Chapter 11

Electroporation – Advantages and Drawbacks for Delivery of Drug, Gene and Vaccine

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1. Introduction

Lack of potent drug and gene delivery is one of the major problems of cancer chemotherapy and biotherapy. Different non-viral approaches have been proposed for drug and gene delivery such as physical and chemical methods. Physical delivery systems are one of the efficient non-viral methods including electroporation, micro-injection, gene gun, tattooing, laser and ultrasound [Bolhassani and Rafati, 2011]. Electroporation (EP) is the formation of aqueous pores in lipid bilayers by the application of a short (microseconds to milliseconds) high-voltage pulse to overcome the barrier of the cell membrane. This transient, permeabilized state can be used to load cells with a variety of different molecules including ions, drugs, dyes, tracers, antibodies, oligonucleotides, RNA and DNA [Faurie et al., 2005]. Electroporation has proven useful both *in vitro*, *in vivo* and in patients, where drug delivery to malignant tumors has been performed. In addition, the data show that electroporation of DNA vaccines *in vivo* is an effective method to increase cellular uptake of DNA and gene expression in tissue leading to marked improvement in immune responses. Electroporation represents a way of increasing the number of DNA-transfected cells and enhancing the magnitude of gene expression, while reducing intersubject variability and requiring less time to reach a maximal immune response compared to conventional intramuscular injection of the vaccine [Monie et al., 2010].

Delivery of DNA vaccines using electroporation has already been tested successfully in a wide range of disease models. Electroporation has been used to enhance immune responses using DNA vaccines directed against infectious diseases such as influenza, HIV, hepatitis C, malaria, anthrax or to treat or prevent the development of tumors including breast cancer, prostate cancer and melanoma [Daemi et al., 2012; Best et al., 2009]. The studies have shown that *in vivo* EP mediated vaccination is a safe and effective modality for the treatment of prostate cancer and has potential to be used as a neo-adjuvant or adjuvant therapy [Ahmad et al.,

2010]. Electroporation has been successfully used to administer HPV DNA vaccine to mice as well as rhesus macaques, which has prompted its use in an ongoing phase I clinical trial such as VGX-3100, a vaccine that includes plasmids targeting E6 and E7 proteins of both HPV subtypes 16 and 18, for treatment of patients with CIN 2 or 3. In addition, electroporation has been used as an effective vaccination technique for the treatment of HPV induced cancers using the pNGVL4a-CRT/E7 (detox) DNA vaccine [Monie et al., 2010]. The application of *in vivo* electroporation to the sites receiving injected plasmid DNA has allowed for dramatic increases in immune responses compared with plasmid DNA injection alone. Among the tissues targeted for *in vivo* electroporation have been skin, liver, tumors and muscle [Widera et al., 2000]. Regarding to *in vivo* EP is predominantly carried out intramuscularly (i.m.), currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ-producing T cells and protective efficacy to infection [Daemi et al., 2012; Brave et al., 2011]. Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+T-cell immune responses while limiting invasiveness of delivery [Brave et al., 2011].

However, this method sometimes leads to cell death, primarily when the electrical fields cause permanent permeabilization of the membrane and the consequent loss of cell homeostasis, in a process known as irreversible electroporation [Rubinsky, 2007]. This is an unusual mode of cell death that is not understood yet. The electroporation procedures used in many laboratories could be optimized with limited effort. Moreover, electroporation, used alone or in combination with other enhancement methods, expands the range of drugs (small to macromolecules, lipophilic or hydrophilic, charged or neutral molecules) that can be delivered transdermally [Escobar-Chávez et al., 2009; Denet et al., 2004]. The efficacy of transport depends on the electrical parameters and the physicochemical properties of drugs. The *in vivo* application of high-voltage pulses is well tolerated, but muscle contractions are usually induced. The electrode and patch design is an important issue to reduce the discomfort of the electrical treatment in humans [Denet et al., 2004]. It was shown that poloxamer 188, added before or immediately after an electrical pulse used for electroporation decreases the number of dead cells and at the same time does not reduce the number of reversible electropores through which small molecules (cisplatin, bleomycin, or propidium iodide) can diffuse. It was suggested that hydrophobic sections of poloxamer 188 molecules are incorporated into the edges of pores and that their hydrophilic parts act as brushy pore structures. The formation of brushy pores may reduce the expansion of pores and delay the irreversible electropermeability. These techniques show a potential for drug and gene delivery. However, site-specific and efficient delivery still remains a difficult problem [Tsoneva et al., 2010]. The voltages generally used for electroporation in animals range from 100 to 1200 V/cm. The investigators have shown that low-voltage electroporation can induce immunity and protect mice effectively [Daemi et al., 2012; Zhou et al., 2008]. In addition, intradermal DNA electroporation is one of the most efficient non-viral methods for the delivery of gene into the skin [Lin et al., 2012]. Previous studies have demonstrated that a combination of a short high voltage pulse (HV) and a long duration low-voltage pulse (LV) was efficient for DNA electroporation in the skin and that intradermal electroporation was suitable to deliver DNA vaccine when a Th1-oriented response is desired [Pavselj

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and Préat, 2005]. Various cell types of the skin are involved in the development of immune response. Langerhans cells (LC) due to their long dendritics and their horizontal orientation, create an almost continuous network that enables them to capture most antigens that enter through the skin. Delivery of DNA into the skin could induce direct-presentation of the encoded antigen by APC or cross-presentation after uptake by keratinocytes. Some studies have indicated that EP induces IgG and Th-cell responses higher than Intramuscular (IM) delivery [Lee et al., 2011]. This chapter is further focused on the use of electroporation-induced delivery of anti-cancer drugs, gene and vaccines in human cancer cells along with description of its advantages and disadvantages.

2. Non-viral delivery systems

Generally, the methods of delivering a gene, vaccine and drug are divided into: **a)** Physical/ non-viral approaches such as tattooing, gene gun, ultrasound, electroporation, laser; **b)** Chemical/ non-viral systems such as: cationic lipids/liposomes, polysaccharides, cationic polymers, cationic peptides, micro-/ nano-particles and **c)** Biological/ viral vectors [Bolhassani et al., 2011]. Non-viral vectors are safe in human body and easy for use. Among them, electroporation can be used to distribute nucleic acid fragments, oligonucleotides, siRNA and plasmids to cells. Studies using electroporation were performed *in vivo*; however electroporation is sometimes harmful to differentiated adult cells [Anwer, 2011; Wang et al., 2012]. Non-viral vectors are attractive tools in gene therapy and vaccine delivery [Draghia-Akli et al., 2005].

3. History and definition of electroporation

Electroporation was introduced in the 1960s and comprises the application of controlled electric fields to facilitate cell permeabilization. The success of *in vitro* delivery by electroporation has led to the development of *in vivo* applications [Takei et al., 2008]. The first *in vitro* and *in vivo* attempts to use electroporation in gene transfer were demonstrated in 1982 and 1991, respectively [Al-Dosari and Gao, 2009]. *In vivo* electroporation depends on electric pulses to drive gene transfer. These pulses generated transient pores in cell membranes followed by intracellular electrophoretic DNA movement. Typically, *in vivo* electroporation is performed by first injecting DNA to the target tissue followed by electric pulses, with varied voltage, pulse duration and number of cycles, from two applied electrodes [Al-Dosari and Gao, 2009, Hao et al., 2012]. This technique is generally safe, efficient and can produce good reproducibility compared to other non-viral methods. When its parameters are optimized, this method can generate transfection efficiency equal to that in viral vectors [Al-Dosari and Gao, 2009]. The initial study of *in vivo* EP was the delivery of chemotherapeutic agents to solid tumors. In the mid-to late 1990s, the efficacy of this approach for drug delivery was demonstrated in a variety of different animal and human tumors. This technique was then tested for enhanced plasmid DNA delivery and subsequently, the initiation of the first clinical trials [Heller and Heller, 2006]. Furthermore, the expression of reporter genes was used to optimize *in vivo* EP param-

eters, to explore the mechanism of EP and to show delivery in a new tissue. The use of *in vivo* EP for gene delivery including immune modulators, cell cycle regulators, suicide genes, anti-angiogenic genes and genes encoding toxins has established its potential for many therapeutic applications [Heller and Heller, 2006]. *In vivo* electroporation as compared to other gene transfer methods, such as viral vectors, has several advantages: **a)** various types of DNA constructs (or RNAi vectors) are readily introduced to the cells without limitation of DNA size; **b)** more than two different DNA constructs can be introduced into the same cells [Matsuda and Cepko, 2007]. Altogether, delivery by electroporation has been performed to a number of tissues including skin, muscle, liver, testes and tumors employing a wide range of electrical conditions and electrodes. While this preclinical research is promising, further optimization of electrical conditions and electrodes would be necessary for clinical use [Fioretti et al., 2013; Heller and Lucas, 2000].

