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Current Progress in Electrotransfection as a Nonviral Method for Gene Delivery

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Abstract

Electrotransfection (ET) is a nonviral method for delivery of various types of molecules into cells both in vitro and in vivo. Close to 90 clinical trials that involve the use of ET have been performed, and approximately half of them are related to cancer treatment. Particularly, ET is an attractive technique for cancer immunogene therapy because treatment of cells with electric pulses alone can induce immune responses to solid tumors, and the responses can be further enhanced by ET of plasmid DNA (pDNA) encoding therapeutic genes. Compared to other gene delivery methods, ET has several unique advantages. It is relatively inexpensive, flexible, and safe in clinical applications, and introduces only naked pDNA into cells without the use of additional chemicals or viruses. However, the efficiency of ET is still low, partly because biological mechanisms of ET in cells remain elusive. In previous studies, it was believed that pDNA entered the cells through transient pores created by electric pulses. As a result, the technique is commonly referred to as electroporation. However, recent discoveries have suggested that endocytosis plays an important role in cellular uptake and intracellular transport of electrotransfected pDNA. This review will discuss current progresses in the study of biological mechanisms underlying ET and future directions of research in this area. Understanding the mechanisms of pDNA transport in cells is critical for the development of new strategies for improving the efficiency of gene delivery in tumors.

Abstract

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Notes

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Keywords

electroporation; electrotransfection; electrogene transfer; gene electroinjection; nonviral gene delivery

INTRODUCTION

Electrotransfection (ET) is a nonviral method that can be used to enhance cellular uptake of exogenous molecules both in vitro and in vivo. In most applications, ET is used to deliver DNA, RNA, and proteins for genome and epigenome editing,^{1–7} generation of human induced pluripotent stem cells for cell and tissue engineering,^{8–11} and cancer gene therapy.^{12,13} It has also been used for delivery of DNA and mRNA vaccines.^{14–16} ET is achieved through the application of a pulsed electric field to cells surrounded by the molecules of interest.¹⁷ It is advantageous for a variety of applications and has had successes in clinical trials.¹² The first clinical trial began in 2004, which involved ET of interleukin-12 plasmid DNA (pDNA) in patients with metastatic melanoma.¹⁸ Since then, there have been close to 90 trials utilizing electric fields for gene delivery (see clinicaltrials.gov),¹² and approximately half of them are related to cancer treatment, in which pDNA and mRNA are delivered to patients or patient-derived samples with electric pulses for immunotherapy or antiangiogenic therapy of cancer. Additionally, ET has been widely used in the veterinary clinic to treat cancer in pets and zoo animals.^{19,20}

ET is particularly useful in cancer immunotherapy applications for two reasons. One is that electric pulse alone can elicit strong immune responses to solid tumors,^{21,22} and the other is that electric pulses can be used to deliver therapeutic genes to solid tumors where the gene expression can further stimulate or enhance the immune responses. For example, the interleukin-12 (IL-12) gene has been delivered to patients with metastatic melanoma that was surgically unresectable.¹⁸ The study showed that ET was “safe, effective, reproducible,

and titratable” in patients. Other cytokine genes, such as interleukin-2 (IL-2), have also been delivered in clinical trials (NCT00223899) to stimulate T-cell responses against melanoma. In addition to the cytokines, ET has been widely used to deliver DNA vaccines to cancer patients. These vaccines are advantageous in that they do not require cultivation of pathogens for gene delivery that may carry the risk of infection, as shown in immunocompromised animal models²³ and in human patients.²⁴ Plasmids, encoding SCIB1 (NCT01138410)²⁵ and tyrosinase (NCT00471133),²⁶ respectively, have been introduced into melanoma patients. Of the 25 patients who received SCIB1, 23 showed that immune responses could be induced by the vaccine and the procedure for ET was safe.²⁵ Besides melanoma, a synthetic plasmid, VGX-3100, has been delivered by ET as the first vaccine showing efficacy against cervical intraepithelial neoplasia grade 2 or 3 lesion (NCT01304524).²⁷ Another DNA vaccine containing two plasmids, encoding HER2 and CEA domains, respectively, has been used to treat patients with various solid tumors (NCT00647114).²⁸ Besides the applications that rely on gene delivery in vivo, ET has been used recently to engineer T-cells ex vivo with a specific chimeric antigen receptor (CAR) gene, CD19, for production of CAR T-cells to treat pediatric acute lymphoblastic leukemia in a clinical trial.²⁹

