrical conductivity arising due electroporation have also been observed experimentally in liver tissue [145]. Tumors can intrinsically have spatial variations in electrical conductivity [146,147]. Variations in electrical conductivity, whether naturally occurring in tissues or induced due permeabi-

Table J					
Electrical	conductivities of	different	tissues.	From	[91,136].

Tissue Type		Conductivity (S/m)
Tumor		0.22-0.4
Fat (Adipose)		0.02-0.04
Muscle	Transversal	0.04-0.14
	Longitudinal	0.3-0.8
Skin	stratum corneum	0.0000125
	Lower skin layers	0.227
Heart		0.06-0.4
Bone		0.01-0.06
Kidney		0.6
Liver		0.023-0.2
Lung (Inflated)		0.024-0.09

Table 3



Fig. 6. Distribution of electric field in tissues for needle and plate electrodes. A voltage difference of 1000 V is applied between the electrodes which are 1 cm apart. The axis represents distances in (cm), and the color bar represents the electric field intensity in (V/cm). Distribution of electric field in a homogenous tissue for plate (A) and needle (C) electrodes. Distribution of electric field in an inhomogeneous tissue for plate (B) and needle (D) electrodes. The inhomogeneous tissue is composed of tissues with conductivities σ_1 and σ_2 , with $\sigma_2 = 3.\sigma_1$. From [148].

lization, lead to inhomogeneous distribution of electric field in the tissue.

Finally, the geometry of the electrodes can also influence the distribution of electric field *in vivo*. Electric field distribution for a homogenous tissue is shown for plate electrodes in Fig. 6 (A) and for needle electrodes in Fig. 6 (C) [148]. The tissue conductivity is same for both the cases and a voltage difference of 1000 V is applied between the electrodes which are 1 cm apart however, one can generally observe that the electric field distribution is more inhomogeneous for needle electrodes than for plate electrodes [130,148,149]. The electric field distribution is further influenced by the diameter of the needle electrodes [150,151]. The influence of tissue electrical conductivity on electric field distribution in an inhomogeneous tissue is shown in Fig. 6 (B) and (D) for plate and needle electrodes, respectively [148].

Orientation of cells with respect to electric field also have an influence on the efficiency of GET. For short $O(1) \ \mu$ s pulses, orientation of cells with respect to the electric field had a negligible effect on electroporation [152]. However, for longer O(1-10) ms pulses, cells oriented parallel to the electric field were electroporated more than cells oriented perpendicular to the electric field [152]. For muscle fibers *in vivo*, a higher electroporation threshold of 200 V/cm was observed for perpendicular orientations of electric field compared to an electroporation threshold of only 80 V/cm for parallel orientations of electric field [153]. The orientations were defined with respect to the long axis of muscle fibers. Anisotropy in the muscle tissue is further evident from different electrical conductivities along longitudinal (parallel) and transversal (perpendicular) directions with respect to the long axis of muscle fibers, as depicted in Table 3.

Therefore, means that can improve the distribution of DNA molecules and electric field in target tissues, making the concentration of DNA and electric field homogenous, have tremendous potential in improving the efficiency and increasing the clinical adoption of GET. For instance, electrolytic damage and cell death due to pH changes [154-158] and muscle contractions and pain associated with GET [159-163] can be minimized by potentially eliminating long mono-polar pulses.

Other PEF-related changes that can influence the interstitial barriers should also be considered. For instance, PEF has shown to directly affect the Gap Junction (GJ) membrane proteins involved in intercellular communication. Application of nanosecond PEF impairs the Gap Junction Intercellular Communication (GJIC), attributed to the disassembly of the membrane proteins involved in the cell-cell communication [164]. However, the GJIC disruption is time and field dependent, with time scale in the O (10) mins [164], similar to the time scale of DNA degradation in the interstitium [11,87]. In another study, cell-cell junctions were altered by the application of PEF in endothelial cells of blood vessels leading to an enhanced permeability to dextrans (70 kDa) [165]. Such alterations of gap junctions and cell-cell junctions imply that PEF alone can modulate the permeability of the interstitium, enabling a more homogenous distribution of solutes in the tissue

4. Cell membrane

Once the DNA molecules overcome the interstitial barriers, they encounter the next barrier - the cell membrane. It is widely accepted that DNA transport across the cell membrane *via* GET is a multi-step process. As mentioned previously in Section 2, DNA transport at the membrane level involves interaction of the DNA with the membrane in the form of DNA aggregates (Fig. 1 B.1) followed by translocation of the aggregates *via* endocytic pathways (Fig. 1 B.2) [63-65]. The understanding that endocytic pathways are involved in DNA translocation does seem to provide a certain degree of control, albeit low. Lack of knowledge on endocytic precursor [52,91] i.e. the DNA-membrane interaction in the form of



Fig. 7. Multi-step process of DNA and cell membrane interaction and internalization in the presence of an electric field. (a) Scheme from [172]; D - DNA, C - unpermeabilized membrane, P - permeabilized membrane, m - maximum number of binding sites, D.C - DNA associated with (or adsorbed to) the unpermeabilized membrane, D.P - DNA associated with (or adsorbed to) the permeabilized membrane, D.P - DNA translocation across the membrane, D_{in} - internalised DNA and TC - final state of transfected cell. (b) Scheme from [174]; D - DNA, C - unpermeabilized membrane, P - permeabilized membrane, DP - DNA anchored to the membrane, DP => PD - DNA translocation across the membrane, Dⁱⁿ - internalised DNA and Dbⁱⁿ - internalised DNA bound to an internal cell structure to initiate genetic cell transformation. (c) Scheme from [41]; I - membrane in native state, P - permeabilized membrane.

DNA aggregation at the cell membrane (Fig. 1 B.1), still limits us to a trial-and-error based optimization using PEF parameters, yielding insufficient improvements.

Another possible way for DNA molecules to overcome the cell membrane barrier is by directly translocating across the permeabilized membrane without the formation of DNA aggregates (Fig. 1 B.3). However, a mechanism of direct DNA translocation through the permeabilized membrane is less widely accepted.

In order to evaluate the role of PEF in mediating DNA transport *via* DNA aggregate formation and subsequent endocytosis (Fig. 1 B.1-2) or in mediating a direct DNA translocation across the permeabilized membrane (Fig. 1 B.3), the existing body of evidence needs to be re-examined. Although such an exercise cannot provide an understanding of a definitive mechanism of DNA aggregate formation and/or DNA translocation, it can still point to the gaps, which when addressed, will lead to improved understanding of how DNA molecules overcome the cell membrane barrier during GET.

Since the initial reports of successful DNA transfection into mammalian cells [39], efforts were dedicated to understand the "motive" force or the mechanism of DNA translocation across the cell membrane [54,62]. Formation of hydrophilic pores (Fig. 2 A), initially described in [39,49,166,167], not only explained the transport of small molecules across the membrane during PEF but also

offered the possibility to explain DNA translocation through the permeabilized membrane.

Various modes of DNA translocation through the hydrophilic pores were considered: diffusion (or electro-diffusion as the authors termed it) through the hydrophilic pores [39], binding of the DNA to the membrane surface and lateral diffusion through the hydrophilic pores [168], translocation of DNA due to flow resulting from colloid-osmotic swelling [169] or from electro-osmotic flux [170] and electrophoretic translocation of DNA through the hydrophilic pores [54,62].

Diffusive translocation through hydrophilic pores was not consistent with the observation that transfection efficiency was drastically reduced when DNA was added only a few seconds after the application of PEF [54]. Further control experiments in the same study also did not support the hypothesis of DNA translocation through hydrophilic pores *via* flow resulting from colloid-osmotic swelling or electro-osmosis [54].

In the meantime, evidence was accumulated that DNA (pre-) adsorption on the cell membrane *via* divalent cations prior to PEF application enhanced DNA transfection [168,171]. In fact, DNA transfection was reduced by two orders of magnitude in the absence of divalent cations [168]. A scheme, shown in Fig. 7 A, was presented which conceptualised the role of divalent cations

in DNA transfection [172]. According to the scheme, DNA molecules (D), in the presence of (m) divalent cations, are adsorbed to the non-permeabilized membrane state (C) or to the permeabilized membrane state (P) as D.C and D.P, respectively. The translocation of DNA molecules across the permeabilized membrane is depicted as D.P \leftrightarrow P.D. Therefore, the scheme in Fig. 7 A assumed the adsorption of DNA on the cell membrane (permeabilized and/or non-permeabilized) as a precursor to DNA translocation. The scheme further assumed a membrane bound or membrane associated DNA translocation across the cell membrane. DNA inside the cytoplasm is depicted as D_{in} and the transfected state of the cell is depicted as TC.

DNA adsorption on the membrane mediated by the divalent cations can take place prior to, and without, the application of PEF in addition to taking place during the application of PEF. However, it was soon understood that there is another DNA membrane interaction that takes place due to, and in the presence of, PEF. This interaction represents the anchoring of the DNA molecule to the permeabilized membrane and is a strong(er) association than the interaction mediated by divalent cations [173].

A theoretical analysis of experimental results also predicted the existence of the step of DNA molecules being anchored to the permeabilized membrane [174]. According to the authors, the interaction is an "electro-diffusive" insertion of the DNA into the permeabilized membrane, and it represents a highly interactive step which takes place in the presence of PEF.

A refined scheme depicting the sequence of events at the membrane level, including the step of DNA anchoring to the membrane, was introduced and is shown in Fig. 7 B [174]. The scheme in Fig. 7 B is conceptually similar to the scheme in Fig. 7 A. However, an additional step of DNA being anchored to the membrane - depicted as DP and encircled in red is introduced in Fig. 7 B. This step of DNA anchoring or "inserting/penetrating" into the membrane is predicted to take place during, and in the presence of, PEF [174]. Whereas, the translocation of DNA across the membrane (DP => PD) is predicted to be a slow process that possibly takes place after the application of PEF and does not require the presence of PEF [40,91,173,174].

With a multi-step transport of DNA across the membrane (Fig. 7) it becomes imperative to understand the role of PEF in mediating this transport, in each of these steps. Meanwhile, other experiments at the time also predicted the DNA transport to be a multi-step process establishing a similar scheme as shown in Fig. 7 C [41]. Although the scheme in Fig. 7 C differs slightly from Fig. 7 A and B, it still acknowledges DNA anchoring or "inserting" into the membrane and DNA translocation across the membrane to be two separate steps. Of note is the fact that the scheme in Fig. 7 C does not assume any pre-adsorption of DNA on the membrane by divalent cations. Rather, it refers to accumulation of DNA at the membrane interface. Nevertheless, the authors were able to infer the role of PEF in this multi-step scheme of DNA transfection through their experiments.

Experiments revealed that the "threshold" electric field intensity required to transfect cells with DNA is the same as that required to permeabilize the cell membrane [41]. Further, DNA transport into the cells takes place only through those parts on the cell membrane that are made permeable [41,175]. However, permeabilization and DNA transport should be considered as two separate events (Fig. 7 C, processes 1,4 and 2,5). This is inferred from the observations that the conditions which optimize permeabilization are not the same as the ones that optimize DNA transfection [41]. In addition, the membrane permeabilization is longlived (O(1-10) minutes after PEF termination), whereas sites on the permeable membrane competent for DNA transport are short lived (within O(1-2) s of PEF termination) [41,54]. Another role of PEF is to electrophoretically accumulate DNA at the membrane (Fig. 7 C, Process 1). This is also discussed in Section 3 of this review.

The authors [41] further acknowledged the existence of the step in which DNA is inserted in (or anchored to) the cell membrane based on the observations that DNA translocation across the membrane is a slow process [173] and that the application of LV pulses after HV pulses improves DNA transfection [62]. The DNA insertion or anchoring step is depicted in Fig. 7 C as DNA_{inserted}. It should be carefully noted that the step of DNA anchoring or insertion to the cell membrane (DNA_{inserted}) is between interfacial accumulation (DNA_{interface}) and DNA 'Translocation' in Fig. 7 C, thus, also indicating a multi-step DNA transport across the cell membrane.

PEF, thus, influences GET at the membrane level in many ways [41]. First, PEF determines permeabilised area/cap where transmembrane potential exceeds a certain "threshold" value and permeabilization takes place; these areas become the competent sites for DNA transport (Fig. 7 C, Process 2). In addition, PEF accumulates DNA near the cell membrane (interfacial accumulation) through electrophoresis (Fig. 7 C, Process 1). Fig. 7 C can be reconciled with Fig. 7 A and B by assuming that the interfacial accumulation could lead to (an enhanced) adsorption of DNA molecules on the membrane *via* divalent cations.

There were no experimental evidences to infer the influence of PEF in anchoring or insertion of the DNA to the membrane (Fig. 7 C, Process 3) [41]. However, as mentioned previously, there were speculations from earlier studies that DNA anchoring or insertion to the membrane takes place in the presence of PEF. For instance, Neumann and co-workers termed this anchoring or insertion of DNA to the membrane as a "highly interactive" "electro-diffusive" process, suggesting that it is influenced by PEF [174].

Although the mechanism of DNA interaction and anchoring to the cell membrane and subsequent translocation were still unknown, such schemes, along with experimental and theoretical analysis [41,172,174], were instrumental in establishing the DNA transport through the permeabilized membrane as a multi-step process involving DNA adsorption to the cell membrane, DNA interaction (anchoring or "insertion") with the cell membrane and DNA translocation across the membrane, as opposed to a direct translocation through permeabilized membrane. While multiple steps involved in the process of GET convoluted the entire process, acknowledging the existence of the multi-step process was crucial in establishing a holistic view of GET and preventing its oversimplification.

It was not until 2002 that experiments confirmed the existence of PEF mediated DNA membrane interaction in the form DNA aggregates using fluorescently labelled DNA molecules and observations at the single-cell level [40]. The observation of DNA aggregates greatly influenced the understanding of DNA transport and DNA translocation across the membrane.

Efforts were then focused on characterizing these DNA aggregates and examining the role of PEF in the formation of these DNA aggregates. The first role of PEF in forming DNA aggregates at the cell membrane was already evident from the initial report [40]. Experiments revealed that only when the membrane was permeabilized (i.e. PEF was above the electropermeabilization threshold) that DNA aggregates were observed at the membrane [40]. This observation was consistent with previous reports of electropermeabilization threshold being same for DNA transfection and uptake of small molecules [41]. Moreover, a longer ms duration pulse was needed to observe DNA aggregate formation at the membrane, whereas the uptake of small propidium iodide was detected even with shorter µs pulses, indicating a role of electrophoresis [40]. However, a few studies have reported formation of DNA aggregates even with short µs pulses but with slightly higher electric field strengths [109]. Further evidence of the involvement of electrophoresis comes from the observations that DNA aggregates are formed on the cathode facing side of the membrane [40,51,52,109].

In an attempt to characterize the kinetics of DNA aggregate formation, it was observed that DNA aggregates take around 1 s to form or become stabilized [52]. Under bi-polar conditions, reversing the polarity of PEF within 1 s does not lead to stable DNA aggregates, resulting in lowered gene expression [52]. Moreover, during the application of a train of pulses, the first pulse led to DNA aggregates on the membrane, whereas subsequent pulses did not lead to new aggregates being formed [51]. Rather, new DNA molecules were electrophoretically added to the existing aggregates [51].

Since DNA molecules interact with the membrane and form aggregates, there are two processes leading to DNA aggregation that need further attention - (i) DNA attaching to the cell membrane followed by (ii) DNA "condensing" on the cell membrane to form aggregates. Both these processes are peculiar since they (might) involve overcoming an electrostatic barrier. For DNA attaching to the cell membrane, the electrostatic barrier arises due to negatively charged DNA molecules attaching to a negatively charged cell membrane. For DNA "condensing" to form aggregates, the electrostatic barrier arises due to negatively charged DNA molecules condensing onto themselves. While a distinct condensed state of DNA molecules in the aggregates is still speculative and has not been explicitly proven, it can still be inferred that DNA molecules in the aggregates represent a state in which strands of DNA molecules are closer to each other than they are in their native "free" state.

We shall now look at the possible role of PEF in overcoming these electrostatic barriers arising due to DNA membrane interaction in the form of DNA aggregation at the cell membrane.

As discussed previously, the process of DNA adsorption to the membrane is mediated by divalent cations, and this process enhances DNA transfection [168,171,172,174]. It should be noted that DNA adsorption to the membrane *via* divalent cations is not equivalent to DNA aggregation at the cell membrane. DNA can adsorb on the membrane irrespective of PEF application whereas DNA will aggregate in the presence of, and possibly due to, PEF [63].

The questions arise – How divalent cations help in overcoming the electrostatic barrier leading to DNA adsorption on the cell membrane? and – How, and if, PEF modulates the process of adsorption of DNA on the cell membrane by divalent cations?