4. Electroporation mechanisms

The development of theoretical models has developed our understanding of electroporation mechanism. Electropermeabilization of cells mainly involves the interaction of the electric field with the lipid domains of the cell membrane. Experimentally measured quantities consist of the membrane lifetimes, the current, the membrane conductance and transmembrane voltage. Regarding to the accumulated evidence, the pores are formed because of the electric field. The transient aqueous pore theory describes the main features of electropermeabilization, which is one major consequence of electroporation. Molecular transport of charged molecules appears to be predominantly due to electrical flow through pores, such that the elevated transmembrane voltage plays two roles: (a) creation of pores and (b) provision of a local driving force [Weaver and Chizmadzhev, 1996]. Electrochemotherapy (ECT) is a cancer therapy that conjugates the administration of a chemotherapy agent to the delivery of permeabilizing pulses released singularly or as bursts. This approach results in higher number of anticancer molecules delivered to their biological targets, but is also associated to undesirable side effects such as pain and muscular spasms. A new electroporator delivering eight biphasic pulses at the voltage of 1,300 V/cm lasting + 50 μsec each, with a frequency of 1 Hz, and with 10-μsec interpulse intervals (total treatment time: 870 μsec/cm^2 of treated area) was tested on the human lung cancer cell line (A549) and both in mice xenografts and rabbits with spontaneous tumors. The tumor cell line treated with electroporation showed efficient drug delivery suggesting further cell death. In addition, *in vivo* data demonstrated that the new permeabilizing protocol adopting biphasic electric pulses displays a significant higher efficacy compared to previous ECT treatments and consequently, substantial reduction of the morbidity [Spugnini et al., 2014].

5. Applications of electroporation

Skin electroporation could be particularly appropriate for topical drug delivery. Skin electroporation temporarily permeabilizes the barrier to drug permeation and therefore could

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broaden topical delivery to drugs not suitable for delivery by passive diffusion (i.e., hydrophilic, charged, and/or large molecular drugs). The use of high-voltage pulses could also enhance the permeability of viable cells as demonstrated by the electrochemotherapy of tumors (e.g., bleomycin) or DNA transfection [Escobar-Chávez et al., 2009]. Indeed, the application of electrical pulses to a cell creates a transient permeability that allows entry of hydrophilic molecules such as drugs and plasmid DNA. The exact mechanism by which the plasmid enters the cell following electroporation is unclear. Although, small molecules such as drugs can enter cells via transient pores, it seems that macromolecules such as plasmid DNA enter by a more complex interaction with the cell membrane. This interaction is enhanced by the application of repeated pulses that brings the plasmid into closer contact with the cell membrane. The voltage required for electroporation varies considerably and is dependent on cell size and shape [Wells, 2010]. It ranges from values of approximately 100 V/cm in large cells up to 1-2 kV/cm in small cells such as bacteria. Plasmid electrotransfer is a multistep process from interaction with the cell membrane, movement into the cell, intracellular trafficking and passage across the nuclear membrane [Wells, 2010; Nakamura and Funahashi, 2013]. A variety of different electrodes could be used depending on the cells to be treated. For *in vitro* studies, electrode patterns vary from a cuvette figure for cells in suspension to complex electrode arrays for adherent cells. An equal variety of electrodes have been developed for *in vivo* use, based on the nature of the tissue being treated [Wells, 2010]. A wide range of pulse patterns have been used both *in vitro* and *in vivo*. Repeated pulses appear better than single pulses. Some authors suggest a combination of one high-voltage pulse with a series of low-voltage pulses. Pulse magnitude and duration also has an effect on the damage caused to the cells. Pretreatment of skeletal muscle *in vivo* with hyaluronidase allows the use of a decreased voltage and so reduces damage while maintaining efficiency. Plasmid size has a significant effect on the efficiency of electroporation with a decreasing efficiency observed with increasing plasmid size using the same expression cassette [Wells, 2010]. The *in vitro* and *in vivo* studies using electroporation have been further described as following:

a. *In vitro* electroporation: Electroporation can be used to transfer a range of genetic materials into cells including DNA, RNA and oligonucleotides. In addition, *in vitro* electroporation is useful for synthetic oligonucleotides which have an uncharged backbone such as the phosphorodiamidate morpholino oligomers [Wells, 2010]. The effects of electrical treatment with high field intensity (200-1000 V/cm) were evaluated on two breast cancer cells (MDA-MB-231 and MCF-7) and one fibroblast cell line 3T3. The degree of electropermeabilization of the adherent cells elevated steadily with the increasing of the field intensity. Furthermore, cell replication of both cancer cell lines was disturbed after electropermeabilization. Altogether, the use of suitable electric pulses could trigger changes in the cytoskeleton organization and cell adhesiveness, led to the enhancement of anti-tumor effects [Pehlivanova et al., 2012].

b. *In vivo* electroporation: *In vivo* electroporation has been shown to be effective for a wide range of tissues, including tumors, skin, liver, lung, kidney, thymus, bladder, adipose tissue, vasculature, retina, cornea, ciliary muscle, brain, spinal cord, skeletal muscle and testis, for delivering a range of genetic material such as DNA, RNA and oligonucleotides

(e.g. siRNA, antisense oligonucleotides). *In vivo* plasmid electroporation has also been used as either a primary or booster vaccination strategy that enhances cell-mediated immune responses. Most of the studies using electroporation have involved local delivery into the target organ but a few have studied local electroporation following systemic (intravenous) delivery of the plasmid. For example, local electroporation targeted plasmid delivery to the liver was effective for liver, kidney and spleen but was not successful for skeletal muscle or skin [Wells, 2010]. Taken together, electroporation has been applied to efficient delivery of drugs, genes and vaccines as described below. Figure 1 shows common application of electroporation.

