# Chapter 5
# Formulations for DNA Delivery via Electroporation In Vivo

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**Abstract** The importance of DNA formulation in safe and efficient electrogene transfer is increasingly recognized as electroporation technology enters into clinical development. A phenomenal increase in naked DNA delivery by electroporation offers new opportunities for nonviral gene therapies previously considered difficult because of insufficient delivery. However, significant tissue damage related to harsh electroporation conditions raises serious safety concerns with the use of electroporation in healthy tissues, which limits its current applications to only nonhealthy tissues such as tumors. DNA formulations designed to minimize tissue damage or enhance expression at weaker electric pulses have been examined to address these concerns. These include formulations fortified with the addition of transfection reagent(s), membrane-permeating agents, tissue matrix modifiers, targeted ligands, or agents modifying electrical conductivity or membrane stability to enhance delivery efficiency or reduce tissue damage. These advancements in DNA formulation could prove to be useful in improving the safety of electroporation protocols for human applications.

**Keywords:** electroporation, gene delivery, polymer, liposomes, DNA formulation

## 1. Introduction

Several DNA formulations for in vivo gene electroporation have been described in the literature. DNA formulation in physiological saline has been the most extensively studied formulation for electroporation. DNA electroporation in saline has been shown to enhance transfection efficiency in several tissues, producing both local and systemic levels of therapeutic proteins. The enhancement of gene electroporation is associated with significant tissue damage directly related to electroporation intensity. Milder electroporation conditions, although less toxic, are transfectionally inefficient. Several formulation strategies have been examined to reduce electroporation toxicity without affecting transfection activity. These approaches include DNA formulation in polymers, liposomes, high salt concentration,

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tissue matrix modifiers, cell permeability enhancers, membrane stabilizers, and cell-targeted systems. This chapter describes the in vivo properties of various DNA formulations used in electroporation.

## 2. Standard Saline Formulation for DNA Electroporation

DNA in saline (naked DNA) is the most commonly used formulation for in vivo gene electroporation (1). In skeletal muscle, electroporation enhancement of *luciferase* gene transfer was 10,000-fold over nonelectroporated control (2, 3). The enhancement of luciferase activity was observed in both small and large animal species. Histochemical analysis of *β-galactosidase* plasmid electroporated muscle showed a larger transfection area per muscle and a higher plasmid copy number per muscle cell when compared with nonelectroporated muscle (4). Muscle electroporation with *$FGF_1$* plasmid also showed significantly larger transfection area in electroporated muscle as compared to nonelectroporated muscle (2). An average of 710 ± 122 muscle fibers were $FGF_1$-positive in electroporated muscle and 4 ± 2 muscle fibers were $FGF_1$-positive in nonelectroporated muscle. In monkeys, three out of four muscle fibers were $FGF_1$ positive in electroporated group and none were $FGF_1$-positive in the nonelectroporated group.

Skeletal muscle has an excellent capacity to express transgene products and secrete them into systemic circulation following gene transfer (5); however, full benefit of this property of muscle has not been fully achieved because of suboptimal delivery (nonviral vectors) (6) or host immune response (viral vectors) (7). A substantial improvement in muscle delivery with the use of electroporation has renewed interest in muscle tissue for systemic protein therapy. Several therapeutic proteins have been expressed from skeletal muscle and secreted into systemic circulation at substantial concentrations with the use of electroporation. Electroporation of mouse muscle with secretory *alkaline phosphatase* (*SEAP*) plasmid produced systemic levels of SEAP that were up to 120-fold higher than those achieved with *SEAP* plasmid alone (3, 8). Intramuscular injection of *erythropoietin* plasmid in mouse leg produced systemic levels of erythropoietin that were 100-fold higher than those from *erythropoietin* plasmid alone (9, 10). Electroporation of *interleukin* (*IL*)-*5* plasmid DNA into mouse tibialis muscle produced 20 ng IL-5/mL while the nonelectroporated delivery produced only 0.2 ng IL-5/mL in the blood (4). Electroporation of mouse muscle with *IL-12* plasmid produced 1,500 pg of IL-12 per injected muscle and 170 pg IL-12/mL in the blood (3). In comparison, only background IL-12 levels were detectable in nonelectroporated group. The huge improvement in muscle delivery (up to 10,000-fold over naked DNA) compared with other nonviral gene delivery systems (at best 10-fold over naked DNA) opens up new product opportunities for muscle-based gene therapy.