Divalent cations can facilitate adsorption of DNA on zwitterionic lipid membranes. This is based on the process of ionexchange and is described in terms of an extended Poisson-Boltzmann framework [176]. Prior to adsorption, divalent cations are bound to the negatively charged phosphate moieties of the DNA molecule. When DNA molecules approach the membrane, the divalent cations that are bound to the DNA phosphate moieties, now bind to the phosphate moieties of the lipid head group. This causes a re-orientation of the lipid head group, exposing the positively charged moieties outward towards the approaching DNA molecule. These positively charged moieties of the lipid headgroup now provide the counter-ion charge to stabilize the phosphate moieties of the DNA molecule. This is the ion exchange pro-



Fig. 8. Different modes of DNA translocation across the cell membrane. DNA is shown in blue, divalent cations are depicted as red circles and cell membrane is depicted in grey (A) DNA aggregation mediated by divalent cations and subsequent endocytosis. The dotted arrows represent ion influx/efflux through permeable sites on the membrane. (B) DNA aggregation mediated by curvature mediated interactions. (C.1–3) DNA translocation through hydrophilic pores without DNA aggregation. (C.1) DNA translocation through large hydrophilic pores without membrane adsorption. (C.2) DNA translocation through large hydrophilic pores with membrane adsorption. (C.3) Single file (i.e., single bp bp) DNA translocation through a small hydrophilic pore. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cess - where divalent cations first neutralizing the phosphate moieties of the DNA molecules, upon DNA adsorption, neutralize the phosphate moieties of the lipid head group. In return, the positively charged moieties of the lipid head group stabilize the phosphate moieties of the DNA molecule. This model was first proposed by McManus *et al.* who experimentally showed that divalent cations adsorbing to the zwitterionic membrane in the presence of DNA molecules effectively render the membrane positively charged [177].

Based on MD simulations it was shown that in the absence of divalent cations, there is a repulsive electrostatic barrier between a DNA molecule and a zwitterionic lipid membrane [178]. However, when enough divalent cations (Ca^{2+}) are adsorbed on the zwitterionic lipid membrane, they render the lipid membrane effectively positive. DNA molecules, on approaching the lipid membrane, now experience an attractive force indicating favourable adsorption to the membrane in the presence of divalent cations. According to the authors, the initial adsorption of DNA on the lipid membrane is driven by the net positive charge of the membrane induced by the divalent cations. Later, a stable adsorption is observed when the adsorbed divalent cations diffuse to the adsorbed DNA and form a tighter bond by bridging the phosphate moieties of the lipids to the phosphate moieties of the DNA [178].

Thus, based on extended Poisson-Boltzmann formalism of ionexchange and/or MD formalism, divalent cations can help reduce the repulsive electrostatic free-energy barrier. At high enough concentrations, divalent cations can eventually overcome the barrier by inducing an attraction between zwitterionic lipid membranes and negatively charged DNA molecules [176-178].

Following plausible explanation of – How divalent cations help in overcoming the electrostatic barrier leading to DNA adsorption on the cell membrane? we shall now look at – How, and if, PEF modulates the process of adsorption of DNA on the membrane by divalent cations?

It appears that PEF could directly contribute to overcoming the electrostatic barrier between negatively charged DNA molecules and (predominantly negatively charged) cell membrane. According to MD simulations. PEF tends to align zwitterionic lipid dipoles along the outward membrane normal at the lipid mono-layer facing the cathode [179], thus creating favourable conditions for negatively charged DNA molecules to interact with the positive moieties of the zwitterionic lipid mono-layer. On the other hand, PEF tends to align zwitterionic lipid dipoles away from the outward membrane normal at the lipid monolayer facing the anode [179], indicating less favourable conditions for DNA molecules to interact with zwitterionic lipid mono-layer. Such orientations of zwitterionic lipid dipoles favour adsorption of DNA on the cathode facing side of the cell membrane based on the ion exchange process described above [176,177]. Moreover, PEF mediated alignment of dipoles at the lipid mono-layers facing the cathode and the anode is also consistent with DNA aggregates being observed at the cell membrane facing the cathode.

DNA can adsorb to the cell membrane even in the absence of PEF [63]. In this case, the role of PEF, at best, could be to accumulate enough DNA molecules near the cell membrane to facilitate their adsorption by divalent cations [41]. Since adsorption of DNA to the cell membrane enhances DNA transfection [168,171,172,174], PEF could indirectly contribute to this enhancement by facilitating DNA adsorption to the cell membrane.

However, it is still not clear how adsorption of DNA molecules to the cell membrane by divalent cations enhances DNA transfection. It is possible that adsorption of DNA to the membrane enhances DNA aggregate formation during PEF. It was shown that a high divalent cation (Mg^{2+}) concentration led to more DNA aggregates being formed [180]. However, the transfection efficiency also decreased with increasing divalent cation concentration (or increasing DNA aggregate formation) [180], or showed bellshaped curve with increasing divalent cation concentration [63]. This is inconsistent with the claim that adsorption of DNA molecules to the membrane enhances DNA transfection *via* DNA aggregate formation.

Further, with the knowledge of DNA aggregates formed at the membrane during PEF, the role and fate of these adsorbed DNA molecules (using divalent cations) is not explicit. A high divalent cation concentration leading to enhanced DNA aggregate formation implicitly implies that the adsorbed DNA molecules predominantly lead to formation of DNA aggregates. *Is there also some contribution of bulk, or non-adsorbed, DNA to the formation of DNA aggregates*? The source of DNA in the DNA aggregates formed on the membrane during the application of PEF has not been explicitly identified.

Divalent cations also offer some explanation to the observation/ speculation that DNA molecules can overcome their own repulsive electrostatic barrier to aggregate at the cell membrane during PEF. Divalent cations have led to condensation of DNA molecules adsorbed on 2-dimensional (2-D) cationic lipid bi-layer [181]. Beyond a critical divalent cation concentration, there was an abrupt transition from a repulsive electrostatic state of adsorbed DNA molecules to an attractive state leading to DNA condensation [181]. The observations of DNA condensation using divalent cations on 2-D lipid surfaces are consistent with the GET experiments in which divalent cations lead to adsorption of DNA molecules at the membrane (2-D). Moreover, increasing the divalent cation (Mg²⁺) concentration lead to an increase in DNA aggregates being formed [63,180], consistent with the possibility of divalent cations mediating DNA condensation on cationic lipid bi-layers [181].

Since the condensed state of DNA aggregates is observed only in the presence of PEF, the role of PEF could be to enhance divalent cation concentration which would trigger the transition of adsorbed DNA molecules to an aggregated and condensed state. For instance, PEF could lead to a locally enhanced divalent cation concentration at the permeable sites on the membrane due to divalent cation influx/efflux. This would lead to aggregation of DNA molecules at (or near) the permeable sites (Fig. 8 A). The aggregated DNA molecules could locally alter membrane properties that could then eventually lead to the translocation of DNA molecules by endocytic pathways (Fig. 8 A).

Other processes that can lead to DNA condensation and DNA aggregate formation are curvature-mediated interactions. It was observed that DNA molecules adsorbed on a cationic lipid bilayer can condense onto themselves [182]. The fraction of condensed DNA molecules increases with increasing cationic lipid concentration. The condensation in this case is driven by curvature mediated interactions [183]. When DNA molecules are adsorbed on the lipid bi-layer membrane, the adsorption can lead to local changes in the membrane curvatures (Fig. 8 B). These local changes can in-turn drive curvature mediated collapse and condensation of DNA molecules at the membrane (Fig. 8 B). Once condensed and aggregated, the increasing membrane curvature at the site of aggregated DNA molecules can further attract incoming DNA molecules to the existing aggregate (Fig. 8 B) [184,185]. This can possibly explain why the number of DNA aggregates remained constant when a train of pulses was applied along with the author's explanation based on lowering of electric field elsewhere in the membrane upon the formation of conducing pores [51]. The high curvature induced by the DNA aggregates due to curvature mediated attraction can naturally lead to translocation of DNA aggregates by vesiculation or endocytic pathways [184].

DNA aggregation can thus be explained based on divalent cation (counter-ion) mediated attraction or curvature mediated interactions. However, it must be noted that these are only speculative attempts to explain DNA aggregate formation. For instance, cationic lipids are not known to be naturally present in cell membranes, and extrapolation of possible mechanisms from the cationic bilayer system to cells undergoing GET should be done with caution. Thus, DNA aggregation mediated by divalent cations and/or curvature mediated interactions only offer a possibility of providing a basis towards a mechanistic understanding of DNA aggregate formation, or they offer possible starting points to investigate and further explore the underlying mechanism of DNA aggregate formation. Attempts to decipher the mechanisms of DNA aggregate formation during PEF/GET have been very scarce.

In view of DNA aggregate formation and the subsequent internalization *via* endocytic pathways (Fig. 1 B.1-2 and Fig. 8 A and B), a mechanism of direct DNA translocation across the permeabilized membrane has still not been completely abandoned (Fig. 1 B.3) [39,54,62,168]. Sukharev et al. showed that permeability of dye-molecules increased in the presence of DNA molecules, indicating that DNA interacts with hydrophilic pores in membrane during their passage across the membrane [62]. Although indirect, it still provided evidence that the interaction of DNA with the hydrophilic pores, which increases the permeability to dye molecules, is electrophoretic in nature and that DNA is directly translocating through the hydrophilic pores due to electrophoresis provided by PEF (Fig. 8 C.1-3).

Since then, various evidence have supported the mechanism of direct entry into the cell by DNA translocation through hydrophilic pores (without the formation of DNA aggregates) during PEF (Fig. 8 C.1-3), but only to minor extent. For instance, siRNA had direct access to the cytoplasm during the application of PEF, and entered the cells from the cell membrane on the cathode facing side of the cell [56]. However, siRNA molecules are small (~25 bp) compared to large pDNA molecules (~5000 bp), and a mechanism of direct translocation via hydrophilic pores for siRNA molecules cannot be trivially extrapolated to large DNA molecules. It was recently shown that irrespective of DNA size, two classes of DNA are observed: aggregated DNA at the membrane and DNA that has direct access to the cytoplasm [55]. Small DNA molecules (25-100 bp) had predominantly direct, and instant, access to the cytoplasm. However, increasing the DNA size shifted the tendency of DNA molecules to aggregate at the cell membrane. Most (but not all) of DNA of size 1000 bp became aggregated at the cell membrane. However, some large DNA still had direct access to the cytoplasm.

Theoretically speaking [186], hydrophilic pores could become large enough to accommodate DNA molecules (Fig. 8 C.1-2), provided that there are no constraints limiting the pore size. For example, in pure lipid systems like giant unilamellar vesicles, one can observe formation pores with diameters of $O(1) \mu m$ [187]. Other experiments with giant unilamellar vesicles and DNA have also indicated the existence of large enough hydrophilic pores allowing free passage to DNA translocation [58,188]. In planar lipid bilayers, pores can expand indefinitely until rupturing the membrane [167].

However, in a cell membrane the dense actin network that attaches to the membrane is thought to limit the pore size [47]. Indeed, formation of macropores was completely supressed in giant unilamellar vesicles encapsulated with actin networks [189]. Estimates of hydrophilic pore size in cells due to application of PEF are of O(1) nm [190]. Some electroporation models in cells have even assumed a maximum hydrophilic pore size of 5–60 nm [186,191-193]. Radius of gyration for pDNA molecules (~5000 bp) is estimated to be ~ 100 nm [194]. Thus, hydrophilic pore sizes of size 10–60 nm can possibly accommodate pDNA molecules of size ~ 100 nm (~5000 bp) and enable a direct DNA translocation through hydrophilic pores. Even for small hydrophilic pores of O(1) nm, a model has been developed in which DNA can

translocate through the hydrophilic pores in a single-file manner (base-pair by base-pair) similar to DNA translocation through *O* (1) nm solid-state nanopores [195] (Fig. 8 C.3). However, single-file DNA translocation models are applicable to linear DNA molecules rather than to circular DNA molecules such as plasmid DNA.

Altogether, these experiments and models do account for a mechanism in which DNA molecules translocate through the hydrophilic pores without the need for DNA aggregates being formed. However, it appears that a direct DNA translocation through hydrophilic pores in the membrane is not the dominant mode of DNA crossing the membrane during GET. A criticism to the mechanism of direct DNA translocation into cell has been the lack of naked DNA distribution inside the cell for large DNA molecules which makes them vulnerable to degradation by intracellular nucleases [68-70]. However, experiments have demonstrated that naked DNA molecules form complexes with intracellular proteins as early as 15 mins post their introduction into the cytoplasm [71]. The protein-DNA complexes can facilitate active trafficking inside the cytoplasm and nuclear import of DNA [71], thus providing a possible pathway for DNA molecules translocating through hydrophilic pores (and directly accessing the cytoplasm) to reach the nucleus.

4.1. Endocytosis

Observations that some of the DNA molecules form large aggregates on cell membrane, which are rapidly protected from degradation by cellular nucleases and appear on the intracellular side only several minutes after PEF treatment [40], pointed to endocytosis as possible mechanism for DNA translocation. DNA molecules in the aggregated form at the cell membrane could be recognized by the cell as cargo for endocytosis. Indeed, several studies have shown that the translocation of DNA molecules across cell membrane is mediated by endocytic-like processes during GET [63,196,197].

Endocytosis is a fundamental cellular process present in all cells. There are various endocytic trafficking pathways that coexist and are active concurrently in the same cell type [198]. However, endocytosis was initially not considered as a possible mechanism by which DNA could cross the cell membrane because of the absence of known cellular receptors for DNA. Nonetheless, it was shown that electrophoretically driven DNA can lead to membrane curvature large enough to initiate membrane invagination which can then activate endocytic vesicle formation where DNA is immobile and rapidly protected from extracellular nucleases degradation [54,62]. Overall, there are several possibilities how endocytosis could be involved in DNA uptake after PEF. For instance, DNA could be internalized by (intrinsic) endocytic pathways that are continuously present in cells. Alternatively, PEF could trigger "electroendocytosis" - an electric-field induced endocytic-like process that was first observed when DNA was internalised into large unilamellar vesicles via the formation of endosome-like vesicles when exposed to PEF [199]. "Electroendocytosis" was later reported in different cells in vitro as well [200-202]. However, it remains unclear whether "electroendocytosis" is specific to PEF or it is simply a native cellular response to membrane damage [203]. Namely, endocytosis is involved in cell membrane repair mechanisms which are activated in response to cell membrane damage. Within 30 s after wounding, the resulting cell membrane damage causes an influx of calcium ions from extracellular space into the cytoplasm triggering exocytosis of lysosomes followed by massive endocytosis. To be internalised into the cell, DNA molecules (aggregated) at the cell membrane could be piggybacked into cell during cell membrane repair after PEF treatment when damaged parts of cell membrane and proteins are being internalized into vesicles for recycling. As

another possibility - negatively charged DNA aggregates on cell membrane could trigger similar effects as do negatively charged PIP2 molecules. PIP2 is an important endocytosis and cytoskeleton regulator. Before endocytosis, PIP2 molecule is present in patches in the cell membrane where it is involved in regulation and recruitment of endocytic proteins to the cell membrane [204]. PIP2 molecules interact with many transmembrane proteins, for instance with Bin-Amphiphysin-Rvs (BAR) domain proteins, which are curvature sensing and are important in regulating membrane shape transitions during endocytosis [205].

In addition to cell membrane repair mechanisms, endocytosis could be linked to cytoskeleton disruption and remodelling following the application of PEF (more details on this are provided later). Cytoskeleton, particularly actin filaments and microtubules, are involved in all stages of endocytosis and post endocytic intracellular transport - from endocytic vesicle formation and early stages of endosomal transport, to transport of vesicles between different cell organelles, and transport to perinuclear space [206]. Shortly after the application of PEF, actin polymerization was observed at the side of the cell where DNA aggregates were formed, but only when DNA was present during PEF treatment [207]. High concentration of PIP2 molecule in cell membrane triggers actin polymerization by recruiting dynamin proteins which polymerize at areas of high membrane curvature [208,209]. Negatively charged DNA aggregates on cell membrane could trigger similar response leading to actin polymerization followed by endocytic vesicle formation [91].

Different endocytic pathways have been reported to participate in GET: caveolae- and clathrin-mediated endocytosis, macropinocytosis, and clathrin- independent carrier/GPI-enriched early endosomal compartment (CLIC/GEEC) pathway, both in vitro [63,64,197,207] and in vivo [210,211]. However, the contribution of each endocytic pathway, or a dominant endocytic pathway, during GET remains elusive. In order to determine which endocytic pathway is involved in DNA internalization during GET, majority of the studies have utilised endocytic inhibitors or have measured the co-localization of DNA and endocytic markers. Endocytic inhibitors are not entirely specific and can interrupt several endocytic mechanisms simultaneously. Even endocytic markers are not entirely specific and can therefore mark several different endocytic pathways. Further, endocytic pathways are complex and diverse. There are many fundamental questions that still remain unanswered including whether key components of specific endocytic pathway are conserved across cell lines and whether there is some overlap in functions of molecules known to participate in specific endocytic pathway [198]. In addition, various sizes of DNA aggregates (100-500 nm) formed on cell membrane during GET could simultaneously trigger multiple endocytic pathways [40,55,212].

4.2. Cytoskeleton disruption and its role in DNA translocation

PEF treatment leads to changes in conformation of all major cytoskeleton components – actin filaments, microtubules and intermediate filaments [213]. Cytoskeleton is highly dynamic and is capable of changing its constitution on a time-scale of O(1-10) mins in response to an external stimulus, mechanical or biochemical in nature. Recovery of cytoskeleton components is reported to be achieved within hours following PEF treatment [214,215].

The cell membrane is connected to actin filaments *via* linker proteins. This provides stability and mechanical support to the lipid bilayer, which is otherwise very fluid. Therefore, disruption of cytoskeleton network due to (and following) PEF application can have a dual effect on GET efficiency. Firstly, it can alter the initiation and transport of endocytic pathways by affecting actin filaments and microtubules as described above. Secondly, it can contribute to mechanisms that participate in the increased membrane permeability. Macropores are observed in the membrane of GUVs when they are treated with PEF [187]. However, macropores are not observed in membranes of cells or actin encapsulated GUVs suggesting that cytoskeletal, and/or associated proteins, can affect membrane permeability, particularly pore expansion and resealing [189,216]. The observation that permeabilized regions of the cell are not laterally mobile hints to the possibility of the cytoskeleton being linked to the permeabilized state and to the influence of cytoskeleton on permeabilization and GET [213,217].