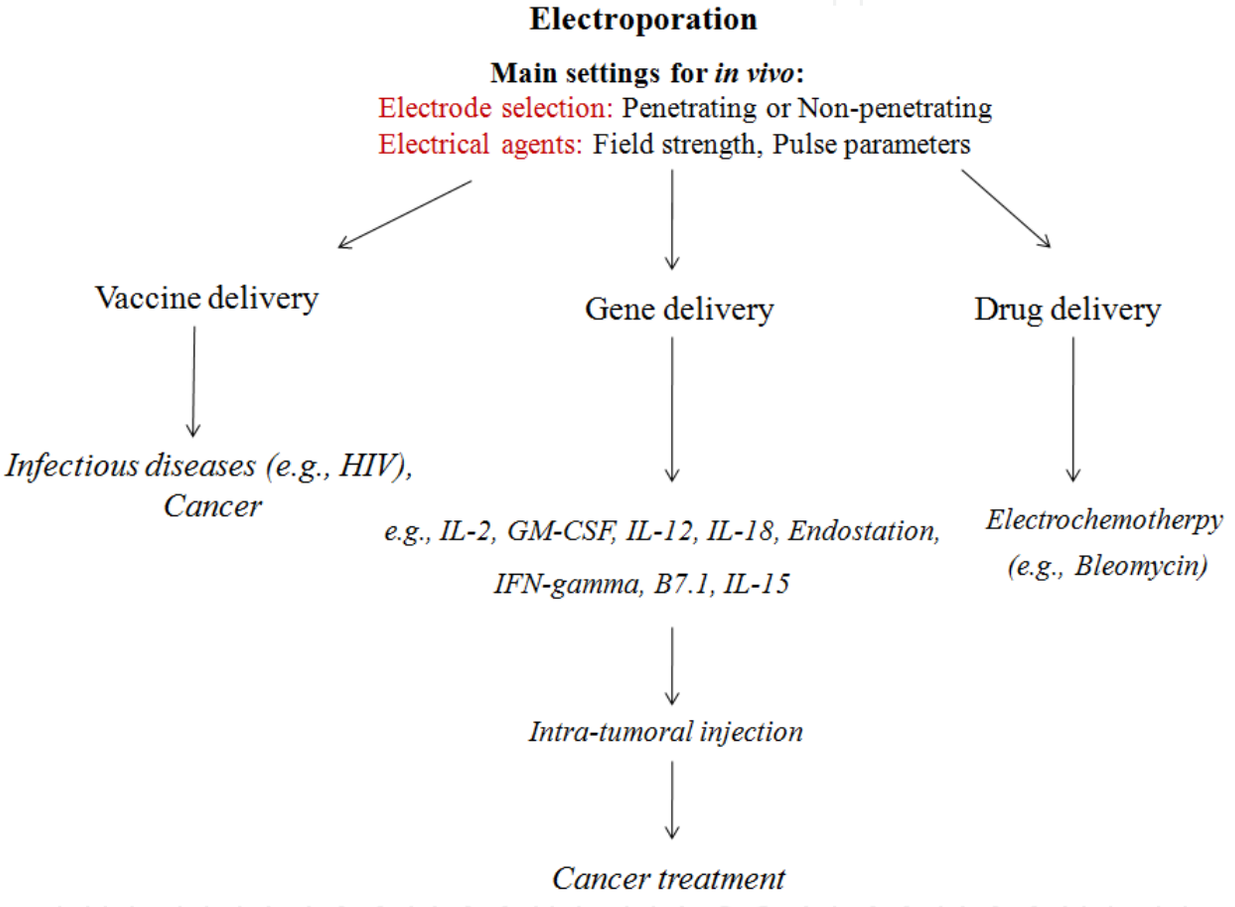

Figure 1. Common applications of electroporation

5.1. Drug delivery

Several studies have investigated the use of electroporation to enhance the efficacy of the drugs especially used for the treatment of various cancer types. Current electroporation protocols are based on preclinical studies. The authors reported the use of 1,000 V/cm (voltage/electrode distance ratio) up to approximately 1,300 V/ cm for electrochemotherapy. One simple way of lowering the applied voltage was to decrease the gap between electrodes, e.g., 0.4 cm [Gehl, 2008]. The threshold potential for transient electric breakdown of cell membranes is about 0.5

V. For a cell with a 10 μm diameter, the field strength needed to reach and exceed a potential of 0.5 V at each end is about 1,000 V/cm [Hui, 2013].

Transdermal drug delivery offers an attractive alternative to the conventional drug delivery methods of oral administration and injection [Escobar-Chávez et al., 2009]. The subcutaneous layer forms the major barrier to most water-soluble and many hydrophobic drugs and contributes the major portion of the electric resistance of the skin. Electroporation is one of the approaches to improve the transdermal delivery by transiently permeabilizing the skin to facilitate drug transport. Transdermal drug delivery has several potential advantages over other parenteral delivery methods. Apart from the convenience and non-invasiveness, the skin also provides a "reservoir" that sustains delivery over a period of days [Hui, 2013]. The authors have shown that if the voltage of the pulses exceeds a voltage threshold at 75–100 V (equivalent to the breakdown threshold of 8–10 lipid bilayers in the SC), microchannels or "local transport regions" are created through the breakdown sites of the SC [Hui, 2013]. Many small-molecule drugs have been successfully delivered through the skin by electroporation. Transport efficiency for small charged molecules (MW ≤ 1000, e.g., protoporphyrin IX), using the same polarity pulses, was higher than that for uncharged molecules (e.g., protoporphyrin IX methyl ester) or charged molecules with opposite polarity pulses. The results indicated that, besides passive diffusion through electropores, electrophoretic force of the pulses also contributes to the electroporation-enhanced transport of these charged molecules [Hui, 2013]. Therefore, the efficacy of transport depends on the electrical parameters and the physicochemical properties of drugs. Some studies indicated that the *in vivo* application of high-voltage pulses is well tolerated, but muscle contractions are generally induced. Furthermore, the electrode and patch design is an important issue to reduce the discomfort of the electrical treatment in humans [Escobar-Chávez et al., 2009]. The electroporation has been first used to enhance the delivery of chemotherapeutic drugs like cisplatin and bleomycin in cancer cells and solid tumors, respectively. This application has been termed electrochemotherapy [Tsoneva et al., 2007; Gehl, 2008].

5.1.1. Anti-cancer drugs

Electrochemotherapy, via cell membrane permeabilizing electric pulses, potentiates the cytotoxicity of non-permeant or poorly permeant anticancer drugs with high intrinsic cytotoxicity, such as bleomycin or cisplatin, at the site of electric pulse. Its advantages are high efficacy on tumors with different histologies, simple application, minimal side effects and the possibility of effective repetitive treatment. In clinical studies, electrochemotherapy has proved to be a highly efficient and safe approach for treating cutaneous and subcutaneous tumor nodules. The treatment response for various tumors (predominantly melanoma) was approximately 75% complete and 10% partial response of the treated nodules [Escobar-Chávez et al., 2009].

5.1.1.1. Bleomycin

A consistent finding is that lipo-or amphiphilic drugs traverse the cell membrane without electroporation, while an enhancement in cytotoxicity is found with drugs that, under normal

circumstances, do not pass the cell membrane easily. The most prominent example is bleomycin, which is a well-known drug. One bleomycin molecule can cause several DNA strand breaks and is highly toxic inside the cell [Gehl, 2008]. Drug doses used in bleomycin-based electrochemotherapy have been variable. Some groups have used intratumoral injection with relatively high doses, while others have applied its lower doses. Also, for *i.v.* administration, bleomycin is generally given in the doses used in standard treatment protocols. The results of the different regimens are comparable, but there may be more necrosis with the higher doses and a better chance to conserve normal tissue with the lower doses [Gehl, 2008]. In bleomycin chemotherapy, treatment was more than 1000 times more effective with electroporation than without electroporation. In comparison with bleomycin, other drugs such as daunorubicin, doxorubincin, 5-fluorouracil and paclitaxel had no electroporation benefits [Hui, 2008]. Bleomycin electrochemotherapy has been successfully applied to treat melanomas, head and neck squamous cell carcinomas, Kaposi's sarcomas, as well as lung, breast, kidney, and bladder cancers. Its cytotoxicity is higher in cancer tissues than in normal tissues, including arteries and nerves. In certain stage II and III clinical trials, 100% complete recovery has been reported. Bleomycin electrochemotherapy induces temporary vasoconstriction, which helps to retain the drug in the tumor tissue [Hui, 2008].

As described above, the bleomycin is used with electroporation (electrochemotherapy) for treatment of tumors in the clinical setting. Calcium electroporation offers several advantages over standard treatment options: calcium is inexpensive and may readily be applied without special precautions mentioned about cytostatic drugs. Therefore, details on the use of calcium electroporation are essential for carrying out clinical trials comparing electrochemotherapy [Frandsen et al., 2014]. Calcium electroporation can induce ATP depletion-associated cellular death. The effects of calcium and bleomycin electroporation (alone or in combination) were compared in three different cell lines (DC-3F, transformed Chinese hamster lung fibroblast; K-562, human leukemia; and murine Lewis Lung Carcinoma) [Frandsen et al., 2014]. Furthermore, the effects of electrical pulsing parameters and calcium compounds on treatment efficacy were determined. The results showed that electroporation with either calcium or bleomycin significantly reduced cell survival, without a synergistic effect at similar voltage parameters. At equimolar concentrations, calcium chloride and calcium glubionate resulted in comparable decreases in cell viability. Indeed, the effect of calcium electroporation is independent of calcium compound [Frandsen et al., 2014]. Briefly, the calcium electroporation can be suggested as a potential cancer therapy in future clinical trial.

5.1.1.2. Poloxamer 188

Poloxamer 188, added before or immediately after an electrical pulse, decreased the number of dead cells as well as it did not reduce the number of reversible electropores. It was suggested that hydrophobic sections of poloxamer 188 molecules are incorporated into the edges of pores and their hydrophilic parts act as brushy pore structures. The formation of brushy pores may reduce the expansion of pores and delay the irreversible electropermeability. Its advantage is the increased uptake and accumulation into reversibly electroporated tumor cells [Tsoneva et al., 2010].

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5.1.2. Analgesic and anti-inflammatory drugs

Electroporation increased the permeation of h-cyclodextrin (BCD) and hydroxy propyl h-cyclodextrin (HPCD), relative to passive transport. The presence of BCD and HPCD enhanced the total transport of the permeants piroxicam and carboxyfluorescein (CF), respectively, from both permeant solutions and suspensions. Another studies demonstrated that electroporation may enhance and control transdermal permeation of nalbuphine (NA) and its prodrugs including nalbuphine benzoate (NAB) and sebacoyl dinalbuphine ester (SDN). The results indicated that the use of iontophoresis or electroporation significantly enhanced the *in vitro* permeation of NA and its prodrugs. In addition, lipophilicity and molecular size had significant effects on skin permeation of NA, NAB, and SDN via passive diffusion or under the electric field. The permeation amounts of NA and its prodrugs may be increased by application of higher pulse voltage, pulse duration and pulse number [Escobar-Chávez et al., 2009].