Gene delivery into solid tumors after direct injection of formulated or naked DNA preparations is generally low because of a multitude of delivery barrier characteristics of tumor complexity. Tumor electroporation significantly enhances DNA

delivery into solid tumors. Electroporation of *luciferase* DNA into mouse and human tumors produced 10- to 1,200-fold increases in luciferase expression when compared with tumors injected with *luciferase* DNA alone (11). In another tumor study, electroporation gave high levels of luciferase expression while naked DNA injection alone failed to produce detectable luciferase activity (12). The magnitude of transfection enhancement in solid tumors is influenced by the electroporation protocol used. Tumor electroporation by six-needle electrodes (100-μs pulses, 1,500 V/cm) produced a 21-fold enhancement over control while tumor electroporation by caliper electrodes (5,000-μs pulses, 800 V/cm) produced a 42-fold enhancement (13). The transfection efficiency of DNA electroporation was compared with that of nonelectroporation methods including, liposome-DNA complexes and integrin-liposome-DNA complexes in different tumors (14). The electroporation delivery was found to be superior to all other test methods. The maximal enhancement in transfection efficiency by electroporation was up to 30-fold over naked DNA, 5- to 10-fold over liposome-DNA complexes, and over 100-fold over integrin-liposome-DNA complexes. Electroporation produced detectable gene expression in every tumor type while nonelectroporated methods were effective only in some tumors. Fluorescent microscopy of GFP-plasmid-transfected tumors shows larger and brighter transfection areas in electroporated tissue than in nonelectroporated tissue (15). In another CT26 study, tumor electroporation with *IL-12* plasmid produced 100 pg of IL-12 per tumor whereas DNA injection alone failed to yield a measurable expression (16). Tumor electroporation with IL-12 and *IL-18* plasmid DNA produced significantly higher tumor inhibition responses than did plasmid administration without electroporation (12). In B16 melanoma, tumor interferon-γ levels in *IL-12*-plasmid-electroporated tumors were 5- to 10-fold higher than in nonelectroporated tumors (17), which suggests that IL-12 receptor signaling pathways are unaltered by electroporation. Tumor electroporation did not increase cytokine concentration in blood circulation, suggesting that the effect of electroporation was highly localized in the tumor. The efficiency of IL-12 delivery by electroporation was quite comparable to adenoviral vector (18); however, the serum IL-2 levels were 50 times lower than in the adenovirus-treated animals, demonstrating highly localized expression in electroporated tissue. Transfection of human dendritic cells by electroporation was comparable to expression obtained with adenoviral or retroviral vectors (19). In another study, the delivery and anticancer efficacy of MBD2 antisense DNA in electroporated tumors were comparable to the adenovirus-treated groups (20).

The benefit of in vivo electroporation was also observed in skin tissue. Electroporation enhancement of luciferase transfection, when compared with that in nonelectroporated group, was up to 16-fold in mouse skin and up to 83-fold in pig skin (21). The enhancement by electroporation was independent of DNA dose and the pulsing protocol. The intradermal delivery of *IL-12* plasmid in mouse skin led to systemic levels of IL-12 (22). The IL-12 levels were 10-fold higher in electroporated animals when compared with those in nonelectroporated animals (22). The erythropoietin levels in blood after plasmid delivery into rat skin were 2-fold higher in electroporated groups when compared with nonelectroporated groups (23).

Compared with that in the solid tissues, gene electroporation in vascular tissues has not been investigated extensively, presumably because of the practical challenges associated with the procedure. Nishi et al., however, have described a novel method of vascular gene delivery by combining in vivo electroporation and intraarterial injection (24). In this method, *β-galactosidase* (*β-gal*) plasmid was injected into an internal carotid artery supplying blood to tumor implants in brain, and then the tumors were directly electroporated with a caliper electrode. The electroporated tumor showed significant β-gal expression while nonelectroporated tumors did not yield transgene expression.

These studies demonstrate that electroporation of solid tissues following injection of DNA in saline results in significant accumulation of therapeutic proteins in the injected tissues and, in some instances, in the systemic circulation depending upon the type of transfected tissue. The dramatic enhancement in naked DNA transfection by electroporation is also associated with considerable tissue damage that undermines the safety of this electroporation protocol. Histochemical analysis of the electroporated tissues shows massive inflammation and significant rise in Creatine Phosphokinase (CPK) levels in blood circulation, indicating muscle damage (25). These undesirable effects of electroporation must be reduced before advancing this procedure into the clinic. Several formulation approaches have been examined to enhance electroporation efficiency and lower toxicity. These formulation approaches are described in the following sections.