To investigate how cytoskeleton structures impact membrane permeability post-PEF application, cytoskeleton disrupting agents were used. They, however, led to contradictory observations regarding the involvement of actin filaments in membrane permeabilization [213]. On one hand, studies showed that exposure to actin inhibiting or disrupting agents led to an increased membrane permeability after PEF application [218-221]. On the other hand, some studies reported significantly decreased PI uptake following PEF application in cells treated with actin inhibiting agents, suggesting a decreased membrane permeability [222,223]. Microtubule inhibition also led to a decreased PI uptake into cells indicative of a decreased membrane permeability. Additionally, shorter resealing time was observed following PEF application in cells treated with microtubule inhibiting agents [218,224]. Use of inhibiting agents thus requires careful attention to the concentration and the exposure of these agents because they can be toxic and can directly affect cell viability and permeability on their own. Additionally, different cell types and pulse parameters may result in different outcomes [213].

Pulses of different durations, from nanosecond to millisecond, can lead to cytoskeletal disruption [213]. However, the mechanism by which PEF leads to cytoskeletal disruption has not yet been elucidated. It was proposed that cytoskeletal disruption could be a consequence of interactions between PEF and cytoskeletal proteins (or associated proteins) in the form of conformation changes, electrophoresis and electromechanical effects. Experiments on actinencapsulated GUVs compared mechanical and electrophoretic forces experienced by actin filaments. It was shown that 4 times higher electrophoretic forces are induced on actin filaments compared to mechanical forces [189]. In actin-encapsulated GUVs, where biological processes can be excluded and direct effects on actin can be investigated, breakdown of actin filaments was observed following PEF application. However, the force of mechanical disruption on the cell membrane was below the threshold that is required for actin filament rupture or depolymerization. This suggests that, upon membrane permeabilization, electrophoretic forces acting on the actin filaments play a major role in actin network disruption [225,226].

Microtubules are composed of α - and β -tubulin which are polar molecules with high negative charge at the c-terminus tail leading to a higher overall electrical charge and a higher dipole moment compared to other proteins. This makes microtubules a (highly susceptible) target for direct modulation by PEF. It has been shown that PEF can directly disrupt microtubules polymerization [227,228]. Further, electrophoretic forces are reported to decrease interactions between microtubules and motor proteins, thus affecting microtubules dynamics [227,228].

Apart from directly influencing cytoskeleton dynamics, PEF can also have secondary effects on cytoskeleton. One of the proposed secondary processes by which PEF application could lead to cytoskeletal disruption is through altering calcium dynamics. Calcium, as a signalling molecule, is involved in a large number of cellular processes and can consequently lead to diversity of cellular responses that can together disrupt the cytoskeleton. For instance, calcium modulates both major components of cytoskeleton - actin and microtubules, and its increased concentration in the cytoplasm can cause actin breakdown and microtubules depolymerization [229,230]. Normally, calcium pumps maintain a 10,000 times lower calcium concentration inside the cells compared to the interstitium (or extracellular space). Following cell membrane permeabilization, extracellular calcium enters the cell by electrophoresis and diffusion through pores and/or through voltage-gated calcium channels. The resulting high concentration of calcium in the cytoplasm can lead to calcium induced calcium release from endoplasmic reticulum, leading to further amplification of calcium signals. In addition, cytoplasmic calcium levels can also increase due to permeabilization of various intracellular calcium stores, such as the endoplasmic reticulum and mitochondria after PEF application [231-233].

Other secondary processes triggered by PEF, such as ATP depletion, cell swelling, pH changes and reactive oxygen species generation may also lead to cytoskeletal disruption. However, respective contributions of each of these mechanisms to cytoskeletal disruption remain poorly characterized. When cell membrane becomes permeabilized, ATP can leak through pores in the cell membrane [234,235]. Additionally, during subsequent membrane resealing, ion concentrations in the cell have to be restored, which is an energy consuming process that requires ATP. Together, this can lead to severe ATP depletion after PEF application [236]. ATP depletion was also shown to inhibit linker proteins and lead to cell membrane separation from the underlying cytoskeleton [237]. Cell blebbing and rounding is often reported after PEF application [219,238,239]. Cell swelling can also lead to cytoskeletal disruption [238,239]. However, cytoskeletal disruption was observed also in the absence of cell swelling suggesting the involvement of alternate mechanisms [240]. In tissues, cell swelling after PEF application is limited compared to cells in suspension, but it could lead to narrowing of the interstitial (i.e., extracellular) space. Further, PEF application can also lead to pH changes in the vicinity of electrodes, with acidic pH at anode and alkaline pH at cathode [156]. Acidic pH was reported to promote actin rearrangement and cellular protrusion [241]. PEF also induce the generation of reactive oxygen species [242-244] which were reported to cause direct oxidation of actin and actin-regulatory proteins [245]. However, the precise role of oxidation of specific proteins in different cellular functions remains to be elucidated.

5. Cytoplasmic barriers

Majority of studies have focused on cell membrane as a barrier to GET. But for GET to be efficient, DNA must overcome additional cellular barriers of the cytoplasm and the nuclear envelope i.e., DNA must travel through cytoplasm to reach the perinuclear space and cross the nuclear envelope to enter the cell nucleus. It was observed that naked DNA diffusion is almost negligible within the dense cytoplasm which consists of cytoskeleton network, various cellular organelles and large amounts of proteins among other molecules. In addition, naked DNA is degraded within minutes by nucleases present in cell cytoplasm [70]. Cytoplasm, thus, presents itself as another significant barrier which cannot be ignored when considering GET optimization. Nevertheless, since GET leads to transgene expression, there must be mechanisms by which DNA circumvents cytoplasmic obstacles.

DNA molecules that have had direct access to the cytoplasm do not stay naked or un-complexed for very long in the cytoplasm. Negatively charged plasmid DNA is quickly coated with cytoplasmic proteins, some of them being sequence-specific DNA-binding proteins, and cations. This, in turn could help in their transport through the cytoplasm. For instance, it was proposed that DNA moves to perinuclear space by active transport, meaning that naked DNA in cell cytoplasm connects with adapter proteins, enabling their binding to the cytoskeleton motor proteins [246]. Proteins that bind to DNA could also act as adaptor proteins to motor proteins which enable transport along cytoskeletal network [70,247]. Results have indeed confirmed that naked DNA, either after endosomal escape or translocation through pores in the cell membrane, complexes with various proteins such as microtubule-directed motor proteins, heterogeneous nuclear ribonucleoproteins (hnRNP), mRNA binding proteins, proteins involved in nuclear import and as well as export and transcription factors. For instance, formation of plasmid DNA-protein complexes has been observed as early as 15 min post PEF application [71]. It should be noted that DNA-protein complex formation could also result in DNA charge neutralization leading to DNA condensation. DNA condensation in turn leads to reduction of its size which could increase diffusion and mobility of DNA. Also, DNA-protein complex formation could protect DNA from degradation.

When DNA is endocytosed, endocytic vesicles containing DNA already possess proteins that interact with motor proteins facilitating their (active) transport [206]. Results have indeed shown that DNA molecules in cell cytoplasm co-localize with early endosomes, recycling endosomes, late endosomes and lysosomes indicative of active transport [64,65]. Contradictory results were obtained regarding endosomal escape of DNA molecules. On one hand, inhibition of endosomal fusion or lysosomal translocation was reported to prevent gene expression [248]. On the other hand, studies reported that DNA molecules must escape endosomes prior to fusion with lysosomes, otherwise they are lost to degradation [249]. Nevertheless, DNA molecules are reported to accumulate in the perinuclear space within 1–2 h following PEF [64].

Importantly, we should note that care must be taken when interpreting results using labelled DNA molecules. DNA markers used for investigating intracellular trafficking of DNA molecules, such as TOTO-1 and BrdU, can change the properties of DNA molecules such as their size, configuration and net effective charge, and consequently DNA transport. For instance, BrdU labelled DNA molecules may become more resistant to lysosomal degradation and might also alter the kinetics of nuclear accumulation of DNA molecules [248]. Further, when DNA is labelled with a high fluorophore to DNA ratio, the ability of DNA to enter the nucleus and its transcriptional activity are reduced [250]. These observations demonstrate that labelling of DNA molecules alters, and possibly interferes with, the behaviour of DNA molecules.

There are only a handful of studies reporting on the role of cytoskeleton network on DNA transport following PEF application. It was observed that pre-treatment with latrunculin B, a molecule that disrupts actin cytoskeleton, decreased the number of cells transfected with DNA molecules as well as the fluorescence intensity (resulting from reporter gene expression) [207]. On the contrary, stabilization of the microtubule network resulted in enhancement of electro-transfected DNA expression. It was also demonstrated that DNA molecules can interact with motor proteins and be actively transported along the microtubule network [66,251]. Cytoskeleton network is a complex and a diverse mesh of various interacting proteins. Different types of cells have different cytoskeleton organization, leading to variations in the cellular responses to external stimuli [237,252]. Various studies have shown that cancerous cells, which have a cytoskeletal network adapted for proliferation and infiltration and consequently have reduced stiffness, have a different response to damage by PEF application compared to healthy cells [252-254]. For instance, following the same PEF treatment, cytoskeleton was conserved in fibroblasts whereas cancer cells showed a loss of cytoskeleton [255].

Transport of DNA thorough cytoplasm remains yet another GET barrier that is still not fully understood. Altogether, above mentioned findings point to different mechanisms which are involved in DNA transport through cytoplasm to perinuclear space. However, further studies are needed to elucidate which mechanisms, and to what extent, or possibly the dominant mechanism, that participate in DNA transport through cytoplasm, lead to successful GET.

6. Nuclear envelope

The nuclear envelope controls the traffic between the cytoplasm and the nucleus for all macromolecules - proteins, DNA, RNA and oligonucleotides. Even in the cells that expressed the transgene, 24 h after GET most of the DNA molecules were visible as aggregates located in the perinuclear space. Apparently, only some DNA molecules crossed the nuclear envelope, but even this small fraction was enough to lead to gene expression [40].

DNA molecules can reach cell nucleus during mitosis when the nuclear envelope is disassembled in a process referred to as nuclear envelope breakdown. DNA transfection efficiency can be increased by synchronization with the mitotic phase [72]. Alternatively, DNA must be transported through the nuclear pore complex to reach cell nucleus where they can be transcribed. In general, nondividing cells are known to be harder to transfect [256]. Majority of cells in tissues are either terminally differentiated or they divide with doubling times ranging from weeks to months. There must then be a way for DNA molecules to enter cell nucleus in the absence of cell division. Indeed, DNA has been delivered to quiescent cells. For instance, pDNA was directly microinjected into the cytoplasm of individual primary skeletal muscle cells, leading to gene expression [257]. In fact, among non-dividing cells, muscle cells seem to be the easiest to transfect with physical methods [258].

In the absence of cell division, the nuclear import of DNA molecules was shown to depend on specific DNA sequences, known as DNA nuclear targeting sequence (DTS), that drive nuclear import better compared to other sequences [259]. The first such sequence discovered was SV40 - a 72 bp long Simian virus 40 enhancer that binds to multiple ubiquitous general transcription factors and facilitates nuclear import of DNA, consequently increasing gene transfer efficiency [260,261]. DTS have binding sites for various transcription factors which are assembled in the cytoplasm and transported to the nucleus with importins - proteins involved in the nuclear import. After binding to DTS, transcription factors interact with importins and enable transport of DNA molecules to the nucleus [71,256,262]. In fact, 30 min after PEF application, over 300 unique proteins were bound to DNA molecules with DTS compared to only 60 proteins which were bound to DNA molecules without DTS [71]. This suggests that DNA molecules with DTS have a higher chance of translocation to the nucleus from the cytoplasm. However, gene transfer and expression have been obtained also with DNA molecules that lack DTS. It was shown that when cytoplasm of a mouse skeletal muscle cell in vivo was injected with DNA molecules, at least some of the DNA molecules were able to cross the nuclear envelope and be imported into the nucleus independent of any DTS [74].

While exposure of cells to micro- and millisecond PEF leads to cell membrane permeabilization, nanosecond PEF have shown to permeabilize cell organelles such as intracellular granules [75], endocytic vesicles [76] and large endocytosed vacuoles [77]. They can also release calcium from the endoplasmic reticulum [80]. Since nanosecond PEF permeabilize intra-cellular organelle membranes, they might also have an effect on the nuclear envelope. In this way GET efficiency could be enhanced by facilitating the access of DNA molecules to the nucleus. However, when cells were exposed to nanosecond PEF after the application of conventional PEF, improvement in GET efficiency in terms of transgene expression and percentage of transfected cells was observed only in some

Table	AT.1			

Parameter	Value	Reference/Justification
R _{cell} M _w (5900 bp DNA molecule)	7.5 μm 3894000 g/mol	[46,267] 5900 bp × 660 g/mol/ bp
c (sub-optimal) c (optimal)	$ \leq 1 \; \mu g/ml \\ \geq 10 \; \mu g/ml $	[107,108] [107,108]
$ ho_{cell}$ (in vitro) $ ho_{cell}$ (in vivo)	$\begin{array}{l} 2\times10^{12} \ cells/m^3 \\ 5.5\times10^{14} \ cells/m^3 \end{array}$	[180,266] *

^{*} Considering a total number of N_{cells} in a space of volume V_{space} , the sphere of volume available to a single cell is V_{space}/N_{cells} or $1/\rho_{cell}$. For extremely dense suspensions or densely packed cells, as is the case for tissues or *in vivo*, the sphere of volume $(1/\rho_{cell})$ available to a single cell equals the volume of a single cell $(\frac{4}{3}\pi R_{cell}^3)$ i.e., $1/\rho_{cell} = \frac{4}{3}\pi R_{cell}^3$. Using $R_{cell} = 7.5 \,\mu\text{m}$, we obtain $\rho_{cell} \sim 5.5 \times 10^{14} \,\text{cells/m}^3$.

cases [80,81]. In other cases, no improvement in GET efficiency was observed [78]. In contrast, applying nanosecond PEF before conventional PEF did show improved GET efficiency, which was attributed to calcium independent and dependent effects of nanosecond PEF on GET [263].

A hypothesis that endosomes with DNA molecules could interact and fuse with endoplasmic reticulum and thus release DNA molecules into the lumen of reticulum was also proposed. DNA molecules could then reach the nucleus through the continuous network between the nuclear and reticulum membranes, bypassing the steps of endosomal escape and of crossing the nuclear envelope [82].

Finally, DNA molecules could be translocated to the nucleus with nuclear envelope-associated endosomes. Nuclear envelopeassociated endosomes are early endosomes that were observed to localize in the perinuclear space and fuse with the nuclear envelope, thus enabling cell surface proteins and extracellular molecules direct access to cell nucleus [83]. Further, it was shown that lipid vesicles can spontaneously fuse with permeabilized membrane [264,265], implying that permeabilization of intracellular organelle membranes could facilitate the fusion of pDNAcontaining endosomes with the endoplasmic reticulum or the nuclear envelope. A theoretical study suggested that permeabilization of the endoplasmic reticulum can occur not only when exposing cells to nsPEF, but also when exposing them to longer and more conventional pulses [191]. However, additional studies are needed to clarify if the above-mentioned mechanisms are involved in the transfer of DNA molecules to the nucleus following PEF application.

7. Conclusion

In vivo barriers of the interstitium, cell-membrane, cytoplasm and nuclear envelope contribute in their own unique ways to prevent DNA molecules from reaching the nucleus of the cell. PEF helps to overcome (some of) these barriers and allow DNA molecules to reach the nucleus. We have revisited the existing literature and formalized the past and the current understanding of the GET process, explaining in detail how DNA molecules interact with each of the barriers and transport through them sequentially. In doing so, we have identified the factors limiting the transport of DNA through the barriers if the transport process is known, such as for the interstitium. We also identified gaps in the understanding of the transport process through the barriers if the transport process is still unknown, such as for the cell membrane, the cytoplasm and the nuclear envelope. Identifying the mechanisms of transport and addressing the transport limitations - each of which have been discussed - will enable further enhancement of GET efficiency in vitro as well as in in vivo across (length) scales, cell types,

Table AT.2

DNA – Cell	distances f	or conce	entrations	ranging	from	sub-optimal	to	optimal.
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Regime	Concentration (µg/ml)	DNA - Cell Distance [D in eq. (A.6)] (µm)				
		in	vitro	i	1 vivo	
sub-optimal	0.01	10.72	O(1-50)	3.28	O(1-10)	
	0.1	4.97		1.52		
	1	2.31		0.71		
	5	1.35		0.41		
optimal	10	1.07	O(0.5-1)	0.33	O(0.1-0.5)	
	50	0.63		0.19		
	100	0.50		0.15		

tissue types and species; a privilege which is not afforded by the current trial-and-error based optimisations of GET.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. DNA – Cell distance

The approach used to calculate the DNA – cell distance is similar to the one in [266]. Considering a total number of N_{cells} in a space of volume V_{space} , the total volume occupied by cells is $N_{cells} \cdot \frac{4}{3}\pi R_{cell}^3$; where R_{cell} is the radius of the cell and $\frac{4}{3}\pi R_{cell}^3$ is the volume of a single cell. The volume left to be occupied by the DNA molecules is:

$$V_{DNA} = V_{space} - N_{cells} \cdot \frac{4}{3} \pi R_{cell}^3 \tag{A.1}$$

The volume space available to single a DNA molecule can be considered as $\frac{4}{3}\pi R_{DNA}^3$; where R_{DNA} is the radius of the sphere available to a DNA molecule. The volume occupied by a total of N_{DNA} molecules is then:

$$V_{DNA} = N_{DNA} \cdot \frac{4}{3} \pi R_{DNA}^3 \tag{A.2}$$

If the concentration of DNA molecules is c (g/ml), then the number of DNA molecules in the space (V_{space}) can be calculated as:

$$N_{DNA} = \frac{c.V_{space}.N_A}{M_w} \tag{A.3}$$

where M_w is the molecular weight (g/mol) of the DNA molecules and N_A is the Avogadro's number.