Compared to other methods of gene delivery, ET has several advantages. It is relatively simple and easy to prepare and modify pDNA molecules; pDNA can be administered multiple times to tissues without inducing significant immune responses; and there is infrequent expression of transgenes at nontarget sites.¹⁷ ET is also advantageous for clinical use because only pDNA solution needs to be administered into patients or patient-derived samples for transfection, without the use of additional chemicals. Despite the wide applications of ET, there are some limitations of the technology. For example, naked pDNA is not protected by delivery vehicles. Thus, it is more vulnerable to the degradation by nucleases,^{30–34} compared to DNA encapsulated in synthetic nanoparticles or packaged in viral vectors. The main limitations of ET are (i) relatively low transfection efficiency, compared to viral delivery methods,³⁵ and (ii) some cell death that may occur, particularly in applications using a high energy electric field.¹⁷

To improve the efficiency and cell viability, one can either use the trial and error approach to optimize transfection parameters or rationally design experimental protocols based on mechanisms of ET.³⁶ Transport of electrotransfected pDNA in solid tumors can be divided into two parts: extracellular and intracellular. Our previous studies have shown that the dominant mode of transport in the extracellular space is electrophoresis.^{17,37–41} Thus, extracellular transport of pDNA can be improved through either enhancing the driving force (i.e., the electric field) or reducing the tissue resistance to the transport. For example, we have observed that treatment of solid tumors with relaxin, which reduces extracellular matrix resistance to pDNA transport by inducing collagen remodeling, can improve the efficiency of ET.³⁷ Another approach to improving the efficiency is to shrink the volume of the cells through the treatment of tumors with hyperosmotic mannitol solution, which reduces cellular resistance to pDNA transport.³⁸ What remains unclear is the mechanisms of cell uptake and intracellular transport of electrotransfected pDNA. Thus, they are the focus of the current review.

MOLECULAR MECHANISMS OF ET

The ET technology was initially developed on the basis of an observation in basic research conducted in the early 1970s, where the permeability of the lipid membrane was increased transiently after the application of short but strong electric pulses.^{42,43} The extent of the permeabilization can be controlled by pulse duration, strength, and frequency. This permeabilization mechanism, referred to as electroporation, has been utilized to deliver various molecules into cells. In 1982, Neumann et al. reported the first transfection of herpes simplex thymidine kinase gene, in both linear and circular DNA forms, into mouse L-cells using a pulsed electric field.⁴⁴ Since then, the ET technique has been widely used for gene delivery both in vitro and in vivo.^{12,24,45–51} It is worth mentioning that, although ET is frequently performed with cells in suspension, it can also be used to transfect adherent cells.^{52–54} During its development, the ET technique has also been referred to as electroporation, gene electrotransfer, and gene electroinjection in different applications.¹⁷

ET of mammalian cells involves transport of pDNA from extracellular medium to the nucleus. In the journey, the transport must overcome three physiological barriers: plasma membrane, cytoplasmic structures, and nuclear envelope (Figure 1).¹⁷ Mechanisms by which the transport of pDNA through these barriers remain largely debated.

Transport across Plasma Membrane.

Two theories have been proposed for transmembrane transport. The traditional theory, commonly referred to as the pore theory, states that application of pulsed electric field creates transient, hydrophilic pores in the plasma membrane, in a process known as electroporation, that permits pDNA to enter the cell.⁴⁴ However, recent studies have shown that endocytic pathways are involved in electric pulse-mediated internalization of pDNA.^{46,55–59} Results from these studies have led to a new theory that the transmembrane transport of pDNA is mediated mainly by endocytosis.