## 3. Formulation Strategies to Improve DNA Electroporation

### *3.1. Polymers*

DNA formulation with certain types of polymers has been found to enhance electroporation efficiency and, in some cases, reduce treatment-related toxicity. Anionic polymers, including poly-L-glutamate, polyacrylic acid, poly-L-aspartate, dextran sulfate, and pectin, have been examined for their ability to enhance electroporation-mediated gene transfer in skeletal muscle (26–30). DNA formulation with poly-L-glutamate increases the electrogene transfer by 4- to 12-fold depending upon the electroporation conditions, animal species, and target tissues. The enhancement of DNA electroporation by poly-L-glutamate is not significantly influenced by the polymer size or concentration; however, a solution of 15–50-kDa poly-L-glutamate (6 mg/mL) has been reported to give the most consistent results (26). In higher species (dogs and pigs), a lower concentration of poly-L-glutamate (0.01 mg/mL) was most effective and least toxic (28). Electroporation of skeletal muscle with *factor IX* or *erythropoietin* plasmid formulated with poly-L-glutamate (6 mg/mL) produced a significant increase in the circulating levels of factor IX and erythropoietin in mice and dogs (26, 27). Plasmid/poly-L-glutamate formulations encoding for the extracellular and transmembrane domains of the protein product of *Her-2/neu* oncogene at 10-week intervals produced complete clearance of neoplastic progression

and protection from mammary carcinoma (29). Combination of this *Her-2-neu*/poly-L-glutamate vaccine with recombinant IL-12 protein therapy further augmented the therapeutic response in cancer-bearing animals (30). The mechanism of poly-L-glutamate enhancement of electroporation has not been fully understood. Poly-L-glutamate decelerates DNA clearance and enhances DNA stability and DNA retention in mouse muscle (26). Facilitation of DNA uptake into the cell and escape from the lysosomal compartment have also been postulated in the mechanism of poly-L-glutamate action (31). Poly-L-glutamate appeared to be nonimmunogenic and nontoxic for in vivo applications in animals and humans (32).

Formulation of *β-gal* plasmid with SP107 polymer significantly increased electroporation efficiency but did not reduce treatment-related inflammatory activity (33). The enhancement of transfection efficiency was higher at low-intensity electric pulses than at high-intensity ones. Formulation of DNA with another nonionic polymer, poloxamer 188, did not influence electroporation efficiency, but significantly reduced treatment-related increase in creatine phosphokinase activity (25), suggesting that the polymer protects tissue from electroporation damage. Poloxamer 188 has been shown to reduce muscle trauma and promote membrane sealing following electric shock (34). Poly(vinylpyrrolidone) (PVP) is another noncondensing polymer that is used in DNA formulation for electroporation. PVP had previously been shown to promote DNA delivery into skeletal muscle (35). The effect of PVP formulation on electrogene transfer of melanoma antigen *gp100* was examined in a mouse melanoma model (36). About 40% of tumor-bearing mice immunized intramuscularly with gp100 plasmid/PVP rejected tumor challenge when compared with only 9% of mice immunized with gp100 plasmid/saline. However, PVP formulation did not enhance electroporation delivery of *factor IX* plasmid in mouse skeletal muscle (27).

## 3.2. *Liposomes*

DNA complexes of cationic liposomes were electroporated into several histologically distinct mouse subcutaneous tumors, and the efficiency of gene transfer was compared with that of naked DNA electroporation (14). Liposomal formulations were transfectionally superior to naked DNA in B16 melanoma, P22 carcinoma, and SaF sarcoma but not in T24 human bladder carcinoma (14) or MC2 mammary carcinoma (37). This variation in tumor response could be due to differences in the state of tumor necrosis, tumor conductivity, or matrix complexity between the different tumors.

Addition of cationic liposomes to transfection medium has been shown to enhance the electroporation of transposon/transposase system in *Spirulina platensis*, a commercially important species of microalgae generally resistant to conventional transformation methods (38). While electroporation alone failed to efficiently transform *S. platensis* with Tn5 transposon/transposase system, addition of 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) into electroporation medium improved the transformation efficiency by 100-fold without affecting the biological

integrity of the transformed algae. These studies demonstrate that the efficiency of electrogene transfer can be improved by addition of cationic liposomes in the electroporation medium. The precise mechanism of liposome action on electrogene transfer has not been investigated. A higher interaction of positively charged lipid-DNA complexes with negatively charged cell surfaces could be one of the underlying mechanisms in the lipid enhancement of the electroporation.