5.1.3. Anti-diuretic drugs

Macromolecules were investigated as chemical enhancers of transdermal transport by skin electroporation. Skin electroporation increased transdermal mannitol delivery [Escobar-Chávez et al., 2009].

5.1.4. Anti-viral drugs

The use of electroporation pulses enhancing the skin permeability to deliver anti-viral drugs is in the early stages of development. A systematic study examining the parameters influencing electroporative transdermal delivery of terazosin hydrochloride to rat skin was previously reported. It was found that voltage, pulse length and number of pulses were the three most important parameters [Escobar-Chávez et al., 2009].

5.1.5. Beta-blocker agents

The studies have shown the effects of electroporation on iontophoretic transport of 2 beta-blockers, timolol (lipophilic) and atenolol (hydrophilic). The iontophoretic transport of timolol was decreased by electroporation because the high accumulation of the lipophilic cation timolol in the *s.c.* resulted in a decrease of electroosmosis. In contrast, electroosmosis was not affected by atenolol, and the iontophoretic transport of atenolol was increased by electroporation. Using two different beta-blockers, the researchers showed that lipophilicity and positive charges affected the electrotransport of drugs [Escobar-Chávez et al., 2009].

5.1.6. Insulin

The data represented that *in vivo*, non-invasive insulin delivery to therapeutic levels and glucose extraction may be achieved by combining electroporation with anionic lipids and electroosmosis [Escobar-Chávez et al., 2009]. These studies confirmed the synergistic effects of electroporation (EP) and iontophoresis (IP) on the *in vivo* percutaneous absorption of human insulin in rats [Escobar-Chávez et al., 2009].

5.1.7. Photosensitizers

Selectivity of photodynamic therapy can be improved with localized photosensitizer delivery, but topical administration is restricted by poor diffusion across the *s.c*. The researchers used the electric pulses to increase transdermal transport of D-aminolevulinic acid (ALA), a protoporphyrin IX (PpIX)-precursor for the photodynamic therapy of superficial skin cancer and cutaneous metastases of internal malignancies. A two-fold enhancement of PpIX production with electroporative delivery was observed compared to passive delivery. The application of iontophoresis also increased the ALA permeation by approximately 15-fold [Escobar-Chávez et al., 2009].

5.1.8. Folic acid antagonists

The topical administration of methotrexate (MTX) for the treatment of psoriasis and neoplastic diseases is restricted by the poor diffusion of MTX across the *s.c*. Some studies showed that electroporation is an efficient method to increase the transdermal transport of MTX. Furthermore, electroporation of MTX with an anion lipid enhancer under a mild hyperthermic environment provided a significant transdermal delivery within a short time [Escobar-Chávez et al., 2009].

5.2. Vaccine delivery

Electroporation-based immunization (especially, EP-mediated DNA vaccine) has been effective in a number of species including mice, rats, rabbits, non-human primates, pigs and sheep.

5.2.1. DNA vaccine

DNA immunization has known as an efficient strategy for vaccination [Bolhassani and Rafati, 2009]. The main disadvantage of plasmid DNA vaccines is their poor immunogenicity when administered as an unformulated intramuscular injection [Anderson and Schneider, 2007]. A number of approaches for enhancing the potency of DNA vaccines have developed over the past few years such as: **a)** Optimization of DNA constructs; **b)** Development of new DNA manufacturing processes and formulations; **c)** Augmentation of immune responses with novel encoded molecular adjuvants; and **d)** Improvement of *in vivo* DNA delivery strategies including electroporation [Sardesai and Weiner, 2011].

Among them, EP-mediated delivery has generated considerable attention and appeared to have a great impact in vaccine immunogenicity and efficacy by increasing antigen delivery up to a 1000 fold versus naked DNA delivery alone [van Drunen Littel-van den Hurk and Hannaman, 2010; Sardesai and Weiner, 2011]. In many cases, the immune responses and protection rates observed following DNA administration via EP were comparable or superior to other vaccine strategies including viral vectors and live/attenuated/inactivated virus vaccines [Sardesai and Weiner, 2011, Daemi et al., 2012; Hosseinzadeh et al., 2013]. An electroporation driven plasmid DNA vaccination strategy was studied in animal models for treatment of prostate cancer. This phPSA plasmid electroporation vaccine strategy could

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effectively activate tumor specific immune responses. Optimization of the approach indicated that a four-dose regimen provided highest tumor protection. Furthermore, the four-dose regimen showed optimal and further tumor protection using co-administration of synthetic oligo CpG. Thus, the *in vivo* EP-mediated vaccination has potential to be use as a neo-adjuvant or adjuvant therapy in cancer treatment [Ahmad et al., 2010]. The effect of electroporation on DNA vaccine potency and gene delivery was studied using skin as a target tissue in larger animal species such as pig, macaque and sheep. In a macaque model, the higher cellular and humoral responses were observed to an HIV DNA vaccine harboring IL-12 gene, with electroporation compared to intradermal DNA injection alone [Hirao et al., 2008]. Furthermore, the safety and lack of integration after immunization with a high dose of a multigene HIV-1 vaccine was studied using a combination of the delivery methods jet-injection and intradermal electroporation. The data showed that plasmids persist in the skin at the site of injection for at least four months after immunization [Brave, et al., 2010]. The researchers demonstrated that mice and guinea pigs vaccinated with single-and multi-gene DNA via EP and then with recombinant gp120 protein (i.e., the synthetic DNA prime-protein boost protocol) induced significantly higher antibody binding titers [Muthumani et al., 2013]. Recently, Minicircle DNA (a new form of DNA containing only gene expression cassette but lacking backbone of bacterial plasmid DNA) is a powerful candidate of gene delivery improving the levels and the duration of transgene expression *in vivo*. A novel vaccine delivery system, including the combined *in vivo* EP and the minicircle DNA carrying codon-optimized HIV-1 gag gene was prepared to evaluate the immunogenicity of this system. The use of EP delivery further increased minicircle-based gag gene expression led to the augmentation of humoral and cellular immune responses. Increased immunogenicity of EP-assisted minicircle-gag may benefit from increasing local antigen expression, up-regulating inflammatory genes and recruiting immune cells [Wang et al., 2014]. In sheep, the significantly higher antibody responses to plasmid-encoded HBsAg were observed after IM delivery followed by electroporation in comparison with conventional IM or ID injection. Importantly, these antibody responses were sustained for 25 weeks after vaccination [van Drunen Littel-van den Hurk et al., 2008]. Moreover, various reports have illustrated that cytokine adjuvants have significant effects on modulating the immune responses to DNA vaccination. Indeed, the co-delivery of plasmid encoded cytokines is able to quantitatively and qualitatively modulate the immune responses in a large animal following *in vivo* electroporation of a DNA vaccine [Yen and Scheerlinck, 2007]. Although, intra-tumor delivery does not generally result in detectable serum transgene expression, intramuscular electroporation does result in serum expression. However, intratumor delivery is more successful than intramuscular delivery in eradicating primary tumors and in generating systemic immunity. For instance, a number of studies have demonstrated long-term, complete tumor regression, using delivery of plasmids encoding IL-12 or IFN-γ as a single agent in melanoma and squamous cell carcinoma (SCC) [Heller and Heller, 2006]. Complete regression after IL-12 gene therapy in combination with herpes simplex virus (HSV) thymidine kinase, bleomycin, or recombinant bacillus Calmette-Guérin (rBCG) has been observed in several experimental models. Electrically mediated bleomycin delivery combined with IL-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF) gene therapy also induced long-term complete regression in a small percentage of mice with

melanomas. Furthermore, complete responses have been observed in a fibrosarcoma model after delivery of a plasmid encoding GM-CSF and B7.1 [Heller and Heller, 2006]. One of the main challenges for efficient electroporation in larger animals is to ensure correct match between the electrical field and the injected DNA. Intramuscular injection of plasmid DNA followed by electrical stimulation (electroporation) is an efficient method for achieving therapeutic levels of encoded proteins or eliciting efficient immune responses in smaller animals such as mice and rats [Tjelle et al., 2006]. Application of short electrical pulses can be used to enhance gene delivery and DNA vaccination in large animals led to improved cellular and humoral immune responses. In addition, lowering the electrical field will therefore be important for reducing electroporation-induced pain. Increasing the number of electrodes and/or injection volume, could enhance the transfection efficiency of the conventional electroporation devices [Tjelle et al., 2006]. It will be interesting to electroporate different plasmids that were mixed together, plasmids mixed with proteins, or mixed proteins to understand the immune response intensity. It was reported that there is no interference with two different DNA vaccines, implying that it is possible to co-administrate vaccines directed against different pathogens at one time [Yuan, 2008; Yuan, 2008]. Induction of a humoral response against amyloid-β peptide may be beneficial for Alzheimer's disease (AD) patients. The potency of an AD DNA epitope vaccine (DepVac) delivered intramuscularly by EP and intradermally by gene gun (GG) was evaluated for treatment and prevention of AD. The results indicated that both delivery methods are effective at promoting potent antibodies specific for Aβ [Davtyan et al., 2012].