Pore Theory.—The pore theory suggests that the electric field creates hydrophilic pores in the plasma membrane that permit pDNA to enter the cell (Figure 2), although the pores have yet to be visualized directly under a microscope. So far, electric field-induced membrane permeabilization has been demonstrated with the use of mathematical modeling, experimental measurements of changes in electrical conductance of cell or synthetic lipid membranes, and direct observation of molecular marker transport across otherwise impermeable membranes.^{17,60} The markers include small fluorescent molecules, siRNA, and some proteins that can enter cells through regions of the plasma membrane facing both negative and positive electrodes;⁶¹ the permeabilization has been found to be approximately symmetrical between the two regions.⁶²

Results from numerical simulations have predicted that the cutoff size of the pores induced in the membrane by pulsed electric fields is on the order of a few hundred nanometers,^{62,63} and the lifetime of the pores that are larger than the size of pDNA is on the order of 10 ms.^{62,64,65} This time scale is several orders of magnitude shorter than the time frame of pDNA uptake, which has been observed to be on the order of 10 min,⁵⁵ suggesting that ET is a slow process compared to the half-life of the transient pores. Furthermore, it has also been

observed that pDNA uptake by cells is preceded by pDNA binding to the plasma membrane, and that the binding is a necessary condition for successful gene transfer.^{55,66,67} The observations discussed above cannot be explained by the pore theory.

A further evidence refuting the pore theory is the observed rate of DNA diffusion in the cytosol. Previous studies have shown that DNA molecules up to 1000 bp can diffuse quickly in the cytosol after direct injection.⁶⁸ However, the diffusion is very slow for DNA larger than 2000 bp and undetectable for DNA larger than 3000 bp.⁶⁸ The slow diffusion is caused mainly by barriers formed by actin filaments.⁶⁹ The size of pDNA molecules used in most studies of ET is larger than 5000 bp, indicating that they cannot diffuse easily through the cytosol. The slow diffusion makes naked pDNA more vulnerable to degradation by nucleases.

DNA degradation by nucleases during intracellular trafficking has been considered as a main biological obstacle to gene delivery.⁷⁰ It is less problematic for polymeric (Figure 3) or other nanoparticle-mediated gene delivery where DNA is protected by materials in the nanoparticles. However, the half-life for both single- and double-stranded circular DNA microinjected into the cytoplasm is only 50–90 min.^{17,27,70} A combination of the short half-life and the slow diffusion suggests that even if electrotransfected pDNA molecules could enter the cytosol through the transient pores in the plasma membrane, they would most likely be degraded by endonucleases before reaching the perinuclear region.

Endocytosis Theory.—Previous studies have shown that following the application of electric pulses, pDNA is electrophoretically pushed toward the plasma membrane and forms a stable complex before internalization, and that this process depends on the topology of the pDNA.⁷¹ The internalization of the pDNA in the complex is likely to be mediated by endocytosis (Figure 4). Previous studies have shown that the application of electric pulses can stimulate endocytosis of macromolecules, such as proteins and dextran.^{72–74} More recently, with the use of inhibitors of endocytosis and endosomal markers in conjugation with imaging techniques, Rosazza et al. reported that 50% of DNA was internalized by caveolin/raft-mediated endocytosis, 25% by clathrin-mediated endocytosis, and 25% by macropinocytosis.⁵⁶ These studies indicate that endocytosis plays an important role in the cellular uptake of electrotransfected pDNA, and that multiple pathways may be involved in the uptake. To demonstrate that electrotransfection efficiency (eTE) is also dependent on endocytosis, our group has utilized pharmacological inhibitors of endocytosis to treat cells prior to ET, and determined effects of the treatments on eTE.^{46,55,57} We observed that treatment of cells with chlorpromazine or dynasore could reduce eTE in all cell lines tested, whereas genistein and amiloride treatments were not effective in reducing eTE, suggesting that clathrin-mediated endocytosis was an important pathway for ET in vitro.^{46,55} The same results have also been observed in vivo, specifically in mouse muscle.⁷⁵

To confirm the results from the pharmacological studies, we have also inhibited the same endocytic pathways, prior to ET, by knocking down the expressions of clathrin heavy chain (CLTC), caveolin-1 (CAV-1), dynamin II, and Rab34, respectively, with small interfering RNA (siRNA).^{46,55} Again, we observed that only the knockdown of CLTC and dynamin II resulted in a significant decrease in eTE.⁴⁶ The knockdown or knockout (achieved with the

CRISPR/cas9 method, unpublished data) of CAV-1 had insignificant effects on eTE, suggesting that although 50% of intracellular pDNA could be internalized by caveolae-mediated endocytosis,⁵⁶ these pDNA molecules did not reach the nucleus for transgene expression.