Addition of anionic liposomes into the electroporation medium has been found to enhance the delivery of macromolecules into cells. For example, dextran uptake during electroporation was enhanced by 80-fold with the addition of phosphatidylglycerol and phosphatidylcholine into the transfection medium (39, 40). The magnitude of liposome enhancement was dependent on the degree of lipid saturation but independent of polar head group. The effect of anionic liposomes on DNA electroporation has not been investigated.

## 3.3. *Targeted Systems*

DNA delivery by electroporation is not target-specific. Several attempts have been made to improve tissue-specific targeting of electroporated DNA with the use of cell-specific ligands (14, 41). Antibodies and other molecular entities that recognize specific cell surface receptors have been conjugated to delivery vehicles to achieve high cell specificity during electroporation. For example, large unilamellar vesicles encapsulating *XGPRT* gene were coated with protein A to target HAL-2-antibody-labeled lymphoid cells prior to electroporation (41). Anti-HLA-antibody-labeled lymphoid cells were electroporated with protein-A-coated liposomes/*XGPRT* DNA complexes to approximately 35% transfection efficiency. In comparison, the same cells electroporated with nontargeted liposomes were transfected to only 5% transfection efficiency. The technical feasibility of in vivo DNA targeting by electroporation has not been fully established. For example, electroporation of integrin-conjugated liposome-DNA complexes yields much lower transfection efficiency than do the nontargeted systems (14). This failure of tumor targeting in vivo could be attributed to poor stability of the targeted complexes in extracellular milieu, altered integrin receptor affinity for integrin ligand, or suboptimal transfection conditions. Hence, the use of targeted ligands is an attractive approach to improve target specificity of electroporation, but its in vivo application has not been fully established.

## 3.4. *Hyaluronidase Treatment*

DNA dispersion in muscle is highly restricted because of the rigid collagen- and hyaluronan-rich matrix surrounding muscle fibers. Pretreatment of tissue with hyaluronidase has been shown to improve gene delivery into liver (42) and skeletal

muscles (43). Hyaluronidase treatment prior to electroporation in skeletal muscle produced a substantial increase both in levels and extent of gene transfer in skeletal muscle (44). Hyaluronidase treatment enhanced transfection efficiency at low electric pulses without significantly damaging the muscle structure or function (44). This tissue-protective effect of hyaluronidase has been observed in ischemic myocardium (45) and tissue edema (46). These results demonstrate that hyaluronidase treatment is a useful approach to improve electrogene transfer in higher species where rigid interstitium is a major limitation to plasmid delivery.

## *3.5. High Salt Formulations*

Since the negative charge of DNA could be one of the important factors affecting transfection efficiency, the presence of salt in the DNA vehicle would alter ionic atmosphere, ionic strength, and conductivity of the medium. Therefore, it is reasonable to assume that changing the salt concentration in plasmid solution would influence electroporation efficiency. Lee et al. have examined the effect of various salts and salt concentrations on the efficiency of electrogene transfer in mouse skeletal muscle (47). Plasmid DNA encoding *luciferase* or *β-galactosidase* gene was formulated in water, saline, or phosphate-buffered saline prior to delivery. In the absence of electric pulse, transfection was highest in phosphate-buffered saline, followed by saline and then water. However, in the presence of electric pulse (125 V/cm, two sets of 4 pulses, 50 ms, 1 Hz), this relationship reversed, with the highest expression observed with water, followed by saline and phosphate-buffered saline. The mechanism of this reversal in gene expression pattern is not fully understood. However, histological examination of the electroporated tissue showed higher degree of tissue damage in water injection group. Varying the salt concentration (0–250 mM NaCl) of plasmid formulation had a pronounced effect on the efficiency of electrogene transfer in skeletal muscle. The transfection activity increased with increasing salt concentration, reaching maximal activity at 75 mM NaCl, and then decreasing as the salt concentration was further increased. Histological examinations show higher tissue damage at salt concentrations below 75 mM. The comparison of electrical conductivity and resistance after intramuscular injection showed higher resistance and lower conductivity with water injection than with normal saline injection. Hence, the efficiency of electrogene transfer increases as the ionic strength decreases. Lowering the ionic strength below a threshold (75 mM) lowers tissue conductivity, enlarges "electroleaks" (48), and exerts tissue damage. In the absence of electroporation, high salt concentration enhances transfection efficiency (49).

Increasing the calcium concentration from 2 to 10 mM in the electroporation medium significantly decreased the efficiency of *lac Z* gene transfer in skin and muscle (50). In comparison, electroporation of FITC-labeled oligonucleotide into mouse ovary was significantly enhanced at higher calcium concentrations (51). These opposite effects of calcium could be due to difference in calcium concentrations, nucleic acid size, or tissue.