Gene delivery into solid tumors after direct injection of formulated or naked DNA preparations is generally low due to a large number of delivery barriers e.g., tumor complexity. Tumor electroporation significantly enhanced DNA delivery into solid tumors. Electroporation of *luciferase* DNA into mouse and human tumors produced 10-to 1200-fold increases in luciferase expression compared to tumors injected with *luciferase* DNA alone [Anwer, 2008]. 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 increase. The transfection efficiency of DNA electroporation was compared with that of non-electroporation methods including, liposome-DNA complexes and integrin-liposome-DNA complexes in different tumors [Anwer, 2008]. 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 non-electroporated methods were effective only in some tumors [Anwer, 2008]. Moreover, electroporation enhancement of luciferase transfection was up to 16-fold in mouse skin and up to 83-fold in pig skin, as compared to that in non-electroporated groups. In another study, the delivery and anticancer efficacy of MBD2 antisense DNA in electroporated tumors were comparable to the *adenovirus*-treated groups [Anwer, 2008].

Intracellular targeting of tumor antigens through its linkage to immunostimulatory molecules such as calreticulin (CRT) can improve antigen processing and presentation through the MHC

class I pathway and increase cytotoxic CD8+T cell production. However, even with these strategies, the efficacy of such immunotherapeutic strategies is dependent on the identification of an effective route and method of DNA administration [Best et al., 2009]. Intramuscular administration of HPV DNA vaccines followed by electroporation increased the number of antigen-loaded dendritic cells resulting in the enhancement of gene expression. In a comparison study of the HPV DNA vaccine administered by different methods, electroporation has been shown to elicit the highest number of E7-specific cytotoxic CD8+T cells and greatest antitumor immune response compared to intramuscular injection and intradermal gene gun delivery [Best et al., 2009; Monie et al., 2010]. Generally, electroporation can be considered as a promising method for delivery of HPV DNA vaccines in human clinical trials [Best et al., 2009]. For instance, electroporation has been successfully used to administer several HPV DNA vaccines to mice as well as rhesus macaques, which has prompted its use in an ongoing Phase I clinical trial of VGX-3100, a vaccine that includes plasmids targeting E6 and E7 proteins of both HPV subtypes 16 and 18, for treatment of patients with CIN 2 or 3 [Monie et al., 2010].

Regarding to *in vivo* EP is predominantly carried out intramuscularly, currently, skin EP is used as an attractive and less invasive option that is able to induce robust adaptive immune responses. To date, studies of DNA EP in skin have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ-producing T cells, and protective efficacy to infection. Plasmid DNA vaccination using skin electroporation (EP) is a promising method able to elicit robust humoral and CD8+T-cell immune responses while limiting invasiveness of delivery [Daemi et al., 2012]. It was shown that subcutaneous administration of HPV16 E7 DNA linked to C-terminal fragment of gp96 followed by electroporation can significantly enhance the potency of DNA vaccines [Daemi et al., 2012, Bolhassani et al., 2011; Bolhassani et al., 2009].

5.2.2. Peptide/ protein vaccine

Larger molecules, including heparin, polylysine, antisense polynucleotides, lactalbumin, and IgG, have been delivered by transdermal electroporation with proper enhancers. The transport of calcium-regulating hormones was found to be increased by applying electroporation and iontophoresis. Anionic lipid formulation has shown significant synergistic effect with electroporation on delivering insulin *in vitro* and *in vivo* and has the potential to lower the voltage threshold to a small level [Hui, 2008]. The reports showed an enhanced transport of human luteinizing hormone releasing hormone through heat-stripped human epidermis by electroporation. Furthermore, the presence of an ionic surfactant such as sodium dodecyl sulfate (SDS) reduced the electroporation threshold and significantly improved the transdermal transport of molecules by electroporation. Indeed, saturated anionic lipids tend to be preferentially retained in the epidermis during electroporation and result in disrupting the lamellar structure of the *sc* lipids, leading to prolonged lifetime of electropores. Using this method, the transport of both charged and neutral macromolecules was enhanced [Hui, 2008]. Recently, peptides and mini-gene vaccines are of particular interest since several epitopes of tumor-associated antigens have been employed as therapeutic and prophylactic cancer vaccines. Although, small molecular size antigens may be delivered into and through the skin by

diffusion or by iontophoresis methods, but, higher molecular weight antigens (>1 kDa), such as peptides, DNA, carbohydrates, as well as vaccine adjuvants need to deliver using an efficient rout of administration. Needle-free non-adjuvant skin immunization by electroporation has been reported [Hui, 2008]. For example, delivering the antigenic peptide MYR to mice by electroporation resulted in mucosal immunity and specific lymph node cell proliferation. Also, the others indicated that antigen-specific CTL response to the peptide vaccine delivered by needle-free electroporation/electroosmosis was equivalent to that delivered by intradermal injection with Freund's Complete Adjuvant. In this experiment, the Kb-binding OVA peptide SIINFEKL was used as an example to induce the peptide-specific cytotoxic T lymphocyte (CTL) response in mice [Hui, 2008; Escobar-Chávez et al., 2009].

Protein-based vaccines have emerged as a potentially promising approach for the generation of antigen-specific immune responses. However, due to their low immunogenicity, there is a need for novel approaches to enhance protein-based vaccine potency. One approach to enhance protein-based vaccine potency is the use of toll-like receptor ligands, such as CpG oligonucleotides, to activate the antigen-specific T cell immune responses [Kang et al., 2011]. Another approach involves employing a method capable of improving the intramuscularly delivery of protein-based vaccine led to the slow release of the protein. The studies showed that intramuscular injection of protein (OVA)-based vaccines in conjunction with CpG followed by electroporation can significantly enhance the antigen-specific CD8+T cell immune responses and antitumor effects in vaccinated mice. Similar results were observed using the HPV-16 E7 protein-based vaccination system [Kang et al., 2011].

5.2.3. RNA-based vaccines

RNA-based vaccines represent an interesting immunization modality, but suffer from poor stability and a lack of efficient and clinically feasible delivery technologies. A study evaluated the immunogenic potential of naked *in vitro* transcribed Semliki Forest virus replicon RNA (RREP) delivered intradermally in combination with electroporation [Johansson et al., 2012]. Replicon-immunized mice showed a strong cellular and humoral response, compared to mice immunized with regular mRNA. RREP-elicited induction of interferon-γ secreting CD8+T cells and antibody responses were significantly increased by electroporation. The immune response during the contraction phase was further increased by a booster immunization, and the proportion of effector memory cells increased significantly. These results demonstrated that naked RREP delivered via intradermal electroporation can constitute an immunogenic, safe and attractive alternative immunization strategy to DNA-based vaccines [Johansson et al., 2012].

5.2.4. DC-based vaccine

Designing effective strategies to load human dendritic cells (DCs) with tumor antigens is a challenging approach for DC-based tumor vaccines. In a study, a cytoplasmic expression system based on mRNA electroporation to efficiently introduce tumor antigens into DCs was described. Preliminary experiments in K562 cells revealed that mRNA electroporation compared to plasmid DNA electroporation showed improved transfection efficiency and

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induced a strikingly lower cell toxicity. Next, mRNA electroporation was used for non-viral transfection of different types of human DCs, including monocyte-derived DCs (Mo-DCs), CD341 progenitor-derived DCs (34-DCs) and Langerhans cells (34-LCs). High-level transgene expression by mRNA electroporation was obtained in more than 50% of all DC types [Van Tendeloo et al., 2001]. In addition, mRNA-electroporated DCs retained their phenotype and maturational potential. Strikingly, a non-specific stimulation of CTL was observed when DCs were transfected with plasmid DNA. The data clearly demonstrated that Mo-DCs electroporated with mRNA efficiently present functional antigenic peptides to cytotoxic T cells. Therefore, electroporation of mRNA-encoding tumor antigens was a powerful technique to charge human dendritic cells with tumor antigens and could serve applications in future DC-based tumor vaccines [Van Tendeloo et al., 2001].