Combining equations (A.1), (A.2) and (A.3), we obtain the radius of the sphere available to single DNA molecules (R_{DNA}) as:

$$R_{DNA} = \left(\frac{V_{space} - N_{cells} \cdot \frac{4}{3}\pi R_{cell}^3}{\frac{4}{3}\pi \cdot \left(\frac{c \cdot V_{space} \cdot N_A}{M_w}\right)}\right)^{1/3}$$
(A.4)

Assuming the cells and DNA molecules are evenly distributed, the average distance between two DNA molecules or a DNA molecule and a cell can be considered as $D = 2.R_{DNA}$, or:

$$D = 2 \cdot \left(\frac{V_{space} - N_{cells} \cdot \frac{4}{3} \pi R_{cell}^3}{\frac{4}{3} \pi . \left(\frac{c \cdot V_{space} \cdot N_A}{M_w} \right)} \right)^{1/3}$$
(A.5)

Considering, $\rho_{cell} = \frac{N_{cell}}{V_{space}}$ to be the cell density, equation (A.5) above can be rewritten as:

$$D = 2 \cdot \left(\frac{1 - \rho_{cell} \cdot \frac{4}{3} \pi R_{cell}^3}{\frac{4}{3} \pi \cdot \left(\frac{cN_A}{M_w} \right)} \right)^{1/3}$$
(A.6)

To get some estimates for DNA – DNA or DNA – cell distance, we consider the values in Table AT.1 for parameters in equation (A.6).

Values and approximate estimates for DNA – cell distances are given below in Table AT.2 below for different concentrations of DNA molecules ranging from sub-optimal to optimal. The calculations are based on Equation (A.6) and parameters in Table AT.1.

Appendix B. Electrophoretic migration

The electrophoretic migration (L) of a DNA molecule under an electric field (E) can be calculated according to:

$$L = \mu ET \tag{B.1}$$

Where μ is the electrophoretic mobility of the DNA molecule and *T* is the duration for which the electric field is applied. We have calculated electrophoretic migration for a DNA molecule with 5900 bp with μ = – 3.75x10⁻⁸ m²V⁻¹s⁻¹ based on [268].

References

- S.L. Ginn, A.K. Amaya, I.E. Alexander, M. Edelstein, M.R. Abedi, Gene therapy clinical trials worldwide to 2017: An update, J. Gene Med. 20 (5) (2018) e3015, https://doi.org/10.1002/jgm.3015.
- [2] X.M. Anguela, K.A. High, Entering the modern era of gene therapy, Annu. Rev. Med. 70 (1) (2019) 273–288, https://doi.org/10.1146/annurev-med-012017-043332.
- [3] F. Capone, F. Nappi, M.C. Galli, Gene Therapy Clinical Trials: Past, Present and Future - ScienceDirect, in: Second Gener. Cell Gene-Based Ther., 2020: pp. 285–301. https://doi.org/10.1016/B978-0-12-812034-7.00011-X.
- [4] A.K. Singh, J.P. McGuirk, CAR T cells: continuation in a revolution of immunotherapy, Lancet Oncol. 21 (3) (2020) e168–e178, https://doi.org/ 10.1016/S1470-2045(19)30823-X.
- [5] E.J. Topol, Messenger RNA vaccines against SARS-CoV-2, Cell. 184 (6) (2021) 1401, https://doi.org/10.1016/j.cell.2020.12.039.
- [6] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines-a new era in vaccinology, Nat. Rev. Drug Discov. 17 (4) (2018) 261–279, https://doi.org/ 10.1038/nrd.2017.243.
- [7] E.N. Gary, D.B. Weiner, DNA vaccines: prime time is now, Curr. Opin. Immunol. 65 (2020) 21–27, https://doi.org/10.1016/j.coi.2020.01.006.
- [8] M.A. Kutzler, D.B. Weiner, DNA vaccines: Ready for prime time?, Nat Rev. Genet. 9 (10) (2008) 776–788, https://doi.org/10.1038/nrg2432.
- [9] M.H. Porteus, A New Class of Medicines through DNA Editing, N. Engl. J. Med. 380 (10) (2019) 947–959, https://doi.org/10.1056/NEJMra1800729.
- [10] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Science 247 (80) (1990) 1465–1468, https://doi.org/ 10.1126/science.1690918.
- [11] M.F. Bureau, S. Naimi, R. Torero Ibad, J. Seguin, C. Georger, E. Arnould, L. Maton, F. Blanche, P. Delaere, D. Scherman, Intramuscular plasmid DNA electrotransfer: Biodistribution and degradation, Biochim. Biophys. Acta -

Gene Struct. Expr. 1676 (2) (2004) 138–148, https://doi.org/10.1016/j. bbaexp.2003.11.005.

- [12] Z. Zhao, A.C. Anselmo, S. Mitragotri, Viral Vector-Based Gene Therapies in the Clinic, Bioeng. Transl. Med. (2021), https://doi.org/10.1002/btm2.10258 e10258.
- [13] J.M. Wilson, T.R. Flotte, Moving Forward After Two Deaths in a Gene Therapy Trial of Myotubular Myopathy, Hum. Gene Ther. 31 (13-14) (2020) 695–696, https://doi.org/10.1089/hum.2020.182.
- [14] H. Vin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Nonviral vectors for gene-based therapy, Nat. Rev. Genet. 15 (8) (2014) 541–555, https://doi.org/10.1038/nrg3763.
- [15] C.M. Wiethoff, C.R. Middaugh, Barriers to nonviral gene delivery, J. Pharm. Sci. 92 (2) (2003) 203–217, https://doi.org/10.1002/jps.10286.
- [16] H. Aihara, J.-I. Miyazaki, Gene transfer into muscle by electroporation in vivo, Nat. Biotechnol. 16 (9) (1998) 867–870, https://doi.org/10.1038/nbt0998-867.
- [17] L.M. Mir, M.F. Bureau, J. Gehl, R. Rangara, D. Rouy, J.-M. Caillaud, P. Delaere, D. Branellec, B. Schwartz, D. Scherman, High-efficiency gene transfer into skeletal muscle mediated by electric pulses, Proc. Natl. Acad. Sci. USA 96 (8) (1999) 4262–4267, https://doi.org/10.1073/pnas.96.8.4262.
- [18] I. Mathiesen, Electropermeabilization of skeletal muscle enhances gene transfer in vivo, Gene Ther. 6 (4) (1999) 508–514, https://doi.org/10.1038/ sj.gt.3300847.
- [19] T. Nishi, K. Yoshizato, S. Yamashiro, H. Takeshima, K. Sato, K. Hamada, I. Kitamura, T. Yoshimura, H. Saya, J.I. Kuratsu, Y. Ushio, High-efficiency in vivo gene transfer using intraarterial plasmid DNA injection following in vivo electroporation, Cancer Res. 56 (1996) 1050–1055.
- [20] R. Heller, M. Jaroszeski, A. Atkin, D. Moradpour, R. Gilbert, J. Wands, C. Nicolau, In vivo gene electroinjection and expression in rat liver, FEBS Lett. 389 (1996) 225–228, https://doi.org/10.1016/0014-5793(96)00590-X.
- [21] R. Heller, L.C. Heller, Gene Electrotransfer Clinical Trials, Adv. Genet. 89 (2015) 235–262, https://doi.org/10.1016/bs.adgen.2014.10.006.
- [22] L. Lambricht, A. Lopes, S. Kos, G. Sersa, V. Préat, G. Vandermeulen, Clinical potential of electroporation for gene therapy and DNA vaccine delivery, Expert Opin. Drug Deliv. 13 (2) (2016) 295–310, https://doi.org/10.1517/ 17425247.2016.1121990.
- [23] B. Geboers, H.J. Scheffer, P.M. Graybill, A.H. Ruarus, S. Nieuwenhuizen, R.S. Puijk, P.M. van den Tol, R.V. Davalos, B. Rubinsky, T.D. de Gruijl, D. Miklavčič, M.R. Meijerink, High-Voltage Electrical Pulses in Oncology : Irreversible Electroporation, Electrochemotherapy, Gene Electrotransfer, Electrofusion, and Electroimmunotherapy, Radiology. 295 (2020) 254–272, https://doi.org/10.1148/radiol.2020192190.
- [24] A.P. Algazi, C.G. Twitty, K.K. Tsai, M. Le, R. Pierce, E. Browning, R. Hermiz, D.A. Canton, D. Bannavong, A. Oglesby, M. Francisco, L. Fong, M.J. Pittet, S.P. Arlauckas, C. Garris, L.P. Levine, C. Bifulco, C. Ballesteros-Merino, S. Bhatia, S. Gargosky, R.H.I. Andtbacka, B.A. Fox, M.D. Rosenblum, A.I. Daud, Phase II Trial of IL-12 Plasmid Transfection and PD-1 Blockade in Immunologically Quiescent Melanoma, Clin. Cancer Res. 26 (12) (2020) 2827–2837, https://doi.org/10.1158/1078-0432.CCR-19-2217.
- [25] A. Algazi, S. Bhatia, S. Agarwala, M. Molina, K. Lewis, M. Faries, L. Fong, L.P. Levine, M. Franco, A. Oglesby, C. Ballesteros-Merino, C.B. Bifulco, B.A. Fox, D. Bannavong, R. Talia, E. Browning, M.H. Le, R.H. Pierce, S. Gargosky, K.K. Tsai, C. Twitty, Al. Daud, Intratumoral delivery of tavokinogene telseplasmid yields systemic immune responses in metastatic melanoma patients, Ann. Oncol. 31 (4) (2020) 532–540, https://doi.org/10.1016/j.annonc.2019.12.008.
- [26] S. Bhatia, N.V. Longino, N.J. Miller, R. Kulikauskas, J.G. Iyer, D. Ibrani, A. Blom, D.R. Byrd, U. Parvathaneni, C.G. Twitty, J.S. Campbell, M.H. Le, S. Gargosky, R. H. Pierce, R. Heller, A.I. Daud, P. Nghiem, Intratumoral delivery of plasmid IL12 via electroporation leads to regression of injected and noninjected tumors in Merkel cell carcinoma, Clin. Cancer Res. 26 (3) (2020) 598–607, https://doi.org/10.1158/1078-0432.CCR-19-0972.
- [27] S.K. Greaney, A.P. Algazi, K.K. Tsai, K.T. Takamura, L. Chen, C.G. Twitty, Li. Zhang, A. Paciorek, R.H. Pierce, M.H. Le, A.I. Daud, L. Fong, Intratumoral Plasmid IL12 Electroporation Therapy in Patients with Advanced Melanoma Induces Systemic and Intratumoral T-cell Responses, Cancer, Immunol. Res. 8 (2) (2020) 246–254, https://doi.org/10.1158/2326-6066.CIR-19-0359.
- [28] C. Aggarwal, R.B. Cohen, M.P. Morrow, K.A. Kraynyak, A.J. Sylvester, D.M. Knoblock, J.M. Bauml, G.S. Weinstein, A. Lin, J. Boyer, L. Sakata, S. Tan, A. Anton, K. Dickerson, D. Mangrolia, R. Vang, M. Dallas, S. Oyola, S. Duff, M. Esser, R. Kumar, D. Weiner, I. Csiki, M.L. Bagarazzi, Immunotherapy targeting HPV16/18 generates potent immune responses in HPV-associated head and neck cancer, Clin. Cancer Res. 25 (1) (2019) 110–124, https://doi.org/10.1158/1078-0432.CCR-18-1763.
- [29] K. Modjarrad, C.C. Roberts, K.T. Mills, A.R. Castellano, K. Paolino, K. Muthumani, E.L. Reuschel, M.L. Robb, T. Racine, M.-D. Oh, C. Lamarre, F.I. Zaidi, J. Boyer, S.B. Kudchodkar, M. Jeong, J.M. Darden, Y.K. Park, P.T. Scott, C. Remigio, A.P. Parikh, M.C. Wise, A. Patel, E.K. Duperret, K.Y. Kim, H. Choi, S. White, M. Bagarazzi, J.M. May, D. Kane, H. Lee, G. Kobinger, N.L. Michael, D.B. Weiner, S.J. Thomas, J.N. Maslow, Safety and immunogenicity of an anti-Middle East respiratory syndrome coronavirus DNA vaccine: a phase 1, open-label, single-arm, dose-escalation trial, Lancet Infect. Dis. 19 (9) (2019) 1013–1022, https://doi.org/10.1016/S1473-3099(19)30266-X.
- [30] M.P. Morrow, K.A. Kraynyak, A.J. Sylvester, X. Shen, D. Amante, L. Sakata, L. Parker, J. Yan, J. Boyer, C. Roh, L. Humeau, A.S. Khan, K. Broderick, K. Marcozzi-Pierce, M. Giffear, J. Lee, C.L. Trimble, J.J. Kim, N.Y. Sardesai, D.B. Weiner, M.L. Bagarazzi, Augmentation of cellular and humoral immune responses to

HPV16 and HPV18 E6 and E7 antigens by VGX-3100, Mol. Ther. - Oncolytics. 3 (2016) 16025, https://doi.org/10.1038/mto.2016.25.