### 3.6. Gold Nanoparticles

Application of electromigration field (3 V for 30 s) has been shown to enhance the uptake of DNA-modified gold nanoparticles during cell electroporation (52). Gold nanoparticles devoid of DNA coating were not taken up by cells during electroporation. Transmission electron microscopy of electroporated cells revealed a higher cell surface density of DNA-modified gold particles in cells subjected to electromigration field than in cells that were not, suggesting that the DNA binding to cell surface is crucial for optimal transfection efficiency from electroporation. Formulations that can enhance DNA binding to cell surface in vivo may also enhance electroporation efficiency at weak electric pulses.

### 3.7. Nucleofection

Transfection of primary cultures of mammalian cells with conventional gene delivery methods, including standard electroporation, is poor. Recently, a gene transfer protocol that combines electroporation with special transfection solutions has been described, which transfects primary cells and other hard-to-transfect cells with high efficiency (53–55). It is believed that this electroporation formulation promotes direct translocation of DNA into the nucleus, producing up to 50% transfection efficiency in otherwise poorly transfectable cells. The chemical composition of this formulation and its in vivo application has not been described.

### 3.8. Other Chemical Formulants

Satyabhama et al. have examined the effect of DNA formulation with several chemical agents on the efficiency of electrogene transfer in human erythroleukemia cells. These chemical agents included membrane perturbation reagents, anesthetics, lipids, polymers, lysosomal inhibitors, macromolecules, nucleic acid precursors, ethanol, polybrene, and various combinations of these agents (56). Addition of ethanol, polybrene, and PEG 6000 resulted in a 3- to 5-fold enhancement in transfection activity, and various combinations of these chemical agents gave up to 15-fold enhancement in electrogene transfer. The mechanisms of these enhancements are attributed to the ability of these agents to influence DNA trafficking.

## 4. Summary

Electroporation is a widely recognized method of gene delivery into mammalian tissues. It is a highly efficient method, with delivery efficiency surpassing that of many nonviral vectors. The preclinical development of electroporation in vivo is

**Table 5.1** Formulation strategies for improvement of DNA electroporation

| Formulant | Gene | Species | Tissue | Effects on electroporation | Reference |
|---|---|---|---|---|---|
| Polyglutamate | *β-gal, Luc, FIX, EPO* | Mouse, rat, dog, pig | Skeletal muscle | Increased gene expression | 26–30 |
| Poloxamers | *β-gal* | Rat | Skeletal muscle | Increased gene expression and less tissue damage | 25, 33 |
| Poly(vinylpyrrolidone) | *FIX* | Mouse | Skeletal muscle | No effect on gene expression | 27, 36 |
| | *gp 100* | Mouse | Solid tumor | Increased gene expression | |
| Liposomes | *GFP, CAT* | Mouse | Solid tumor | Variable results (tumor-dependent) | 14, 37 |
| Targeted ligands (antibody,integrins) | *XGPRT* | Mouse | Lymphoid cells, tumor | Increased gene expression in lymphoid cells, no effect on tumor | 14, 41 |
| Hyaluronic acid | *β-gal* | Mouse | Skeletal muscle, liver | Increased gene expression | 44 |
| Salt concentration | | | | | |
| NaCl | *Luc, β-gal* | Mouse | Muscle | Optimal expression at 75 m M $Na^+$ | 47 |
| $CaCl_2$ | *β-gal, Oligos* | Mouse | Skin, muscle, ovary | Variable results with $Ca^{2+}$ | 50, 51 |
| Nucleofection | *GFP, CAT* | Human, mouse | Leukemia, endothelial and smooth muscle cells | Increased expression | 53–55 |
| Chemical stimulants | *CAT* | Mouse, human | Lymphoid and myeloid cells | Moderate enhancement | 56 |
| Gold nanoparticles | *Oligos* | Mouse | Osteoblast cells | Increased expression | 52 |

focused on tissues that are easily accessible to electroporation and can electric pulsation. The standard DNA formulation for electroporation i physiological saline. Under optimal conditions, DNA electroporation yields a 10- to 10,000-fold enhancement in gene delivery efficiency ove troporated controls. This enormous increase in transfection activity, accompanies significant tissue damage and local inflammation, which mig a bad thing to have if the target is cancer. However, for applications expression from normal tissues is desired, tissue damage and infla response are not conducive to therapeutic objectives and, therefore, must mized. Several formulation strategies have been designed to enhance elec tion efficiency and minimize toxicity (Table 5.1). Encouraging results ha obtained with some approaches, which must be further developed into c viable formulations for noncancer applications.

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