5.3. Gene therapy

Much intensive research has gone into the development of safe and efficient methods for the delivery of therapeutic genes [Tamura and Sakata, 2003]. Recently, an improved electroporation protocol was established by optimizing the electroporation parameters including plasmid concentration, voltage and pulse duration, to deliver DNA into dental follicle cells to study the roles of candidate genes in regulating tooth eruption [Yao et al., 2009]. Using this approach, highly efficient gene transfer has already been achieved in muscle and liver as well as in tumors [Tamura and Sakata, 2003]. 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. Intramuscular injection of *erythropoietin* plasmid in mouse leg produced systemic levels of erythropoietin that were 100-fold higher than those from *erythropoietin* plasmid alone. Electroporation of IL-5 plasmid DNA into mouse tibialis muscle produced 20 ng IL-5/mL while the non-electroporated delivery generated only 0.2 ng IL-5/mL in the blood. Electroporation of mouse muscle with IL-12 plasmid produced 1500 pg of IL-12 per injected muscle and 170 pg IL-12/ mL in the blood. The huge improvement in muscle delivery (up to 10,000-fold over naked DNA) compared with other non-viral gene delivery systems (10-fold over naked DNA) opens new opportunities for muscle-based gene therapy [Anwer, 2008].

5.3.1. DNA delivery

Numerous studies on gene transfer have been published in a wide variety of tissues from animal models [Gehl, 2008]. Most of the studies investigated the treatment of protein deficiencies and cancers using cytokines. DNA formulations were designed to minimize tissue damage or enhance expression at weaker electric pulses. These formulations were prepared with the addition of transfection reagents, 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 [Anwer, 2008]. In addition, several DNA formulations have been described for *in vivo* gene electroporation. DNA electroporation in saline were 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 [Anwer, 2008]. Naked DNA in saline is the most commonly used formulation for *in vivo* gene electroporation. In skeletal muscle, electroporation enhancement of *luciferase* gene transfer was 10,000-fold over non-electroporated control. The enhancement of luciferase activity was observed in both small and large animal species. Histochemical analysis of *b-galactosidase* plasmid electroporated muscle showed a larger transfection area per muscle and a higher plasmid copy number per muscle cell when compared with non-electroporated muscle. Muscle electroporation with *FGF*1 plasmid also indicated significantly larger transfection area in electroporated muscle as compared to non-electroporated muscle [Anwer, 2008]. Also, electroporation enhanced intra-arterial administration of a transgenic construct in rats resulted in expression in mesengial cells [Stokman et al., 2010]. A report demonstrated the feasibility of electroporating genes into intact nerve to modify Schwann cell gene expression [Aspalter et al., 2009]. Gene therapy may represent a promising alternative strategy for cardiac muscle regeneration. *In vivo* electroporation with an optimized protocol was also a safe and effective tool for non-viral gene delivery to the beating heart [Ayuni et al., 2010]. This method was used to examine whether introduction and expression of PPARγ gene could differentiate skeletal muscle satellite cells to adipocytes *in vivo* [Bonamassa and Liu, 2010]. The studies indicated that the cricket (Gryllus bimaculatus) is a hemimetabolous insect that is emerging as a model organism for the study of neural and molecular mechanisms of behavioral traits. However, research strategies have been limited by a lack of genetic manipulation techniques that target the nervous system of the cricket. The development of a new method for efficient gene delivery into cricket brains was studied using *in vivo* electroporation. Plasmid DNA harboring an enhanced green fluorescent protein (EGFP) gene was injected into adult cricket brains, followed by electroporation at a sufficient voltage. Expression of EGFP was observed within the brain tissue [Matsumoto et al., 2013]. Gene therapies for cancer utilizing *in vivo* electroporation have been proved effective in a number of experimental murine tumor models. The therapeutic genes delivered in those cases were diverse including cytokine genes (IL-12) and cytotoxic genes (TRAIL), making a wide range of therapeutic strategies [Tamura and Sakata, 2003]. Generally, cancer gene therapy has been studied using *in vivo* electroporation including suicide genes (e.g., combination of HSV-TK and prodrug GCV: TK-GCV), apoptosis inducing genes (e.g., TRAIL), immuno-stimulatory genes (e.g., IFN-gamma, IL-12 and IL-18) and anti-angiogenic genes (e.g., Endostatin) [Tamura and Sakata, 2003].

5.3.2. Protein delivery

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 [Tamura and Sakata, 2003].

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5.3.3. SiRNA delivery

There are increasing interests in physical methods for delivery of siRNA [Oh and Park, 2009]. Among physical methods, electroporation has been frequently studied to stimulate the cellular and *in vivo* localized delivery of siRNA by electric pulses [Oh and Park, 2009]. An electroporation method was established to involve a constant voltage and ''plate and fork'' type electrodes and use it for *in vivo* delivery of siRNA. The electric current correlated to the microvascular density and vascular endothelial growth factor (VEGF) expression and exhibited a threshold that assures efficient delivery. VEGF siRNA electroporation suppressed the growth of tumors exhibiting high VEGF expression to less than 10% of the control level, but it had no effect on low VEGF-expressing tumors. Notably, a long interval (20 days) of electroporation was enough to obtain a satisfactory effect. Systemically injected siRNA could also be delivered into tumors by this method [Valero et al., 2008]. In atopic dermatitis mouse model, the intradermal delivery of cyclooxygenase specific siRNA into the skin by electroporation resulted in the silencing of the target gene in the skin, and reduced the scratching behavior of mice [Oh and Park, 2009]. The delivery of tumor necrosis factor α-specific siRNA via electroporation was shown to inhibit inflammation in mice with collagen-induced arthritis. Moreover, the *in vivo* silencing of target genes by electrically mediated siRNA delivery was reported in mice bearing solid tumors [Oh and Park, 2009]. Some studies reported the successful use of electroporation of siRNA delivery to renal tissue. In rats, injection of siRNA into the renal artery followed by electroporation led to predominant knockdown of the target protein in the glomeruli [Stokman et al., 2010]. A number of studies have demonstrated the feasibility of targeted delivery of oligonucleotides, small interfering RNA (siRNA), plasmid DNA, and viral vectors to the corneal cells *in vivo*, specifically stromal keratocytes and corneal epithelial cells, via intrastromal injection, iontophoresis, electroporation, and gene gun. The combination of iontophoresis and electroporation was found to be effective in delivering siRNA but not plasmid DNA into the corneal epithelium [Hao et al., 2010]. Altogether, there is great interest in platforms which efficiently deliver RNA molecules such as messenger RNA and small interfering RNA (siRNA) to mammalian tissues [Broderick et al., 2012]. However, the *in vivo* delivery of RNA enhanced by EP has not been extensively characterized.

6. Efficient agents involved in electroporation

The type of a nucleic acid and the type of the transfected cell generally affect the efficiency of electroporation [Stroh et al., 2010]. Skeletal muscle is a preferable target tissue for a number of reasons including long-term secretion of therapeutic proteins for systemic distribution and promotion of strong humoral and cellular immune responses post-vaccination. Numerous factors impact plasmid uptake and expression after intramuscular injection followed by EP. Briefly, they include: species, targeted muscle, age, plasmid formulation, plasmid concentration and dose, pulse pattern, electric field intensity (current, voltage and resistance), pulse length, lag time, electrode configuration and orientation. These improvements in the conditions of EP can increase the efficacy of plasmid transfer and lower the total amount of plasmid

and DNA vaccines required to generate targeted levels of biologically active proteins or antibodies [Draghia-Akli et al., 2005].