- [31] M.P. Morrow, K.A. Kraynyak, A.J. Sylvester, M. Dallas, D. Knoblock, J.D. Boyer, J. Yan, R. Vang, A.S. Khan, L. Humeau, N.Y. Sardesai, J.J. Kim, S. Plotkin, D.B. Weiner, C.L. Trimble, M.L. Bagarazzi, Clinical and immunologic biomarkers for histologic regression of high-grade cervical dysplasia and clearance of HPV16 and HPV18 after immunotherapy, Clin. Cancer Res. 24 (2) (2018) 276–294, https://doi.org/10.1158/1078-0432.CCR-17-2335.
- [32] C.L. Trimble, M.P. Morrow, K.A. Kraynyak, X. Shen, M. Dallas, J. Yan, L. Edwards, R.L. Parker, L. Denny, M. Giffear, A.S. Brown, K. Marcozzi-Pierce, D. Shah, A.M. Slager, A.J. Sylvester, A. Khan, K.E. Broderick, R.J. Juba, T.A. Herring, J. Boyer, J. Lee, N.Y. Sardesai, D.B. Weiner, M.L. Bagarazzi, Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: A randomised, double-blind, placebo-controlled phase 2b trial, Lancet. 386 (10008) (2015) 2078–2088, https://doi.org/10.1016/S0140-6736(15)00239-1.
- [33] A.I. Daud, R.C. DeConti, S. Andrews, P. Urbas, A.I. Riker, V.K. Sondak, P.N. Munster, D.M. Sullivan, K.E. Ugen, J.L. Messina, R. Heller, Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma, J. Clin. Oncol. 26 (36) (2008) 5896–5903, https://doi.org/ 10.1200/JCO.2007.15.6794.
- [34] T.R.F. Smith, A. Patel, S. Ramos, D. Elwood, X. Zhu, J. Yan, E.N. Gary, S.N. Walker, K. Schultheis, M. Purwar, Z. Xu, J. Walters, P. Bhojnagarwala, M. Yang, N. Chokkalingam, P. Pezzoli, E. Parzych, E.L. Reuschel, A. Doan, N. Tursi, M. Vasquez, J. Choi, E. Tello-Ruiz, I. Maricic, M.A. Bah, Y. Wu, D. Amante, D.H. Park, Y. Dia, A.R. Ali, F.I. Zaidi, A. Generotti, K.Y. Kim, T.A. Herring, S. Reeder, V. M. Andrade, K. Buttigieg, G. Zhao, J.M. Wu, D. Li, L. Bao, J. Liu, W. Deng, C. Qin, A.S. Brown, M. Khoshnejad, N. Wang, J. Chu, D. Wrapp, J.S. McLellan, K. Muthumani, B. Wang, M.W. Carroll, JJ. Kim, J. Boyer, D.W. Kulp, L.M.P.F. Humeau, D.B. Weiner, K.E. Broderick, Immunogenicity of a DNA vaccine candidate for COVID-19, Nat. Commun. 11 (2020) 1–13, https://doi.org/10.1038/s41467-020-16505-0.
- [35] P. Tebas, ShuPing Yang, J.D. Boyer, E.L. Reuschel, A. Patel, A. Christensen-Quick, V.M. Andrade, M.P. Morrow, K. Kraynyak, J. Agnes, M. Purwar, A. Sylvester, J. Pawlicki, E. Gillespie, I. Maricic, F.I. Zaidi, K.Y. Kim, Y. Dia, D. Frase, P. Pezzoli, K. Schultheis, T.R.F. Smith, S.J. Ramos, T. McMullan, K. Buttigieg, M. W. Carroll, J. Ervin, M.C. Diehl, E. Blackwood, M.P. Mammen, J. Lee, M.J. Dallas, A.S. Brown, J.E. Shea, J.J. Kim, D.B. Weiner, K.E. Broderick, L.M. Humeau, Safety and immunogenicity of INO-4800 DNA vaccine against SARS-CoV-2: A preliminary report of an open-label, Phase 1 clinical trial, EClinicalMedicine. 31 (2021) 100689, https://doi.org/10.1016/j. eclinm.2020.100689.
- [36] A.K. Fajrial, Q.Q. He, N.I. Wirusanti, J.E. Slansky, X. Ding, A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing, Theranostics. 10 (12) (2020) 5532–5549, https://doi.org/10.7150/thno.43465.
- [37] H.-X. Wang, M. Li, C.M. Lee, S. Chakraborty, H.-W. Kim, G. Bao, K.W. Leong, CRISPR/Cas9-Based Genome Editing for Disease Modeling and Therapy: Challenges and Opportunities for Nonviral Delivery, Chem. Rev. 117 (15) (2017) 9874–9906, https://doi.org/10.1021/acs.chemrev.6b00799.
- [38] A. Patel, M.A. Bah, D.B. Weiner, In Vivo Delivery of Nucleic Acid-Encoded Monoclonal Antibodies, BioDrugs. 34 (3) (2020) 273–293, https://doi.org/ 10.1007/s40259-020-00412-3.
- [39] E. Neumann, M. Schaefer-Ridder, Y. Wang, P.H. Hofschneider, Gene transfer into mouse lyoma cells by electroporation in high electric fields, EMBO J. 1 (7) (1982) 841–845, https://doi.org/10.1002/j.1460-2075.1982.tb01257.x.
- [40] M. Golzio, J. Teissie, M.-P. Rols, Direct visualization at the single-cell level of electrically mediated gene delivery, Proc. Natl. Acad. Sci. U. S. A. 99 (3) (2002) 1292–1297, https://doi.org/10.1073/pnas.022646499.
- [41] H. Wolf, M.P. Rols, E. Boldt, E. Neumann, J. Teissié, Control by pulse parameters of electric field-mediated gene transfer in mammalian cells, Biophys. J. 66 (2) (1994) 524–531, https://doi.org/10.1016/S0006-3495(94) 80805-7.
- [42] T. Kotnik, L. Rems, M. Tarek, D. Miklavčič, Membrane Electroporation and Electropermeabilization: Mechanisms and Models, Annu. Rev. Biophys. 48 (1) (2019) 63–91, https://doi.org/10.1146/annurev-biophys-052118-115451.
- [43] K. Kinosita, T.Y. Tsong, Voltage-induced conductance in human erythrocyte membranes, Biochim. Biophys. Acta - Biomembr. 554 (2) (1979) 479–497, https://doi.org/10.1016/0005-2736(79)90386-9.
- [44] J. Li, H. Lin, Numerical simulation of molecular uptake via electroporation, Bioelectrochemistry. 82 (1) (2011) 10–21, https://doi.org/10.1016/j. bioelechem.2011.04.006.
- [45] K.A. DeBruin, W. Krassowska, Modeling electroporation in a single cell. I. Effects of field strength and rest potential, Biophys. J. 77 (3) (1999) 1213– 1224, https://doi.org/10.1016/S0006-3495(99)76973-0.
- [46] M. Schmeer, T. Seipp, U. Pliquett, S. Kakorin, E. Neumann, Mechanism for the conductivity changes caused by membrane electroporation of CHO cellpellets, Phys. Chem. Chem. Phys., The Royal Soc. Chem. (2004) 5564–5574, https://doi.org/10.1039/b411037d.
- [47] J.C. Weaver, Y.A. Chizmadzhev, Theory of electroporation: A review, Bioelectrochemistry Bioenerg. 41 (2) (1996) 135–160, https://doi.org/ 10.1016/S0302-4598(96)05062-3.
- [48] L.V. Chernomordik, S.I. Sukharev, S.V. Popov, V.F. Pastushenko, A.V. Sokirko, I. G. Abidor, Y.A. Chizmadzhev, The electrical breakdown of cell and lipid membranes: the similarity of phenomenologies, BBA - Biomembr. 902 (3) (1987) 360–373, https://doi.org/10.1016/0005-2736(87)90204-5.

- [49] K. Kinosita, T.Y. Tsong, Formation and resealing of pores of controlled sizes in human erythrocyte membrane, Nature 268 (5619) (1977) 438–441, https:// doi.org/10.1038/268438a0.
- [50] T. Batista Napotnik, D. Miklavčič, In vitro electroporation detection methods An overview, Bioelectrochemistry. 120 (2018) 166–182, https://doi.org/ 10.1016/j.bioelechem.2017.12.005.
- [51] J.-M. Escoffre, T. Portet, C. Favard, J. Teissié, D.S. Dean, M.-P. Rols, Electromediated formation of DNA complexes with cell membranes and its consequences for gene delivery, Biochim. Biophys. Acta - Biomembr. 1808 (6) (2011) 1538–1543, https://doi.org/10.1016/j.bbamem.2010.10.009.
- [52] C. Faurie, M. Rebersek, M. Golzio, M. Kanduser, J.-M. Escoffre, M. Pavlin, J. Teissie, D. Miklavcic, M.-P. Rols, Electro-mediated gene transfer and expression are controlled by the life-time of DNA/membrane complex formation, J. Gene Med. 12 (1) (2010) 117–125, https://doi.org/10.1002/jgm.1414.
- [53] M. Breton, L. Delemotte, A. Silve, L.M. Mir, M. Tarek, Transport of siRNA through lipid membranes driven by nanosecond electric pulses: An experimental and computational study, J. Am. Chem. Soc. 134 (34) (2012) 13938–13941, https://doi.org/10.1021/ja3052365.
- [54] V.A. Klenchin, S.I. Sukharev, S.M. Serov, L.V. Chernomordik, Chizmadzhev YuA, Chizmadzhev YuA, Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis, Biophys. J. 60 (4) (1991) 804–811, https://doi.org/10.1016/S0006-3495(91)82115-4.
- [55] S. Sachdev, S. Feijoo Moreira, Y. Keehnen, L. Rems, M.T. Kreutzer, P.E. Boukany, DNA-membrane complex formation during electroporation is DNA size-dependent, Biochim. Biophys. Acta - Biomembr. 1862 (2) (2020) 183089, https://doi.org/10.1016/j.bbamem.2019.183089.
- [56] A. Paganin-Gioanni, E. Bellard, J.M. Escoffre, M.P. Rols, J. Teissie, M. Golzio, Direct visualization at the single-cell level of siRNA electrotransfer into cancer cells, Proc. Natl. Acad. Sci. USA 108 (26) (2011) 10443–10447, https:// doi.org/10.1073/pnas.1103519108.
- [57] O.V. Bondar, D.V. Saifullina, I.I. Shakhmaeva, I.I. Mavlyutova, T.I. Abdullin, Monitoring of the Zeta Potential of Human Cells upon Reduction in Their Viability and Interaction with Polymers, Acta Naturae. 4 (2012) 78–81, https://doi.org/10.32607/20758251-2012-4-1-78-81.
- [58] S. Sachdev, A. Muralidharan, D.K. Choudhary, D.L. Perrier, L. Rems, M.T. Kreutzer, P.E. Boukany, DNA translocation to giant unilamellar vesicles during electroporation is independent of DNA size, Soft Matter. 15 (45) (2019) 9187– 9194, https://doi.org/10.1039/C9SM01274E.
- [59] D.A. Zaharoff, F. Yuan, Effects of pulse strength and pulse duration on in vitro DNA electromobility, Bioelectrochemistry. 62 (1) (2004) 37–45, https://doi. org/10.1016/j.bioelechem.2003.10.011.
- [60] D.A. Zaharoff, R.C. Barr, C.-Y. Li, F. Yuan, Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery, Gene Ther. 9 (19) (2002) 1286–1290, https://doi.org/10.1038/sj.gt.3301799.
- [61] J.W. Henshaw, D.A. Zaharoff, B.J. Mossop, F. Yuan, Electric field-mediated transport of plasmid DNA in tumor interstitium in vivo, Bioelectrochemistry. 71 (2) (2007) 233–242, https://doi.org/10.1016/j.bioelechem.2007.07.005.
- [62] S.I. Sukharev, V.A. Klenchin, S.M. Serov, L.V. Chernomordik, Chizmadzhev YuA, Chizmadzhev YuA, Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores, Biophys. J. 63 (5) (1992) 1320–1327, https://doi.org/10.1016/S0006-3495(92)81709-5.
- [63] M. Wu, F. Yuan, J. Rao, Membrane binding of plasmid DNA and endocytic pathways are involved in electrotransfection of mammalian cells, PLoS One. 6 (6) (2011) e20923, https://doi.org/10.1371/journal.pone.0020923.
- [64] C. Rosazza, H. Deschout, A. Buntz, K. Braeckmans, M.-P. Rols, A. Zumbusch, Endocytosis and Endosomal Trafficking of DNA After Gene Electrotransfer In Vitro, Mol. Ther. - Nucleic Acids. 5 (2016) e286, https://doi.org/10.1038/ mtna.2015.59.
- [65] C. Rosazza, A. Buntz, T. Rieß, D. Wöll, A. Zumbusch, M.-P. Rols, Intracellular tracking of single-plasmid DNA particles after delivery by electroporation, Mol. Ther. 21 (12) (2013) 2217–2226, https://doi.org/10.1038/mt.2013.182.
- [66] E.E. Vaughan, D.A. Dean, Intracellular trafficking of plasmids during transfection is mediated by microtubules, Mol. Ther. 13 (2) (2006) 422– 428, https://doi.org/10.1016/j.ymthe.2005.10.004.
- [67] E. Dauty, A.S. Verkman, Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: A new barrier for non-viral gene delivery, J. Biol. Chem. 280 (9) (2005) 7823–7828, https://doi.org/10.1074/ jbc.M412374200.
- [68] G.L. Lukacs, P. Haggie, O. Seksek, D. Lechardeur, N. Freedman, A.S. Verkman, Size-dependent DNA mobility in cytoplasm and nucleus, J. Biol. Chem. 275 (3) (2000) 1625–1629, https://doi.org/10.1074/jbc.275.3.1625.
- [69] L.D. Cervia, F. Yuan, Current Progress in Electrotransfection as a Nonviral Method for Gene Delivery, Mol. Pharm. 15 (9) (2018) 3617–3624, https://doi. org/10.1021/acs.molpharmaceut.8b00207.
- [70] D. Lechardeur, K.-J. Sohn, M. Haardt, P.B. Joshi, M. Monck, R.W. Graham, B. Beatty, J. Squire, H. O'Brodovich, G.L. Lukacs, Metabolic instability of plasmid DNA in the cytosol: A potential barrier to gene transfer, Gene Ther. 6 (4) (1999) 482–497, https://doi.org/10.1038/sj.gt.3300867.
- [71] M.A. Badding, J.D. Lapek, A.E. Friedman, D.A. Dean, Proteomic and functional analyses of protein-DNA complexes during gene transfer, Mol. Ther. 21 (4) (2013) 775–785, https://doi.org/10.1038/mt.2012.231.
- [72] S. Brunner, E. Fürtbauer, T. Sauer, M. Kursa, E. Wagner, Overcoming the nuclear barrier: Cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation, Mol. Ther. 5 (1) (2002) 80–86, https:// doi.org/10.1006/mthe.2001.0509.

- [73] M. Golzio, J. Teissié, M.-P. Rols, Cell synchronization effect on mammalian cell permeabilization and gene delivery by electric field, Biochim. Biophys. Acta – Biomembr. 1563 (1-2) (2002) 23–28, https://doi.org/10.1016/S0005-2736 (02)00369–3.
- [74] J.K. Utvik, A. Nja, K. Gundersen, DNA injection into single cells of intact mice, Hum. Gene Ther. 10 (2) (1999) 291–300, https://doi.org/10.1089/ 10430349950019075.
- [75] K.H. Schoenbach, S.J. Beebe, E.S. Buescher, Intracellular effect of ultrashort electrical pulses, Bioelectromagnetics. 22 (6) (2001) 440–448, https://doi.org/ 10.1002/bem.71.
- [76] T.B. Napotnik, M. Reberšek, T. Kotnik, E. Lebrasseur, G. Cabodevila, D. Miklavčič, Electropermeabilization of endocytotic vesicles in B16 F1 mouse melanoma cells, Med. Biol. Eng. Comput. 48 (5) (2010) 407–413, https://doi.org/10.1007/s11517-010-0599-9.
- [77] E. Tekle, H. Oubrahim, S.M. Dzekunov, J.F. Kolb, K.H. Schoenbach, P.B. Chock, Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses, Biophys. J. 89 (1) (2005) 274–284, https://doi.org/ 10.1529/biophysj.104.054494.
- [78] L. Chopinet, T. Batista-Napotnik, A. Montigny, M. Rebersek, J. Teissié, M.-P. Rols, D. Miklavčič, Nanosecond electric pulse effects on gene expression, J. Membr. Biol. 246 (11) (2013) 851–859, https://doi.org/10.1007/s00232-013-9579-v.
- [79] N. Chen, K.H. Schoenbach, J.F. Kolb, R. James Swanson, A.L. Garner, J. Yang, R.P. Joshi, S.J. Beebe, Leukemic cell intracellular responses to nanosecond electric fields, Biochem. Biophys. Res. Commun. 317 (2) (2004) 421–427, https://doi.org/10.1016/j.bbrc.2004.03.063.
- [80] S.J. Beebe, J. White, P.F. Blackmore, Y. Deng, K. Somers, K.H. Schoenbach, Diverse Effects of Nanosecond Pulsed Electric Fields on Cells and Tissues, DNA Cell Biol. 22 (12) (2003) 785–796, https://doi.org/10.1089/ 104454903322624993.
- [81] S.J. Beebe, P.F. Blackmore, J. White, R.P. Joshi, K.H. Schoenbach, Physiol. Meas., Physiol Meas. 25 (4) (2004) 1077–1093, https://doi.org/10.1088/0967-3334/ 25/4/023.
- [82] A. Elouahabi, J.-M. Ruysschaert, Formation and intracellular trafficking of lipoplexes and polyplexes, Mol. Ther. 11 (3) (2005) 336–347, https://doi.org/ 10.1016/j.ymthe.2004.12.006.
- [83] A. Chaumet, G.D. Wright, S.H. Seet, K.M. Tham, N.V. Gounko, F. Bard, Nuclear envelope-associated endosomes deliver surface proteins to the nucleus, Nat. Commun. 6 (1) (2015), https://doi.org/10.1038/ncomms9218.
- [84] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Molecular Biology of the Cell, 4th Editio, Garland Science, New York, 2002.
- [85] A.G. Baranovskii, V.N. Buneva, G.A. Nevinsky, Human deoxyribonucleases, Biochem. 69 (6) (2004) 587–601, https://doi.org/10.1023/B: BIRY.0000033731.50496.01.
- [86] L. Lauková, B. Konečná, L. Janovičová, B. Vlková, P. Celec, Deoxyribonucleases and their applications in biomedicine, Biomolecules. 10 (2020) 1–20, https:// doi.org/10.3390/biom10071036.
- [87] T. Nomura, S. Nakajima, K. Kawabata, F. Yamashita, Y. Takakura, M. Hashida, Intratumoral Pharmacokinetics and in Vivo Gene Expression of Naked Plasmid DNA and Its Cationic Liposome Complexes after Direct Gene Transfer, Cancer Res. 57 (1997).
- [88] I. Danko, P. Williams, H. Herweijer, G. Zhang, U.S. Latendresse, I. Bock, J.A. Wolff, High expression of naked plasmid DNA in muscles of young rodents, Hum. Mol. Genet. 6 (1997) 1435–1443, https://doi.org/10.1093/hmg/ 6.9.1435.
- [89] D.J. Wells, G. Goldspink, Age and sex influence expression of plasmid DNA directly injected into mouse skeletal muscle, FEBS Lett. 306 (1992) 203–205, https://doi.org/10.1016/0014-5793(92)81000-C.
- [90] S. Jiao, P. Williams, R.K. Berg, B.A. Hodgeman, L. Liu, G. Repetto, J.A. Wolff, Direct gene transfer into nonhuman primate myofibers in vivo, Hum. Gene Ther. 3 (1) (1992) 21–33, https://doi.org/10.1089/hum.1992.3.1-21.
- [91] C. Rosazza, S. Haberl Meglic, A. Zumbusch, M.-P. Rols, D. Miklavcic, Gene Electrotransfer: A Mechanistic Perspective, Curr. Gene Ther. 16 (2) (2016) 98– 129, https://doi.org/10.2174/1566523216666160331130040.
- [92] J.M. McMahon, E. Signori, K.E. Wells, V.M. Fazio, D.J. Wells, Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase - Increased expression with reduced muscle damage, Gene Ther. 8 (16) (2001) 1264–1270, https://doi.org/10.1038/sj.gt.3301522.
 [93] M. Cemazar, M. Golzio, G. Sersa, J.-M. Escoffre, A. Coer, S. Vidic, J. Teissie,
- [93] M. Cemazar, M. Golzio, G. Sersa, J.-M. Escoffre, A. Coer, S. Vidic, J. Teissie, Hyaluronidase and collagenase increase the transfection efficiency of gene electrotransfer in various murine tumors, Hum. Gene Ther. 23 (1) (2012) 128–137, https://doi.org/10.1089/hum.2011.073.
- [94] L. Wasungu, J.-M. Escoffre, A. Valette, J. Teissie, M.-P. Rols, A 3D in vitro spheroid model as a way to study the mechanisms of electroporation, Int. J. Pharm. 379 (2) (2009) 278–284, https://doi.org/10.1016/j. ijpharm.2009.03.035.
- [95] L. Chopinet, L. Wasungu, M.-P. Rols, First explanations for differences in electrotransfection efficiency in vitro and in vivo using spheroid model, Int. J. Pharm. 423 (1) (2012) 7–15, https://doi.org/10.1016/j.ijpharm.2011.04.054.
- [96] M. Pavlin, N. Pavselj, D. Miklavcic, Dependence of induced transmembrane potential on cell density, arrangement, and cell position inside a cell system, IEEE Trans. Biomed. Eng. 49 (6) (2002) 605–612, https://doi.org/10.1109/ TBME.2002.1001975.
- [97] R. Susil, D. Šemrov, D. Miklavčič, Electric field-induced transmembrane potential depends on cell density and organization, Electro- and

Magnetobiology. 17 (3) (1998) 391–399, https://doi.org/10.3109/ 15368379809030739.