Trafficking in the Cytoplasm.

Role of Microtubules in Transport.—Very little is known regarding the mechanisms by which pDNA travels through the cytoplasm to the nucleus. Recent mathematical models have shown an implicated role of microtubules in the delivery of pDNA from the plasma membrane to the nucleus.⁷⁶ Experimentally, it has been found that dynein, a molecular motor that moves on the tracks of microtubules, is essential for the movement of organelles and transporting pDNA to the nucleus.⁷⁷ It has also been observed that the pDNA transport is mediated by endocytic vesicles because pDNA trajectories are co-localized with the bio-markers for endosomes.⁵⁶ As a result, the eTE can be improved through increasing the speed of the vesicular transport of pDNA to the nucleus, which can be achieved by knocking down the expression of histone deacetylase 6 (HDAC6) that increases acetylation of microtubules.⁷⁸

Endosomal Escape.—It has been suggested that nucleic acid transfection efficiency is limited by the ability to release pDNA from the early endosomes.⁷⁹ If the pDNA is not released, it may be degraded enzymatically in lysosomes after endosomes fuse with lysosomes, thereby limiting the eTE. Although endosomal escape is considered to be crucial for gene delivery, an important question is, when is the optimal time for the escape to occur? Different pathways for gene trafficking in the cytoplasm may commonly involve transition from early endosome (EE) to endosomal carrier vesicle (ECV) or multivesicular body (MVB), to late endosome (LE), and to lysosome (Figure 5).^{56,59,80–82} The release of pDNA from endosomes avoids degradation in lysosomes and can potentially increase efficiency of gene delivery. Indeed, data in the literature have shown that transfection efficiency can be increased when pDNA is released from early endosomes immediately after cell transfection with polyethylenimine (PEI) or poly-L-lysine (PLL) as carriers.^{80,81} However, the same approach reduced eTE.⁵⁹ In fact, we observed that endosomal escape should be discouraged for successful delivery of electroporated pDNA to the nucleus. This observation was similar to those in previous reports of gene delivery with cationic lipid-based vectors. The parallels between lipid-based vectors and ET imply that these two approaches to gene delivery might share similar mechanisms of intracellular transport.⁵⁹

Nuclear Entry.

Molecules typically enter the nucleus through nuclear pore complexes (NPCs) (see Figure 1). The NPCs allow free exchange of molecules by diffusion between the nucleus and the cytosol if the molecular size is significantly smaller than 9 nm,^{83,84} which is approximately 40 kDa in molecular weight for proteins. For larger molecules up to 39 nm in diameter, the exchange is mediated by active transport, which requires signals on the imported/exported molecules that can interact with the nuclear transport system.^{47,85–88} However, the radius of gyration of naked pDNA is ~100 nm,⁸⁹ which is significantly larger than the cutoff size of the channel in the NPCs. The question is how does pDNA enter the nucleus? Previous

studies have shown that pDNA can enter the nucleus via the NPCs with the help from nuclear localization signals (NLSs) in the form of peptides or proteins,^{47,88} indicating that the active transport can provide enough energy to push/pull pDNA into the NPCs through conformational changes in both pDNA and NPCs. In this approach, a specific nuclear targeting sequence (e.g., SV40) is incorporated into the pDNA that allows binding of pDNA with NLSs.⁴⁷ However, a drawback of this approach is that there is no universal nuclear targeting sequence that can facilitate the nuclear entry of pDNA in all cell types,⁵⁸ which makes it laborious to design and synthesize cell specific pDNA.⁸⁶ As a result, the nuclear targeting approach has achieved a limited success.⁸⁷ For all dividing cells, the nuclear envelope breaks down during mitosis, which allows for nuclear entry of pDNA with little resistance. Therefore, one can synchronize the cell cycle at the G2-M phase prior to ET to achieve high nuclear entry.⁵⁸