7. Advantages, disadvantages and solutions

The electroporation can be applied equally to all cell types and at all stages of the cell cycle [Escobar-Chávez et al., 2009]. Collateral damage by electroporation can be serious, compared with some other physical methods. When electroporation field is applied through the skin using surface plate electrodes, the major potential drop develops across the skin instead of across the targeted subcutaneous tissues. Skin edema is a common consequence. Most electroporation protocols aim to permeate only the plasma membranes. Electroporation of the nucleus requires a further step, using higher threshold voltage and shorter pulse length (nucleoporation) [Hui, 2008]. Although the principle of electroporation is applicable to all cell types, its efficiency depends on the electrical properties of the cells. Smaller cells require higher field to permeate. This is an important consideration for *ex vivo* gene delivery especially to hematopoietic cells. Cells with less conductive contents (such as adipocytes) are less susceptible. The thresholds for different cells in a heterogeneous tissue would thus vary [Hui, 2008].

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. In addition, 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 [Anwer, 2008, Lai et al., 2008]. Liposomal formulations were transfectionally superior to naked DNA in B16 melanoma, P22 carcinoma, and SaF sarcoma but not in T24 human bladder carcinoma or MC2 mammary carcinoma. 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 [Anwer, 2008]. 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. The magnitude of liposome enhancement was dependent on the degree of lipid saturation but independent of polar head group [Anwer, 2008]. 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. Antibodies and other molecular entities that recognize specific cell surface receptors have been conjugated to delivery vehicles to achieve high cell specificity during electroporation. 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 non-targeted systems [Anwer, 2008]. 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.

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 and skeletal muscles [Anwer, 2008]. Hyaluronidase treatment prior to electroporation in skeletal muscle produced a substantial increase both in levels and extent of gene transfer in skeletal muscle. Hyaluronidase treatment enhanced transfection efficiency at low electric pulses without significantly damaging the muscle structure or function. This tissue-protective effect of hyaluronidase has been observed in ischemic myocardium and tissue edema. These results demonstrated that hyaluronidase treatment is a useful approach to improve electrogene transfer in higher species where rigid interstitium is a major limitation to plasmid delivery [Anwer, 2008]. Application of electromigration field (3 V for 30 s) has been shown to enhance the uptake of DNA-modified gold nanoparticles during cell electroporation. Gold nanoparticles devoid of DNA coating were not taken up by cells during electroporation. Formulations that can enhance DNA binding to cell surface *in vivo* may also enhance electroporation efficiency at weak electric pulses [Anwer, 2008].

Currently used methods to introduce foreign DNA into mammalian cells are based on bulk procedures in which large cell numbers are simultaneously transfected, electroporated or virally infected. All of these methods have a number of specific limitations, such as limited control over the amount of DNA uptake, the intracellular half-life and fate of the introduced DNA, and site of genomic integration [Valero et al., 2008]. These limitations represent a serious drawback in situations where genetically modified stem cells have to be produced for therapeutic application, including gene therapy and regenerative medicine, especially when these cells are hard to isolate in large enough numbers. Recently, microfluidic devices have shown great benefits for studying a variety of cell processes. Of particular importance is the use of such devices for electroporation, enabling high efficiency transfer of a variety of macromolecules into cells [Valero et al., 2008]. However, further optimization of DNA vaccine delivery is needed for this vaccine modality to ultimately be efficacious in humans [Hallengärd et al., 2012]. The "plate and fork" electrodes were used for the transfer of a plasmid vector for erythropoietin expression into rat skin and were compared with needle-type and disc-type electrodes. Therefore, the electroporation conditions for significant efficacy vary with the molecule to be delivered [Takei et al., 2008].

In general, there are differences in effective variables between a drug and a gene for delivery by electroporation. High field strength and a short pulse length gave good results, at least with some of the drugs investigated (e.g., bleomycin), whereas electroporation for genes benefits from a combination of a low electric field and a long pulse length [Takei et al., 2008].

Membrane poration methods, such as electroporation and sonoporation, are an attractive alternative in some applications. Indeed, electroporation has demonstrated its efficacy in a number of DNA and RNA delivery applications for previously difficult-to-transfect primary

cells. However, this method can cause cell death and has been shown to damage sensitive materials such as quantum dots, which aggregate due to exposure to electric fields. There have also been limited reports of successful protein delivery by this mechanism [Sharei et al., 2013].

Electroporation is a technique that increases the permeability of cell membranes by changing the transmembrane potential and subsequently disrupting the lipid bilayer integrity to allow transportation of molecules across the cell membrane *via* nano-size pores. This process when used in a *reversible* fashion has been used in medicine and research for drug or macromolecule delivery into cells [Guo et al., 2010; Heish et al., 2011; Phillips et al., 2012; Li et al., 2012; Niessen et al., 2013; Narayanan et al., 2013]. Irreversible electroporation (IRE) is a new minimally invasive tumor ablation technique which induces irreversible disruption of cell membrane integrity by changing the transmembrane potential resulting in cell death. Irreversible electroporation is currently undergoing clinical investigation as local tumor therapy for malignant liver and lung lesions [Niessen et al., 2013].

The use of *irreversible* electroporation (IRE) has been introduced by Rubinsky's group as a method to induce irreversible disruption of cell membrane integrity subsequently causing cell death. IE can effectively create tissue death in micro-to millisecond ranges of treatment time compared to conventional ablation techniques, which require at least 30 minutes to hours. Additionally, it is possible to treat a considerably larger lesion with shorter treatment times than available with current techniques [Guo et al., 2010; Heish et al., 2011; Phillips et al., 2012; Li et al., 2012; Niessen et al., 2013; Narayanan et al., 2013]. A higher electric voltage leading to a larger potential gradient to create irreversible electroporation has been studied using *in vitro* and *in vivo* studies. Irreversible electroporation is technically simple to use and suitable for minimally invasive surgery [Rubinsky, 2007]. Irreversible electroporation is an innovative local-regional therapy that involves delivery of intense electrical pulses to induce nano-scale cell membrane defects for tissue ablation. The purpose of this study was to investigate the feasibility of using irreversible electroporation as a liver-directed ablation technique for the treatment of hepatocellular carcinoma (HCC) in the N1-S1 rodent model. The findings suggested that IRE was effective for targeted ablation of liver tumors in the N1-S1 rodent model; IRE may offer a promising new approach for liver-directed treatment of HCC [Guo et al., 2010]. The advantage of this technique is that it is drug-free and is targeted [Heish et al., 2011]. In an experiment, it was shown that direct IRE completely ablated the tumor cells in osteosarcoma-bearing rats. A significant increase in peripheral lymphocytes, especially CD3+and CD4+cells, as well as an increased ratio of CD4+/CD8+were detectable after the IE application. As compared to the surgical resection group, the IRE group exhibited a stronger cellular immune response. These findings indicated that IRE could not only locally destroy the tumor but also change the status of cellular immunity in osteosarcoma-bearing rats [Li et al., 2012]. Some reports indicate that this novel procedure can be used for abdominal cancer treatment while minimising collateral damage to adjacent tissues because of the unique ability of the ablation method to target the cell membrane [Phillips et al., 2012]. Irreversible electroporation (IRE) is a new ablative technology that uses high-voltage, low-energy DC current to create nanopores in the cell membrane, disrupting the homeostasis mechanism and inducing

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cell death by initiating apoptosis in patients with Hepatocellular carcinoma (HCC) [Narayanan et al., 2013].