- [98] P.J. Canatella, M.M. Black, D.M. Bonnichsen, C. McKenna, M.R. Prausnitz, Tissue Electroporation: Quantification and Analysis of Heterogeneous Transport in Multicellular Environments, Biophys. J. 86 (5) (2004) 3260– 3268, https://doi.org/10.1016/S0006-3495(04)74374-X.
- [99] M. Madi, M.-P. Rols, L. Gibot, Efficient In Vitro Electropermeabilization of Reconstructed Human Dermal Tissue, J. Membr. Biol. 248 (5) (2015) 903–908, https://doi.org/10.1007/s00232-015-9791-z.
- [100] M.F. Bureau, J. Gehl, V. Deleuze, L.M. Mir, D. Scherman, Importance of association between permeabilization and electrophoretic forces for intramuscular DNA electrotransfer, Biochim. Biophys. Acta - Gen. Subj. 1474 (3) (2000) 353–359, https://doi.org/10.1016/S0304-4165(00)00028-3.
- [101] S. Satkauskas, M.F. Bureau, M. Puc, A. Mahfoudi, D. Scherman, D. Miklavcic, L. M. Mir, Mechanisms of in vivo DNA electrotransfer: Respective contribution of cell electropermeabilization and DNA electrophoresis, Mol. Ther. 5 (2002) 133–140, https://doi.org/10.1006/mthe.2002.0526.
- [102] G.L. Andreason, G.A. Evans, Optimization of electroporation for transfection of mammalian cell lines, Anal. Biochem. 180 (2) (1989) 269–275, https://doi. org/10.1016/0003-2697(89)90429-6.
- [103] S. Šatkauskas, F. André, M.F. Bureau, D. Scherman, D. Miklavčič, L.M. Mir, Electrophoretic component of electric pulses determines the efficacy of in vivo DNA electrotransfer, Hum. Gene Ther. 16 (10) (2005) 1194–1201, https://doi.org/10.1089/hum.2005.16.1194.
- [104] N. Pavšelj, V. Préat, DNA electrotransfer into the skin using a combination of one high- and one low-voltage pulse, J. Control. Release. 106 (3) (2005) 407– 415, https://doi.org/10.1016/j.jconrel.2005.05.003.
- [105] F. Andre, J. Gehl, G. Sersa, V. Preat, P. Hojman, J. Eriksen, M. Golzio, M. Cemazar, N. Pavselj, M.-P. Rols, D. Miklavcic, E. Neumann, J. Teissie, L.M. Mir, Efficiency of high and low voltage pulse combinations for gene electrotransfer in muscle, liver, tumor and skin, Hum. Gene Ther. 19 (2008) 1261–1271, https://doi.org/10.1089/hum.2008.060.
- [106] M.C. Diehl, J.C. Lee, S.E. Daniels, P. Tebas, A.S. Khan, M. Giffear, N.Y. Sardesai, M.L. Bagarazzi, Tolerability of intramuscular and intradermal delivery by CELLECTRA[®] adaptive constant current electroporation device in healthy volunteers, Hum. Vaccines Immunother. 9 (10) (2013) 2246–2252, https:// doi.org/10.4161/hv.24702.
- [107] M. Kandušer, D. Miklavčič, M. Pavlin, Mechanisms involved in gene electrotransfer using high- and low-voltage pulses - An in vitro study, Bioelectrochemistry. 74 (2) (2009) 265–271, https://doi.org/10.1016/j. bioelechem.2008.09.002.
- [108] M. Pavlin, K. Flisar, M. Kandušer, The role of electrophoresis in gene electrotransfer, J. Membr. Biol. 236 (1) (2010) 75–79, https://doi.org/ 10.1007/s00232-010-9276-z.
- [109] M. Pavlin, M. Kandušer, New Insights into the Mechanisms of Gene Electrotransfer - Experimental and Theoretical Analysis, Sci. Rep. 5 (1) (2015), https://doi.org/10.1038/srep09132.
- [110] M. Thomsen, A. Hernandez-Garcia, J. Mathiesen, M. Poulsen, D.N. Sørensen, L. Tarnow, R. Feidenhans'l, J.C.D. Alamo, Model study of the pressure build-up during subcutaneous injection, PLoS One. 9 (8) (2014) e104054, https://doi.org/10.1371/journal.pone.0104054.
- [111] K. Comley, N. Fleck, Deep penetration and liquid injection into adipose tissue, J. Mech. Mater. Struct. 6 (1-4) (2011) 127–140, https://doi.org/10.2140/ jomms.2011.6.127.
- [112] P.D. Fisher, C.J. Brambila, J.R. McCoy, W.B. Kiosses, J.M. Mendoza, J. Oh, B.S. Yung, K. Schultheis, T.R.F. Smith, K.E. Broderick, Adipose tissue: A new target for electroporation-enhanced DNA vaccines, Gene Ther. 24 (12) (2017) 757– 767, https://doi.org/10.1038/gt.2017.96.
- [113] M. Dupuis, K. Denis-Mize, C. Woo, C. Goldbeck, M.J. Selby, M. Chen, G.R. Otten, J.B. Ulmer, J.J. Donnelly, G. Ott, D.M. McDonald, Distribution of DNA Vaccines Determines Their Immunogenicity After Intramuscular Injection in Mice, J. Immunol. 165 (5) (2000) 2850–2858, https://doi.org/10.4049/ jimmunol.165.5.2850.
- [114] M.A. Swartz, M.E. Fleury, Interstitial flow and its effects in soft tissues, Annu. Rev. Biomed. Eng. 9 (1) (2007) 229–256, https://doi.org/10.1146/annurev. bioeng.9.060906.151850.
- [115] S. McGuire, D. Zaharoff, F. Yuan, Nonlinear dependence of hydraulic conductivity on tissue deformation during intratumoral infusion, Ann. Biomed. Eng. 34 (7) (2006) 1173–1181, https://doi.org/10.1007/s10439-006-9136-2.
- [116] X.-Y. Zhang, J. Luck, M.W. Dewhirst, F. Yuan, Interstitial hydraulic conductivity in a fibrosarcoma, Am. J. Physiol. - Hear. Circ. Physiol. 279 (6) (2000) H2726–H2734, https://doi.org/10.1152/ajpheart.2000.279.6.H2726.
- [117] H. Wiig, M.A. Swartz, Interstitial fluid and lymph formation and transport: Physiological regulation and roles in inflammation and cancer, Physiol. Rev. 92 (3) (2012) 1005–1060, https://doi.org/10.1152/physrev.00037.2011.
- [118] P.A. Netti, D.A. Berk, M.A. Swartz, A.J. Grodzinsky, R.K. Jain, Role of extracellular matrix assembly in interstitial transport in solid tumors, Cancer Res. 60 (2000) 2497–2503.
- [119] F. Xiao, C. Nicholson, J. Hrabe, S. Hrabětová, Diffusion of flexible random-coil dextran polymers measured in anisotropic brain extracellular space by integrative optical imaging, Biophys. J. 95 (3) (2008) 1382–1392, https://doi. org/10.1529/biophysj.107.124743.
- [120] T. Stylianopoulos, B. Diop-Frimpong, L.L. Munn, R.K. Jain, Diffusion anisotropy in collagen gels and tumors: The effect of fiber network orientation, Biophys. J. 99 (10) (2010) 3119–3128, https://doi.org/10.1016/j.bpj.2010.08.065.

- [121] H. Kim, H. Park, S.J. Lee, Effective method for drug injection into subcutaneous tissue, Sci. Rep. 7 (2017) 1–11, https://doi.org/10.1038/ s41598-017-10110-w.
- [122] G. Vandermeulen, E. Staes, M.L. Vanderhaeghen, M.F. Bureau, D. Scherman, V. Préat, Optimisation of intradermal DNA electrotransfer for immunisation, J. Control. Release. 124 (1-2) (2007) 81–87, https://doi.org/10.1016/j. jconrel.2007.08.010.
- [123] A. Gothelf, F. Mahmood, F. Dagnaes-Hansen, J. Gehl, Efficacy of transgene expression in porcine skin as a function of electrode choice, Bioelectrochemistry. 82 (2) (2011) 95–102, https://doi.org/10.1016/j. bioelechem.2011.06.001.
- [124] C.Y. Calvet, J. Thalmensi, C. Liard, E. Pliquet, T. Bestetti, T. Huet, P. Langlade-Demoyen, L.M. Mir, Optimization of a gene electrotransfer procedure for efficient intradermal immunization with an hTERT-based DNA vaccine in mice, Mol. Ther. - Methods Clin. Dev. 1 (2014) 14045, https://doi.org/ 10.1038/mtm.2014.45.
- [125] T. Forjanič, D. Miklavčič, Numerical study of gene electrotransfer efficiency based on electroporation volume and electrophoretic movement of plasmid DNA, Biomed. Eng. Online. 17 (1) (2018), https://doi.org/10.1186/s12938-018-0515-3.
- [126] L.C. Heller, M.J. Jaroszeski, D. Coppola, A.N. McCray, J. Hickey, R. Heller, Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode, Gene Ther. 14 (3) (2007) 275–280, https://doi.org/10.1038/ si.gt.3302867.
- [127] X. Ding, M.P. Stewart, A. Sharei, J.C. Weaver, R.S. Langer, K.F. Jensen, Highthroughput nuclear delivery and rapid expression of DNA via mechanical and electrical cell-membrane disruption, Nat. Biomed. Eng. 1 (3) (2017), https:// doi.org/10.1038/s41551-017-0039.
- [128] T. Akerstrom, K. Vedel, J. Needham, P. Hojman, E. Kontou, Y. Hellsten, J.F.P. Wojtaszewski, Optimizing hyaluronidase dose and plasmid DNA delivery greatly improves gene electrotransfer efficiency in rat skeletal muscle, Biochem. Biophys. Reports. 4 (2015) 342–350, https://doi.org/10.1016/j. bbrep.2015.10.007.
- [129] M. Manthorpe, F. Cornefert-Jensen, J. Hartikka, J. Felgner, A. Rundell, M. Margalith, V. Dwarki, Gene Therapy by Intramuscular Injection of Plasmid DNA: Studies on Firefly Luciferase Gene Expression in Mice, Hum. Gene Ther. 4 (4) (1993) 419–431, https://doi.org/10.1089/hum.1993.4.4-419.
- [130] J.W. Henshaw, F. Yuan, Field distribution and DNA transport in solid tumors during electric field-mediated gene delivery, J. Pharm. Sci. 97 (2) (2008) 691– 711, https://doi.org/10.1002/jps.21000.
- [131] A. Zupanic, S. Corovic, D. Miklavcic, M. Pavlin, Numerical optimization of gene electrotransfer into muscle tissue, Biomed. Eng. Online. 9 (2010) 66, https://doi.org/10.1186/1475-925X-9-66.
- [132] J. Grove, M. Marsh, The cell biology of receptor-mediated virus entry, J. Cell Biol. 195 (2011) 1071–1082, https://doi.org/10.1083/jcb.201108131.
 [133] D.S. Dimitrov, Virus entry: Molecular mechanisms and biomedical
- [133] D.S. Dimitrov, Virus entry: Molecular mechanisms and biomedical applications, Nat. Rev. Microbiol. 2 (2) (2004) 109–122, https://doi.org/ 10.1038/nrmicro817.
- [134] A. Srivastava, In vivo tissue-tropism of adeno-associated viral vectors, Curr. Opin. Virol. 21 (2016) 75–80, https://doi.org/10.1016/j.coviro.2016.08.003.
- [135] G. McFadden, M.R. Mohamed, M.M. Rahman, E. Bartee, Cytokine determinants of viral tropism, Nat. Rev. Immunol. 9 (9) (2009) 645–655, https://doi.org/10.1038/nri2623.
- [136] D. Miklavčič, N. Pavšelj, F.X. Hart, Electric Properties of Tissues, Wiley Encycl. Biomed. Eng., Am. Cancer Soc. (2006), https://doi.org/10.1002/ 9780471740360.ebs0403.
- [137] S. Čorović, L.M. Mir, D. Miklavčič, In vivo muscle electroporation threshold determination: Realistic numerical models and in vivo experiments, J. Membr. Biol. 245 (9) (2012) 509–520, https://doi.org/10.1007/s00232-012-9432-8.
- [138] D. Miklavcic, S. Corovic, G. Pucihar, N. Pavselj, Importance of tumour coverage by sufficiently high local electric field for effective electrochemotherapy, Eur. J. Cancer, Suppl. 4 (11) (2006) 45–51, https://doi.org/10.1016/j. eicsup.2006.08.006.
- [139] G. Serša, M. Čemažar, D. Šemrov, D. Miklavčič, Changing electrode orientation improves the efficacy of electrochemotherapy of solid tumors in mice, Bioelectrochemistry Bioenerg. 39 (1) (1996) 61–66, https://doi.org/10.1016/ 0302-4598(95)01866-2.
- [140] J. Dermol-Černe, E. Pirc, D. Miklavčič, Mechanistic view of skin electroporation-models and dosimetry for successful applications: an expert review, Expert Opin. Drug Deliv. 17 (5) (2020) 689–704, https://doi. org/10.1080/17425247.2020.1745772.
- [141] N. Pavšelj, V. Préat, D. Miklavčič, A numerical model of skin electropermeabilization based on in vivo experiments, Ann. Biomed. Eng. 35 (2007) 2138–2144, https://doi.org/10.1007/s10439-007-9378-7.
- [142] N. Pavselj, D. Miklavcic, Numerical models of skin electropermeabilization taking into account conductivity changes and the presence of local transport regions, IEEE Trans. Plasma Sci. 36 (4) (2008) 1650–1658, https://doi.org/ 10.1109/TPS.2008.928715.
- [143] M. Kranjc, B. Markelc, F. Bajd, M. Čemažar, I. Serša, T. Blagus, D. Miklavčič, In situ monitoring of electric field distribution in mouse tumor during electroporation, Radiology. 274 (1) (2015) 115–123, https://doi.org/ 10.1148/radiol.14140311.
- [144] S. Corovic, I. Lackovic, P. Sustaric, T. Sustar, T. Rodic, D. Miklavcic, Modeling of electric field distribution in tissues during electroporation, Biomed. Eng. Online. 12 (2013) 1–27, https://doi.org/10.1186/1475-925X-12-16.