APPROACHES TO IMPROVING ET

Attempts have been made to increase ET efficiency by modifying electric field settings, pulsing buffers, and electrodes. In most studies, ET is commonly improved through optimization of electric field settings.^{90–92} The optimization is often performed through the trial-and-error approach. In other studies, new electrodes are designed for specific applications. Some examples of electrodes include penetrating needle arrays, nonpenetrating parallel needles (Genetrode electrodes, Genetronics, San Diego, CA, USA), plate electrodes (Tweezertrodes, BTX, Hollister, MA), balloon catheter-based electrodes, spoon electrodes (for vascular electroporation), conformable defibrillator pads, and multielectrode arrays.⁸⁸ Additionally, different materials have been considered for making the electrodes. Among them, aluminum is a common choice, which is relatively inexpensive and easier for manufacturing. However, metal electrodes, except for platinum, can cause pDNA precipitation in extracellular medium and low cell viability. Thus, nonmetal materials, such as carbon, have been used to make electrodes.⁹³ In addition to the control of the experimental conditions and materials for electrodes, one important issue is how to improve transport and reduce degradation of pDNA in cells.

Without help, few naked pDNA molecules can penetrate across the membrane, and less than 1/1000 of naked pDNA molecules microinjected into the cytosol are effectively trafficked into the nucleus.^{94–96} With respect to ET, the nuclear envelope is the main physical barrier in cells that has critically limited eTE. Dilation of the NPCs can increase the expression level of transgenes,⁵⁸ but the pore dilating agents can be toxic to cells. Furthermore, the dilation only increases the transgene expression level but not the eTE.⁵⁸ Two better approaches to facilitate the nuclear entry of pDNA are incorporation of the nuclear targeting sequence into pDNA and synchronization of nuclear envelope breakdown prior to ET (see the discussion above).⁵⁸ These approaches are more effective for improving gene delivery without significantly compromising cell viability. To protect pDNA from degradation, one strategy is to accelerate pDNA transport in cells, which can reduce the likelihood of degradation. Additionally, delay of endosomal escape may offer more protection of naked pDNA against endonuclease degradation in the cytosol.^{58,59} We expect that more strategies will be proposed to enhance pDNA delivery in cells with the development of the endocytosis theory.

SUMMARY

ET is a promising nonviral approach that has been widely used for gene delivery, especially to some difficult-to-transfect cells. Although its efficiency is still low, recent studies have suggested that the eTE can be significantly improved by understanding mechanisms of pDNA transport in cells. The new insights into the mechanisms will facilitate the development of completely new strategies to improve eTE and cell viability. We expect that with the improvement, ET will play an important role in gene delivery involved in the treatment of different diseases,^{47,97,98} especially cancer.^{12,13,19,20,29}

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Figure 1.

Physiological barriers to transport electrotransfected pDNA into the nucleus. The main barriers are the plasma membrane, cytoplasmic structures, and nuclear envelope. NPC: nuclear pore complex.

Figure 2.

Schematic of pore theory. Transient pores are induced in the plasma membrane by a pulsed electric field (from left to right). Extracellular pDNA enters the cell, denoted by the red circle, through the pores from the side facing the cathode. The pores are resealed after the pulse application. The symbols "+" and "-" represent cations and anions, respectively.

Figure 3. Effects of DNA degradation on intracellular gene delivery. Polymer forms a complex with pDNA (polyplex) that protects pDNA from degradation by endonucleases. Naked pDNA, however, is vulnerable to degradation by nucleases in the cytosol.

Figure 4.

Proposed mechanism of ET. When the cell is exposed to a pulsed electric field, electrophoretic force will push pDNA toward the cell surface and form a complex with the plasma membrane. Then, pDNA in the complex will be internalized via endocytic pathways and move toward the perinuclear region via vesicular trafficking. Thereafter, pDNA will escape from endosomes to enter the cytosol and eventually enter the nucleus for transgene expression.

Figure 5.

Schematic of possible pathways for intracellular trafficking of naked pDNA introduced into the cell via ET. During ET, extracellular pDNA is internalized via endocytic pathways. The internalized pDNA may travel from early endosomes to lysosomes, recycle back to the cell surface, or escape from endosomes. In the cytosol, the naked pDNA may enter the nucleus for transgene expression.