8. Clinical trials

One of the methods that improve DNA penetration of the cell is electroporation [Bolhassani and Rafati, 2011]. EP itself works as an adjuvant to enhance the necessary "danger signals" that become detectable by the immune system. The tissue damage caused by the application of EP causes inflammation and recruits DCs, macrophages and lymphocytes to the injection site inducing significant immune responses, including antibody and T-cell responses [Fioretti et al., 2014; Saade and Petrovsky, 2012]. *In vivo* use of electroporation is done by injecting naked DNA followed by electric pulses from electrodes that are located *in situ* in the target tissues. Successful use of electroporation was observed in transfecting muscles, brain, skin, liver, and tumors. Since every tissue is specific and has its own characteristics, there are no generally accepted optimal conditions of electroporation that are suitable for effective transfection. These are dependent both on the amplitude and duration of the electric pulses and on the amount and concentration of DNA [Bolhassani and Rafati, 2011]. Up to now, several clinical trials have been planned using the electroporation with DNA vaccines for cancer therapy such as: a) Intratumoral IL-12 DNA plasmid (pDNA) [ID: NCT00323206, phase I clinical trials in patients with malignant melanoma, Heller and Heller, 2006; Daud et al., 2008]; 2) Intratumoral VCL-IM01 (encoding IL-2) [ID: NCT00223899; phase I clinical trials in patients with metastatic melanoma]; 3) Xenogeneic tyrosinase DNA vaccine [ID: NCT00471133, phase I clinical trials in patients with melanoma]; 4) VGX-3100 [ID: NCT00685412, phase I clinical trials for HPV infections], and 5) IM injection prostate-specific membrane antigen (PSMA)/ pDOM fusion gene [ID: UK-112, phase I/II clinical trials for prostate cancer, Low et al., 2009; Fioretti et al., 2010] [Saade and Petrovsky, 2012; Bolhassani and Rafati, 2011]. Furthermore, Hepatitis C virus DNA vaccine showed acceptable safety when delivered by Inovio Biomedical's electroporation delivery system in phase I/II clinical study at Karolinska University Hospital. ChronVac-C is a therapeutic DNA vaccine being given to individuals already infected with hepatitis C virus with the aim to clear the infection by boosting a cell-mediated immune response against the virus. This vaccination was among the first infectious disease DNA vaccine to be delivered in humans using electroporation based DNA delivery [Bolhassani and Rafati, 2011]. Recent patents have been focused on the use of genetic immunomodulators, such as “universal” T helper epitopes derived from tetanus toxin, *E. coli* heat labile enterotoxin and vegetable proteins, as well as cytokines, chemokines or co-stimulatory molecules such as IL-6, IL-15, IL-21 to amplify immunity against cancer. Electroporation-based DNA delivery technology dramatically enhances cellular uptake of DNA vaccines [Fioretti et al., 2014]. Preliminary data from an ongoing clinical trial showed electroporation enhanced the frequency and the magnitude of the anti-HIV-1 T-cell response [Saade and Petrovsky, 2012].

Hemorrhagic fever with renal syndrome (HFRS) is endemic in Asia, Europe and Scandinavia, and is caused by infection with the hantaviruses Hantaan (HTNV), Seoul (SEOV), Puumala (PUUV), or Dobrava (DOBV) viruses. The candidate DNA vaccines were developed for HFRS

expressing Gn and Gc genes of HTNV or PUUV and evaluated in a Phase I study. Three groups of nine subjects each were vaccinated on days 0, 28 and 56 with the DNA vaccines for HTNV, PUUV, or mixture of both vaccines using the Ichor Medical Systems TriGrid™ Intramuscular Delivery System (TDS-IM) [Hooper et al., 2012]. All vaccinations consisted of a total dose of 2.0 mg DNA in an injected volume of 1 mL saline. For the combined vaccine, the mixture contained equal amounts (1.0 mg) of each DNA vaccine. There were no study-related serious adverse events (SAEs). Neutralizing antibody responses were detected in 5/9 and 7/9 of individuals who completed all three vaccinations with the HTNV or PUUV DNA vaccines, respectively. In the combined vaccine group, 7/9 of the volunteers receiving all three vaccinations developed neutralizing antibodies to PUUV. The three strongest responders to the PUUV vaccine also had strong neutralizing antibody responses to HTNV. These results demonstrated that the HTNV and PUUV DNA vaccines delivered by electroporation separately or as a mixture are safe. In addition, both vaccines were immunogenic, although when mixed together, more subjects responded to the PUUV than to the HTNV DNA vaccine [Hooper et al., 2012]. Figue 2 shows several important EP-mediated DNA vaccines used in clinical trials.

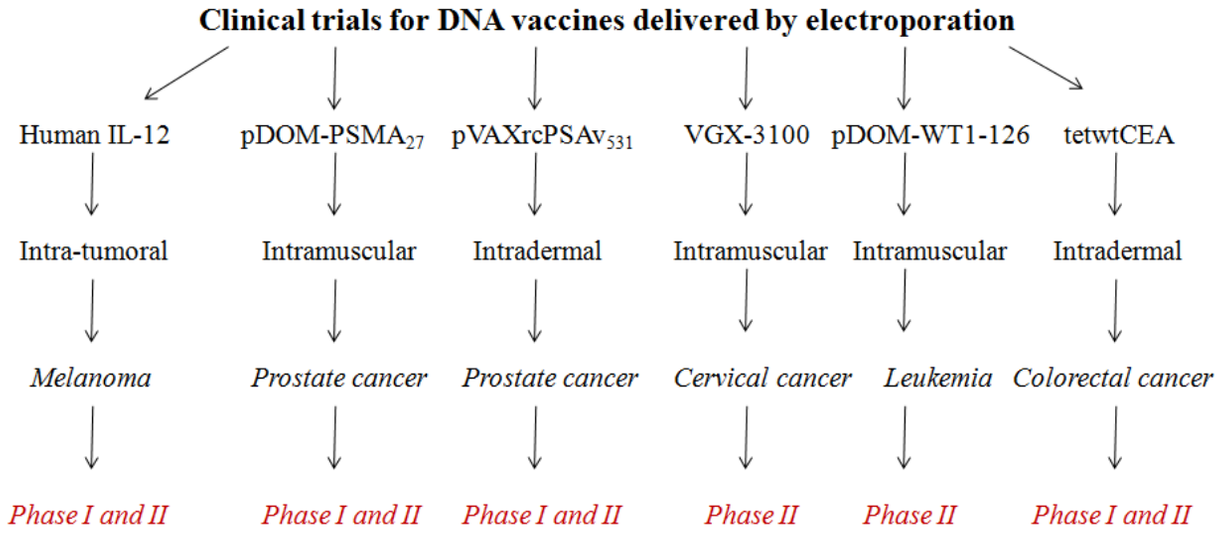

Figure 2. EP-mediated DNA vaccines used in cancer clinical trials

Drug delivery by electroporation has been in experimental use for cancer treatment since 1991 as shown in 11 studies of electrochemotherapy (ECT) of malignant cutaneous or subcutaneous lesions, e.g., metastases from melanoma, breast or head-and neck cancer. The treatment was well tolerated and could be performed on an out-patient basis [Gothelf et al., 2003]. At the Institut Gustave-Roussy, France, the fist clinical trial of ECT with bleomycin in eight patients with recurrent or progressive head and neck squamous cell carcinoma was published in 1991. After that, several clinical studies have been performed in different tumors. Clinical trials have been performed in the treatment of basal cell carcinoma, head and neck cancer (squamous cell carcinoma, adenocarcinoma and adenoid cystic carcinoma), adenocarcinoma of the breast, and malignant melanoma. In addition, a case report was published in which metastatic lesions from a bladder cancer have been successfully treated [Gothelf et al., 2003].

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9. Conclusions

Electroporation is a widely recognized method of gene delivery into mammalian tissues. It is a highly efficient method, with delivery efficiency better than many non-viral vectors. The preclinical development of electroporation in vivo is focused on tissues that are easily accessible to electroporation and can resist to electric pulsation. The standard DNA formulation for electroporation is DNA in physiological saline. Under optimal conditions, DNA electroporation in saline yields a 10- to 10,000-fold enhancement in gene delivery efficiency over non-electroporated controls. This enormous increase in transfection activity, however, accompanies significant tissue damage and local inflammation, which might not be a disadvantage, if the target is cancer. However, for applications in which expression from normal tissues is desired, tissue damage and inflammatory response are not favorable to therapeutic objectives and, therefore, must be minimized. Several formulation strategies have been designed to enhance electroporation efficiency and minimize toxicity. Hopeful results have been obtained with some approaches, which must be further developed into clinically viable formulations for non-cancer applications. Some progresses, such as HIV vaccine, West Nile virus vaccine have been made; however, these also propose some questions: What are the differences for best parameters when conduct electroporation on various muscle cells with distinct morphology and membrane properties that are also different among species? How to reduce the pain during electroporation? How long can gene expression be maintained after electrotransfer? Many experiments showed that electroporation is a safe and potent method, thus electroporation-mediated anticancer gene therapy represents a great therapeutic potential. The further improvements of electrodes including shape or arrangement of electrodes and electric conditions, by which more efficient and reliable gene transfer is achieved, are important especially in clinical trials. Furthermore, electroporation is an efficient method for enhancing transdermal drug delivery in vitro and in vivo and expands the range of compounds delivered transdermally. The combined use of electroporation with other physical enhancers such as iontophoresis is likely to yield useful and interesting data, to further explore electroporation as an efficient method of transdermal drug delivery. The technique of electroporation to enhance anticancer drug (such as bleomycin) delivery to tumor cells, so-called as electrochemotherapy, is already being applied clinically against head and neck cancers with little or no side effects. In summary, electroporation is one of the physicochemical methods for gene and drug delivery. It is superior in some aspects but also has several drawbacks. Pulse protocol and electrode design need to be optimized to reduce the main side effects e.g., muscle contraction.

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