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- [145] M. Kranjc, F. Bajd, I. Serša, D. Miklavčič, Magnetic resonance electrical impedance tomography for measuring electrical conductivity during electroporation, Physiol. Meas. 35 (6) (2014) 985–996, https://doi.org/ 10.1088/0967-3334/35/6/985.
- [146] L.T. Muftuler, M.J. Hamamura, O. Birgul, O. Nalcioglu, In Vivo MRI Electrical Impedance Tomography (MREIT) of tumors, Technol. Cancer Res. Treat. 5 (2006) 381–387 (accessed October 5, 2021) https://europepmc.org/article/ med/16866568.
- [147] Y. Wang, Q.i. Shao, P.-F. Van de Moortele, E. Racila, J. Liu, J. Bischof, B. He, Mapping electrical properties heterogeneity of tumor using boundary informed electrical properties tomography (BIEPT) at 7T, Magn. Reson. Med. 81 (1) (2019) 393–409, https://doi.org/10.1002/mrm.27414.
- [148] B. Kos, Treatment planning for electrochemotherapy and irreversible electroporation of deep-seated tumors, Handb. Electroporation, Springer, Cham (2017) 1001–1017, https://doi.org/10.1007/978-3-319-32886-7_2.
- [149] J. Gehl, T.H. Sørensen, K. Nielsen, P. Raskmark, S.L. Nielsen, T. Skovsgaard, L.M. Mir, In vivo electroporation of skeletal muscle: Threshold, efficacy and relation to electric field distribution, Biochim. Biophys. Acta - Gen. Subj. 1428 (2-3) (1999) 233–240, https://doi.org/10.1016/S0304-4165(99)00094-X.
- [150] D. Miklavčič, D. Šemrov, H. Mekid, L.M. Mir, A validated model of in vivo electric field distribution in tissues for electrochemotherapy and for DNA electrotransfer for gene therapy, Biochim. Biophys. Acta - Gen. Subj. 1523 (1) (2000) 73–83, https://doi.org/10.1016/S0304-4165(00)00101-X.
- [151] D. Sel, D. Cukjati, D. Batiuskaite, T. Slivnik, L.M. Mir, D. Miklavcic, Sequential finite element model of tissue electropermeabilization, IEEE Trans. Biomed. Eng. 52 (5) (2005) 816–827, https://doi.org/10.1109/TBME.2005.845212.
- [152] J. Dermol-Černe, T. Batista Napotnik, M. Reberšek, D. Miklavčič, Short microsecond pulses achieve homogeneous electroporation of elongated biological cells irrespective of their orientation in electric field, Sci. Rep. 10 (1) (2020), https://doi.org/10.1038/s41598-020-65830-3.
- [153] S. Čorović, A. Županič, S. Kranjc, B. Al Sakere, A. Leroy-Willig, L.M. Mir, D. Miklavčič, The influence of skeletal muscle anisotropy on electroporation: In vivo study and numerical modeling, Med. Biol. Eng. Comput. 48 (7) (2010) 637–648, https://doi.org/10.1007/s11517-010-0614-1.
- [154] P. Turjanski, N. Olaiz, F. Maglietti, S. Michinski, C. Suárez, F.V. Molina, G. Marshall, B. Rubinsky, The Role of pH Fronts in Reversible Electroporation, PLoS One. 6 (4) (2011) e17303, https://doi.org/10.1371/journal. pone.0017303.
- [155] F. Maglietti, S. Michinski, N. Olaiz, M. Castro, C. Suárez, G. Marshall, M.R. Scarfi, The Role of Ph Fronts in Tissue Electroporation Based Treatments, PLoS One. 8 (11) (2013) e80167, https://doi.org/10.1371/journal.pone.0080167.
- [156] N. Olaiz, E. Signori, F. Maglietti, A. Soba, C. Suárez, P. Turjanski, S. Michinski, G. Marshall, Tissue damage modeling in gene electrotransfer: The role of pH, Bioelectrochemistry. 100 (2014) 105–111, https://doi.org/10.1016/j. bioelechem.2014.05.001.
- [157] M. Phillips, L. Rubinsky, A. Meir, N. Raju, B. Rubinsky, Combining Electrolysis and Electroporation for Tissue Ablation: Technol, Cancer Res. Treat. 14 (4) (2015) 395–410, https://doi.org/10.1177/1533034614560102.
- [158] N. Klein, E. Guenther, F. Botea, M. Pautov, S. Dima, D. Tomescu, M. Popescu, A. Ivorra, M. Stehling, I. Popescu, M.R. Scarfi, The combination of electroporation and electrolysis (E2) employing different electrode arrays for ablation of large tissue volumes, PLoS One. 14 (8) (2019) e0221393, https://doi.org/10.1371/ journal.pone.0221393.
- [159] C.B. Arena, R.V. Davalos, Advances in Therapeutic Electroporation to Mitigate Muscle Contractions, J. Membr. Sci. Technol. 2 (2012) 1–3, https://doi.org/ 10.4172/2155-9589.1000E102.
- [160] B. Mercadal, C.B. Arena, R.V. Davalos, A. Ivorra, Avoiding nerve stimulation in irreversible electroporation: a numerical modeling study, Phys. Med. Biol. 62 (20) (2017) 8060–8079, https://doi.org/10.1088/1361-6560/aa8c53.
- [161] M. Wallace, B. Evans, S. Woods, R. Mogg, L. Zhang, A.C. Finnefrock, D. Rabussay, M. Fons, J. Mallee, D. Mehrotra, F. Schödel, L. Musey, Tolerability of Two Sequential Electroporation Treatments Using MedPulser DNA Delivery System (DDS) in Healthy Adults, Mol. Ther. 17 (5) (2009) 922–928, https://doi.org/10.1038/mt.2009.27.
- [162] A. Županič, S. Ribarič, D. Miklavčič, Increasing the repetition frequency of electric pulse delivery reduces unpleasant sensations that occur in electrochemotherapy, Neoplasma. 54 (2007) 246–250.
- [163] R. Fusco, E. Di Bernardo, V. D'Alessio, S. Salati, M. Cadossi, Reduction of muscle contraction and pain in electroporation-based treatments: An overview, World J. Clin. Oncol. 12 (5) (2021) 367–381, https://doi.org/ 10.5306/wjco.v12.i5.367.
- [164] A. Steuer, A. Schmidt, P. Labohá, P. Babica, J.F. Kolb, Transient suppression of gap junctional intercellular communication after exposure to 100nanosecond pulsed electric fields, Bioelectrochemistry. 112 (2016) 33–46, https://doi.org/10.1016/j.bioelechem.2016.07.003.
- [165] B. Markelc, E. Bellard, G. Sersa, T. Jesenko, S. Pelofy, J. Teissié, R. Frangez, M.P. Rols, M. Cemazar, M. Golzio, Increased permeability of blood vessels after reversible electroporation is facilitated by alterations in endothelial cell-tocell junctions, J. Control. Release. 276 (2018) 30–41, https://doi.org/10.1016/ j.jconrel.2018.02.032.
- [166] J.C. Weaver, R.A. Mintzer, Decreased bilayer stability due to transmembrane potentials, Phys. Lett. A. 86 (1) (1981) 57–59, https://doi.org/10.1016/0375-9601(81)90688-5.
- [167] I.G. Abidor, V.B. Arakelyan, L.V. Chernomordik, Y.A. Chizmadzhev, V.F. Pastushenko, M.P. Tarasevich, Electric breakdown of bilayer lipid membranes. I. The main experimental facts and their qualitative

discussion, J. Electroanal. Chem. 104 (1979) 37–52, https://doi.org/10.1016/ S0022-0728(79)81006-2.

- [168] T.D. Xie, L. Sun, T.Y. Tsong, Study of mechanisms of electric field-induced DNA transfection. I. DNA entry by surface binding and diffusion through membrane pores, Biophys. J. 58 (1) (1990) 13–19, https://doi.org/10.1016/ S0006-3495(90)82349-3.
- [169] H. Stopper, H. Jones, U. Zimmermann, Large scale transfection of mouse Lcells by electropermeabilization, BBA - Biomembr. 900 (1) (1987) 38–44, https://doi.org/10.1016/0005-2736(87)90275-6.
- [170] D.S. Dimitrov, A.E. Sowers, Membrane electroporaton fast molecular exchange by electroosmosis, BBA - Biomembr. 1022 (3) (1990) 381–392, https://doi.org/10.1016/0005-2736(90)90289-Z.
- [171] T.-K. Wong, E. Neumann, Electric field mediated gene transfer, Biochem. Biophys. Res. Commun. 107 (2) (1982) 584–587, https://doi.org/10.1016/ 0006-291X(82)91531-5.
- [172] E. Neumann, Membrane electroporation and direct gene transfer, Bioelectrochemistry Bioenerg. 28 (1-2) (1992) 247–267, https://doi.org/ 10.1016/0302-4598(92)80017-B.
- [173] N. Eynard, S. Sixou, N. Duran, J. Teissie, Fast kinetics studies of Escherichia coli electrotransformation, Eur. J. Biochem. 209 (1) (1992) 431–436, https://doi. org/10.1111/j.1432-1033.1992.tb17306.x.
- [174] 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. 71 (2) (1996) 868–877, https://doi.org/10.1016/ S0006-3495(96)79288-3.
- [175] K. Schwister, B. Deuticke, Formation and properties of aqueous leaks induced in human erythrocytes by electrical breakdown, BBA - Biomembr. 816 (2) (1985) 332–348, https://doi.org/10.1016/0005-2736(85)90501-2.
- [176] D.H. Mengistu, K. Bohinc, S. May, Binding of DNA to zwitterionic lipid layers mediated by divalent cations, J. Phys. Chem. B. 113 (36) (2009) 12277–12282, https://doi.org/10.1021/jp904986j.
- [177] J.J. McManus, J.O. R\u00e4dler, K.A. Dawson, Does Calcium Turn a Zwitterionic Lipid Cationic?, J Phys. Chem. B. 107 (36) (2003) 9869–9875, https://doi.org/ 10.1021/jp034463d.
- [178] A.Y. Antipina, A.A. Gurtovenko, Molecular-level insight into the interactions of DNA with phospholipid bilayers: Barriers and triggers, RSC Adv. 6 (43) (2016) 36425-36432, https://doi.org/10.1039/c6ra05607e.
- [179] R.A. Böckmann, B.L. de Groot, S. Kakorin, E. Neumann, H. Grubmüller, Kinetics, statistics, and energetics of lipid membrane electroporation studied by molecular dynamics simulations, Biophys. J. 95 (4) (2008) 1837–1850, https://doi.org/10.1529/biophysj.108.129437.
- [180] S. Haberl, D. Miklavčič, M. Pavlin, Effect of Mg ions on efficiency of gene electrotransfer and on cell electropermeabilization, Bioelectrochemistry. 79 (2) (2010) 265–271, https://doi.org/10.1016/j.bioelechem.2010.04.001.
- [181] I. Koltover, K. Wagner, C.R. Safinya, DNA condensation in two dimensions, Proc. Natl. Acad. Sci. U. S. A. 97 (26) (2000) 14046–14051, https://doi.org/ 10.1073/pnas.97.26.14046.
- [182] C. Herold, P. Schwille, E.P. Petrov, DNA condensation at freestanding cationic lipid bilayers, Phys. Rev. Lett. 104 (14) (2010), https://doi.org/10.1103/ PhysRevLett.104.148102.
- [183] A.G. Cherstvy, E.P. Petrov, Modeling DNA condensation on freestanding cationic lipid membranes, Phys. Chem. Chem. Phys. 16 (5) (2014) 2020–2037, https://doi.org/10.1039/C3CP53433B.
- [184] B.J. Reynwar, G. Illya, V.A. Harmandaris, M.M. Müller, K. Kremer, M. Deserno, Aggregation and vesiculation of membrane proteins by curvature-mediated interactions, Nature. 447 (7143) (2007) 461–464, https://doi.org/ 10.1038/nature05840.
- [185] I. Koltover, J.O. R\u00e4dler, C.R. Safinya, Membrane mediated attraction and ordered aggregation of colloidal particles bound to giant phospholipid vesicles, Phys. Rev. Lett. 82 (9) (1999) 1991–1994, https://doi.org/10.1103/ PhysRevLett.82.1991.
- [186] W. Krassowska, P.D. Filev, Modeling electroporation in a single cell, Biophys. J. 92 (2) (2007) 404–417, https://doi.org/10.1529/biophysj.106.094235.
- [187] T. Portet, R. Dimova, A new method for measuring edge tensions and stability of lipid bilayers: Effect of membrane composition, Biophys. J. 99 (10) (2010) 3264–3273, https://doi.org/10.1016/j.bpj.2010.09.032.
- [188] T. Portet, C. Favard, J. Teissié, D.S. Dean, M.P. Rols, Insights into the mechanisms of electromediated gene delivery and application to the loading of giant vesicles with negatively charged macromolecules, Soft Matter. 7 (2011) 3872–3881, https://doi.org/10.1039/c0sm01389g.
- [189] D.L. Perrier, A. Vahid, V. Kathavi, L. Stam, L. Rems, Y. Mulla, A. Muralidharan, G.H. Koenderink, M.T. Kreutzer, P.E. Boukany, Response of an actin network in vesicles under electric pulses, Sci. Rep. 9 (1) (2019), https://doi.org/ 10.1038/s41598-019-44613-5.
- [190] G. Saulis, R. Saulė, Size of the pores created by an electric pulse: Microsecond vs millisecond pulses, Biochim. Biophys. Acta - Biomembr., Elsevier 1818 (12) (2012) 3032–3039, https://doi.org/10.1016/j.bbamem.2012.06.018.
- [191] A.T. Esser, K.C. Smith, T.R. Gowrishankar, Z. Vasilkoskl, J.C. Weaver, Mechanisms for the intracellular manipulation of organelles by conventional electroporation, Biophys. J. 98 (2010) 2506–2514, https://doi. org/10.1016/j.bpj.2010.02.035.
- [192] K.C. Smith, R.S. Son, T.R. Gowrishankar, J.C. Weaver, Emergence of a large pore subpopulation during electroporating pulses, Bioelectrochemistry. 100 (2014) 3–10, https://doi.org/10.1016/j.bioelechem.2013.10.009.
- [193] R.S. Son, T.R. Gowrishankar, K.C. Smith, J.C. Weaver, Modeling a Conventional Electroporation Pulse Train: Decreased Pore Number, Cumulative Calcium

Transport and an Example of Electrosensitization, IEEE Trans. Biomed. Eng. 63 (3) (2016) 571–580, https://doi.org/10.1109/TBME.2015.2466234.

- [194] R.M. Robertson, D.E. Smith, Self-diffusion of entangled linear and circular DNA molecules: Dependence on length and concentration, Macromolecules. 40 (2007) 3373–3377, https://doi.org/10.1021/ma070051h.
- [195] M. Yu, W. Tan, H. Lin, A stochastic model for DNA translocation through an electropore, Biochim. Biophys. Acta - Biomembr. 1818 (11) (2012) 2494– 2501, https://doi.org/10.1016/j.bbamem.2012.05.025.
- [196] C. Rosazza, E. Phez, J.-M. Escoffre, L. Cézanne, A. Zumbusch, M.-P. Rols, Cholesterol implications in plasmid DNA electrotransfer: Evidence for the involvement of endocytotic pathways, Int. J. Pharm. 423 (1) (2012) 134–143, https://doi.org/10.1016/j.ijpharm.2011.05.024.
- [197] L. Wang, S.E. Miller, F. Yuan, Ultrastructural analysis of vesicular transport in electrotransfection, Microsc. Microanal. 24 (5) (2018) 553–563, https://doi. org/10.1017/S143192761801509X.
- [198] S. Kumari, S. MG, S. Mayor, Endocytosis unplugged: Multiple ways to enter the cell, Cell Res. 20 (3) (2010) 256–275, https://doi.org/10.1038/cr.2010.19.
- [199] L.V. Chernomordik, A.V. Sokolov, V.G. Budker, Electrostimulated uptake of DNA by liposomes, BBA - Biomembr. 1024 (1) (1990) 179–183, https://doi. org/10.1016/0005-2736(90)90222-A.
- [200] Y. Antov, A. Barbul, H. Mantsur, R. Korenstein, Electroendocytosis: Exposure of cells to pulsed low electric fields enhances adsorption and uptake of macromolecules, Biophys. J. 88 (3) (2005) 2206–2223, https://doi.org/ 10.1529/biophysj.104.051268.
- [201] N. Ben-Dov, I. Rozman Grinberg, R. Korenstein, A.M. Delprato, Electroendocytosis Is Driven by the Binding of Electrochemically Produced Protons to the Cell's Surface, PLoS One. 7 (11) (2012) e50299, https://doi.org/ 10.1371/journal.pone.0050299.
- [202] R. Lin, D.C. Chang, Y.-K. Lee, Single-cell electroendocytosis on a micro chip using in situ fluorescence microscopy, Biomed. Microdevices. 13 (6) (2011) 1063–1073, https://doi.org/10.1007/s10544-011-9576-9.
- [203] T.Y. Tsong, Electroporation of cell membranes, Biophys. J. 60 (2) (1991) 297– 306, https://doi.org/10.1016/S0006-3495(91)82054-9.
- [204] H.N. Farrer, F.J.C. Rossotti, Proton-fluoride association in sodium perchlorate media, J. Inorg. Nucl. Chem. 26 (11) (1964) 1959–1965, https://doi.org/ 10.1016/0022-1902(64)80020-8.
- [205] B. Sorre, A. Callan-Jones, J.-B. Manneville, P. Nassoy, J.-F. Joanny, J. Prost, B. Goud, P. Bassereau, Curvature-driven lipid sorting needs proximity to a demixing point and is aided by proteins, Proc. Natl. Acad. Sci. U. S. A. 106 (14) (2009) 5622–5626, https://doi.org/10.1073/pnas.0811243106.
- [206] J.W. Murray, A.W. Wolkoff, Roles of the cytoskeleton and motor proteins in endocytic sorting, Adv. Drug Deliv. Rev. 55 (11) (2003) 1385–1403, https:// doi.org/10.1016/j.addr.2003.07.008.
- [207] C. Rosazza, J.-M. Escoffre, A. Zumbusch, M.-P. Rols, The actin cytoskeleton has an active role in the electrotransfer of plasmid DNA in mammalian cells, Mol. Ther. 19 (5) (2011) 913–921, https://doi.org/10.1038/mt.2010.303.
- [208] D.A. Schafer, Regulating actin dynamics at membranes: A focus on dynamin, Traffic. 5 (2004) 463–469, https://doi.org/10.1111/j.1600-0854.2004.00199.
- [209] A. Roux, G. Koster, M. Lenz, B. Sorre, J.-B. Manneville, P. Nassoy, P. Bassereau, Membrane curvature controls dynamin polymerization, Proc. Natl. Acad. Sci. U. S. A. 107 (9) (2010) 4141–4146, https://doi.org/10.1073/pnas.0913734107.
- [210] S. Šatkauskas, M.F. Bureau, A. Mahfoudi, L.M. Mir, Slow accumulation of plasmid in muscle cells: Supporting evidence for a mechanism of DNA uptake by receptor-mediated endocytosis, Mol. Ther. 4 (4) (2001) 317–323, https:// doi.org/10.1006/mthe.2001.0465.
- [211] B. Markelc, E. Skvarca, T. Dolinsek, V.P. Kloboves, A. Coer, G. Sersa, M. Cemazar, Inhibitor of endocytosis impairs gene electrotransfer to mouse muscle in vivo, Bioelectrochemistry. 103 (2015) 111–119, https://doi.org/ 10.1016/j.bioelechem.2014.08.020.
- [212] C. Faurie, E. Phez, M. Golzio, C. Vossen, J.-C. Lesbordes, C. Delteil, J. Teissié, M.-P. Rols, Effect of electric field vectoriality on electrically mediated gene delivery in mammalian cells, Biochim. Biophys. Acta Biomembr. 1665 (1-2) (2004) 92–100, https://doi.org/10.1016/j.bbamem.2004.06.018.
 [213] P.M. Graybill, R.V. Davalos, Cytoskeletal disruption after electroporation and
- [213] P.M. Graybill, R.V. Davalos, Cytoskeletal disruption after electroporation and its significance to pulsed electric field therapies, Cancers (Basel). 12 (5) (2020) 1132, https://doi.org/10.3390/cancers12051132.
- [214] C. Kanthou, S. Kranjc, G. Sersa, G. Tozer, A. Zupanic, M. Cemazar, The endothelial cytoskeleton as a target of electroporation-based therapies, Mol. Cancer Ther, 5 (12) (2006) 3145–3152, https://doi.org/10.1158/1535-7163. MCT-06-0410.
- [215] A. Steuer, K. Wende, P. Babica, J.F. Kolb, Elasticity and tumorigenic characteristics of cells in a monolayer after nanosecond pulsed electric field exposure, Eur. Biophys. J. 46 (6) (2017) 567–580, https://doi.org/10.1007/ s00249-017-1205-y.
- [216] H. Krassen, U. Pliquett, E. Neumann, Nonlinear current-voltage relationship of the plasma membrane of single CHO cells, Bioelectrochemistry. 70 (1) (2007) 71–77, https://doi.org/10.1016/j.bioelechem.2006.03.033.
- [217] A.E. Sowers, The long-lived fusogenic state induced in erythrocyte ghosts by electric pulses is not laterally mobile, Biophys. J. 52 (6) (1987) 1015–1020, https://doi.org/10.1016/S0006-3495(87)83294-0.
- [218] M.-P. Rols, J. Teissié, Experimental evidence for the involvement of the cytoskeleton in mammalian cell electropermeabilization, BBA - Biomembr. 1111 (1) (1992) 45–50, https://doi.org/10.1016/0005-2736(92)90272-N.
- [219] G.L. Thompson, C. Roth, G. Tolstykh, M. Kuipers, B.L. Ibey, Disruption of the actin cortex contributes to susceptibility of mammalian cells to nanosecond

pulsed electric fields, Bioelectromagnetics. 35 (4) (2014) 262–272, https://doi.org/10.1002/bem.21845.

- [220] M. Stacey, P. Fox, S. Buescher, J. Kolb, Nanosecond pulsed electric field induced cytoskeleton, nuclear membrane and telomere damage adversely impact cell survival, Bioelectrochemistry. 82 (2) (2011) 131–134, https://doi. org/10.1016/j.bioelechem.2011.06.002.
- [221] A. Muralidharan, L. Rems, M.T. Kreutzer, P.E. Boukany, Actin networks regulate the cell membrane permeability during electroporation, Biochim. Biophys. Acta - Biomembr. 1863 (1) (2021) 183468, https://doi.org/10.1016/j. bbamem.2020.183468.
- [222] H.B. Kim, S. Lee, J.H. Chung, S.N. Kim, C.K. Sung, K.Y. Baik, Effects of Actin Cytoskeleton Disruption on Electroporation In Vitro, Appl. Biochem. Biotechnol. 191 (4) (2020) 1545–1561, https://doi.org/10.1007/s12010-020-03271-4.
- [223] D. Xiao, L. Tang, C. Zeng, J. Wang, X. Luo, C. Yao, C. Sun, Effect of actin cytoskeleton disruption on electric pulse-induced apoptosis and electroporation in tumour cells, Cell Biol. Int. 35 (2) (2011) 99–104, https:// doi.org/10.1042/CBI20100464.
- [224] G.L. Thompson, C. Roth, G. Tolstykh, M. Kuipers, B.L. Ibey, Role of cytoskeleton and elastic moduli in cellular response to nanosecond pulsed electric fields, in, Terahertz Ultrashort Electromagn. Pulses Biomed. Appl. (2013) 85850T, https://doi.org/10.1117/12.2005114.
- [225] R. Dimova, K.A. Riske, Electrodeformation, electroporation, and electrofusion of giant unilamellar vesicles, in, Handb. Electroporation (2017) 235–252, https://doi.org/10.1007/978-3-319-32886-7_199.
- [226] K.A. Riske, R. Dimova, Electro-deformation and poration of giant vesicles viewed with high temporal resolution, Biophys. J. 88 (2) (2005) 1143–1155, https://doi.org/10.1529/biophysj.104.050310.
- [227] D.E. Chafai, V. Sulimenko, D. Havelka, L. Kubínová, P. Dráber, M. Cifra, Reversible and Irreversible Modulation of Tubulin Self-Assembly by Intense Nanosecond Pulsed Electric Fields, Adv. Mater. 31 (39) (2019) 1903636, https://doi.org/10.1002/adma.201903636.
- [228] P. Marracino, D. Havelka, J. Průša, M. Liberti, J. Tuszynski, A.T. Ayoub, F. Apollonio, M. Cifra, Tubulin response to intense nanosecond-scale electric field in molecular dynamics simulation, Sci. Rep. 9 (1) (2019), https://doi.org/ 10.1038/s41598-019-46636-4.
- [229] F.-C. Tsai, G.-H. Kuo, S.-W. Chang, P.-J. Tsai, Ca2+ signaling in cytoskeletal reorganization, cell migration, and cancer metastasis, Biomed Res. Int. 2015 (2015) 1–13, https://doi.org/10.1155/2015/409245.
- [230] E.T. O'Brien, E.D. Salmon, H.P. Erickson, How calcium causes microtubule depolymerization, Cell Motil. Cytoskeleton. 36 (1997) 125–135, https://doi. org/10.1002/(SICI)1097-0169(1997)36:2<125::AID-CM3>3.0.CO;2-8.
- [231] A. Görlach, K. Bertram, S. Hudecova, O. Krizanova, Calcium and ROS: A mutual interplay, Redox Biol. 6 (2015) 260–271, https://doi.org/10.1016/j. redox.2015.08.010.
- [232] I. Semenov, S. Xiao, A.G. Pakhomov, Primary pathways of intracellular Ca2 + mobilization by nanosecond pulsed electric field, Biochim. Biophys. Acta -Biomembr. 1828 (3) (2013) 981–989, https://doi.org/10.1016/j. bbamem.2012.11.032.
- [233] J.A. White, P.F. Blackmore, K.H. Schoenbach, S.J. Beebe, Stimulation of capacitative calcium entry in HL-60 cells by nanosecond pulsed electric fields, J. Biol. Chem. 279 (22) (2004) 22964–22972, https://doi.org/10.1074/ jbc.M311135200.
- [234] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon, Biophys. J. 58 (5) (1990) 1089–1098, https://doi. org/10.1016/S0006-3495(90)82451-6.
- [235] T. Polajzer, T. Jarm, D. Miklavcic, Analysis of damage-associated molecular pattern molecules due to electroporation of cells in vitro, Radiol. Oncol. 54 (2020) 317–328, https://doi.org/10.2478/raon-2020-0047.
- [236] W.E. Ford, W. Ren, P.F. Blackmore, K.H. Schoenbach, S.J. Beebe, Nanosecond pulsed electric fields stimulate apoptosis without release of pro-apoptotic factors from mitochondria in B16f10 melanoma, Arch. Biochem. Biophys. 497 (1-2) (2010) 82–89, https://doi.org/10.1016/j.abb.2010.03.008.
- [237] I. Titushkin, M. Cho, Regulation of cell cytoskeleton and membrane mechanics by electric field: Role of linker proteins, Biophys. J. 96 (2) (2009) 717–728, https://doi.org/10.1016/j.bpj.2008.09.035.
- [238] A.G. Pakhomov, S. Xiao, O.N. Pakhomova, I. Semenov, M.A. Kuipers, B.L. Ibey, Disassembly of actin structures by nanosecond pulsed electric field is a downstream effect of cell swelling, Bioelectrochemistry. 100 (2014) 88–95, https://doi.org/10.1016/j.bioelechem.2014.01.004.
- [239] M.A. Rassokhin, A.G. Pakhomov, Electric field exposure triggers and guides formation of pseudopod-like blebs in U937 monocytes, J. Membr. Biol. 245 (9) (2012) 521–529, https://doi.org/10.1007/s00232-012-9433-7.
- [240] L. Carr, S.M. Bardet, R.C. Burke, D. Arnaud-Cormos, P. Leveque, R.P. O'Connor, Calcium-independent disruption of microtubule dynamics by nanosecond pulsed electric fields in U87 human glioblastoma cells, Sci. Rep. 7 (1) (2017), https://doi.org/10.1038/srep41267.
- [241] S. Li, N. Xiong, Y. Peng, K. Tang, H. Bai, X. Lv, Y. Jiang, X. Qin, H. Yang, C. Wu, P. Zhou, Y. Liu, Acidic pHe regulates cytoskeletal dynamics through conformational integrin β1 activation and promotes membrane protrusion, Biochim. Biophys. Acta Mol. Basis Dis. 1864 (7) (2018) 2395–2408, https://doi.org/10.1016/j.bbadis.2018.04.019.
- [242] L.C. Benov, P.A. Antonov, S.R. Ribarov, Oxidative damage of the membrane lipids after electroporation, Gen. Physiol. Biophys. (1994).
- [243] B. Gabriel, J. Teissie, Generation of reactive-oxygen species induced by electropermeabilization of Chinese hamster ovary cells and their

consequence on cell viability, Eur. J. Biochem. 223 (1) (1994) 25-33, https://doi.org/10.1111/j.1432-1033.1994.tb18962.x.

- [244] M. Maccarrone, M.R. Bladergroen, N. Rosato, A.F. Agro, Role of lipid peroxidation in electroporation-induced cell permeability, Biochem. Biophys. Res. Commun. 209 (2) (1995) 417–425, https://doi.org/10.1006/ bbrc.1995.1519.
- [245] Q. Xu, L.P. Huff, M. Fujii, K.K. Griendling, Redox regulation of the actin cytoskeleton and its role in the vascular system, Free Radic. Biol. Med. 109 (2017) 84–107, https://doi.org/10.1016/j.freeradbiomed.2017.03.004.
- [246] J. Suh, D. Wirtz, J. Hanes, Real-time intracellular transport of gene nanocarriers studied by multiple particle tracking, Biotechnol. Prog. 20 (2) (2004) 598–602, https://doi.org/10.1021/bp034251y.
- [247] G.L. Wilson, B.S. Dean, G. Wang, D.A. Dean, Nuclear import of plasmid DNA in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA sequences, J. Biol. Chem. 274 (31) (1999) 22025–22032, https://doi.org/ 10.1074/jbc.274.31.22025.
- [248] A. Coonrod, F.-Q. Li, M. Horwitz, On the mechanism of DNA transfection: Efficient gene transfer without viruses, Gene Ther. 4 (12) (1997) 1313–1321, https://doi.org/10.1038/sj.gt.3300536.
- [249] A. El Ouahabi, M. Thiry, V. Pector, R. Fuks, J.M. Ruysschaert, M. Vandenbranden, The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids, FEBS Lett. 414 (1997) 187–192, https://doi.org/10.1016/S0014-5793(97)00973-3.
- [250] H. Bai, G.M.S. Lester, L.C. Petishnok, D.A. Dean, Cytoplasmic transport and nuclear import of plasmid DNA, Biosci. Rep. 37 (2017), https://doi.org/ 10.1042/BSR20160616.
- [251] E.E. Vaughan, R.C. Geiger, A.M. Miller, P.L. Loh-Marley, T. Suzuki, N. Miyata, D. A. Dean, Microtubule acetylation through HDAC6 inhibition results in increased transfection efficiency, Mol. Ther. 16 (11) (2008) 1841–1847, https://doi.org/10.1038/mt.2008.190.
- [252] M. Stacey, J. Stickley, P. Fox, V. Statler, K. Schoenbach, S.J. Beebe, S. Buescher, Differential effects in cells exposed to ultra-short, high intensity electric fields: Cell survival, DNA damage, and cell cycle analysis, Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 542 (1-2) (2003) 65–75, https://doi.org/10.1016/j. mrgentox.2003.08.006.
- [253] W. Yang, Y.-H. Wu, D. Yin, H.P. Koeffler, D.E. Sawcer, P.T. Vernier, M.A. Gundersen, Differential sensitivities of malignant and normal skin cells to nanosecond pulsed electric fields, Technol. Cancer Res. Treat. 10 (3) (2011) 281–286, https://doi.org/10.7785/tcrt.2012.500204.
- [254] J.W. Ivey, E.L. Latouche, M.B. Sano, J.H. Rossmeisl, R.V. Davalos, S.S. Verbridge, Targeted cellular ablation based on the morphology of malignant cells, Sci. Rep. 5 (1) (2015), https://doi.org/10.1038/srep17157.
- [255] V.N. Pehlivanova, I.H. Tsoneva, R.D. Tzoneva, Multiple effects of electroporation on the adhesive behaviour of breast cancer cells and fibroblasts, Cancer Cell Int. 12 (1) (2012), https://doi.org/10.1186/1475-2867-12-9.

- [256] R. Zhou, R.C. Geiger, D.A. Dean, Intracellular trafficking of nucleic acids, Expert Opin. Drug Deliv. 1 (1) (2004) 127–140, https://doi.org/10.1517/ 17425247.1.1.127.
- [257] M.E. Dowty, P. Williams, G. Zhang, J.E. Hagstrom, J.A. Wolff, Plasmid DNA entry into postmitotic nuclei of primary rat myotubes, Proc. Natl. Acad. Sci. U. S. A. 92 (10) (1995) 4572–4576, https://doi.org/10.1073/pnas.92.10.4572.
- [258] D.A. Dean, Nonviral gene transfer to skeletal, smooth, and cardiac muscle in living animals, Am. J. Physiol. - Cell Physiol. 289 (2) (2005) C233-C245, https://doi.org/10.1152/ajpcell.00613.2004.
- [259] D.A. Dean, B.S. Dean, S. Muller, L.C. Smith, Sequence requirements for plasmid nuclear import, Exp. Cell Res. 253 (2) (1999) 713–722, https://doi.org/ 10.1006/excr.1999.4716.
- [260] P. Blomberg, M. Eskandarpour, S. Xia, C. Sylvén, K.B. Islam, Electroporation in combination with a plasmid vector containing SV40 enhancer elements results in increased and persistent gene expression in mouse muscle, Biochem. Biophys. Res. Commun. 298 (4) (2002) 505–510, https://doi.org/ 10.1016/S0006-291X(02)02486-5.
- [261] J.B. Martin, J.L. Young, J.N. Benoit, D.A. Dean, Gene transfer to intact mesenteric arteries by electroporation, J. Vasc. Res. 37 (2000) 372–380, https://doi.org/10.1159/000025753.
- [262] J.L. Young, J.N. Benoit, D.A. Dean, Effect of a DNA nuclear targeting sequence on gene transfer and expression of plasmids in the intact vasculature, Gene Ther. 10 (17) (2003) 1465–1470, https://doi.org/10.1038/sj.gt.3302021.
- [263] S. Guo, D.L. Jackson, N.I. Burcus, Y.J. Chen, S. Xiao, R. Heller, Gene electrotransfer enhanced by nanosecond pulsed electric fields, Mol. Ther. -Methods Clin. Dev. 1 (2014) 14043, https://doi.org/10.1038/mtm.2014.43.
- [264] C. Ramos, D. Bonato, M. Winterhalter, T. Stegmann, J. Teissié, Spontaneous lipid vesicle fusion with electropermeabilized cells, FEBS Lett. 518 (2002) 135–138, https://doi.org/10.1016/S0014-5793(02)02676-5.
- [265] D.L. Perrier, L. Rems, P.E. Boukany, Lipid vesicles in pulsed electric fields: Fundamental principles of the membrane response and its biomedical applications, Adv. Colloid Interface Sci. 249 (2017) 248–271, https://doi. org/10.1016/j.cis.2017.04.016.
- [266] S. Chopra, P. Ruzgys, M. Maciulevičius, M. Jakutavičiūtė, S. Šatkauskas, Investigation of Plasmid DNA Delivery and Cell Viability Dynamics for Optimal Cell Electrotransfection In Vitro, Appl. Sci. 10 (2020) 6070, https:// doi.org/10.3390/app10176070.
- [267] Y.i. Han, X.-M. Liu, H. Liu, S.-C. Li, B.-C. Wu, L.-L. Ye, Q.-W. Wang, Z.-L. Chen, Cultivation of recombinant Chinese hamster ovary cells grown as suspended aggregates in stirred vessels, J. Biosci. Bioeng. 102 (5) (2006) 430–435, https://doi.org/10.1263/jbb.102.430.
- [268] N.C. Stellwagen, C. Gelfi, P.G. Righetti, The free solution mobility of DNA, Biopolymers. 42 (1997) 687–703, https://doi.org/10.1002/(SICI)1097-0282 (199711)42:6.