

Different physical delivery systems: An important approach for delivery of biological molecules *in vivo*

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ABSTRACT

Delivery of exogenous materials such as nucleic acids, peptides, proteins, and drugs into cells is an important strategy in modern cellular and molecular biology. Recently, the development of gene carriers for efficient gene transfer into cells has attracted a great attention. Furthermore, lack of effective drug delivery is one of the major problems of cancer chemotherapy. Many physical methods have been studied to enhance the efficiency of gene and drug delivery. These strategies help to cross the materials from membranes including needle injection, photodynamic therapy, jet injection, gene gun, electroporation, hydrodynamic injection, laser, magnetofection, and tattooing. The physical systems improve the transfer of genes from extracellular to nucleus by creating transient membrane pores using physical forces including local or rapid systemic injection, particle impact, electric pulse, ultrasound, and laser irradiation. The recent optimization techniques of transdermal patches could improve the transdermal drug delivery through the skin. Among different physical carriers, electroporation and gene gun are the most potent methods for gene transfection and drug delivery *in vivo*. However, the researchers have focused on enhancing their potency with the structural modifications. Regarding to numerous barriers for biomolecules delivery in cells, this review is concentrated on description and optimization of different physical delivery systems for gene or drug transfer across membrane.

Keywords: Non-viral delivery system; Physical carriers; Drug delivery; Gene transfer

INTRODUCTION

Development of an efficient delivery system is important for basic research of therapeutic materials such as peptides, proteins, DNA, siRNA and drugs. The ideal vectors for gene delivery would have at least four properties including: a) specificity for the targeted cells; b) resistance to metabolic degradation and/or attack by the immune system; c) safety; and d) an ability to express efficiently [1]. Among different carriers, non-viral vectors have benefits due to simple use, easy production in large-scale, and lack of specific immune response to vector [2]. Non-viral gene delivery system depends on chemical (*e.g.*, cationic polymer and lipid) or physical (*e.g.*, electroporation and gene gun) transfer of the genetic material and thus relies on cellular transport systems for uptake and expression in the host cell [2, 3]. Physical

approaches such as needle injection, electroporation, gene gun, and ultrasound have been efficiently used to transfer gene into cells *in vitro* and *in vivo*. These methods utilize a physical force that permeates the cell membrane and facilitates intracellular gene transfer [4]. Figure. 1 shows some major physical delivery systems. However, physical techniques for gene delivery into cells, with and without adjuvants, should be significantly optimized for different goals such as local injection, systemic administration, and organ specific delivery [2]. Recently, a variety of strategies have been developed to enhance DNA vaccine efficacy [5]. The researchers explained different delivery systems that make the epidermal and dermal layers of the skin accessible for vaccine administration. Depending on the device, the desired vaccine can be used either as a liquid formulation or as solid vaccine particles ⁶. For

instance, tattooing can be applied to deliver liquid vaccine formulations into the dermal layer of the skin; and/or microneedles are made of vaccine-coated solid or biodegradable microneedles. Delivery devices using liquid vaccine formulations are useful, because established vaccine formulations can be used without the need for re-formulation. However, approaches that deliver vaccines in a solid form may also prove to be promising such as the ballistic approaches [6]. Regarding to the studies, different physical approaches need to be optimized for efficient drug and gene delivery in targeted cells especially tumor cells *e.g.*, injection sites (skin, muscle, systemic-mediated delivery ...), device properties (pulse, electric field ...), cell type and animal model. In this review, we will briefly discuss and evaluate the efficiency of main physical methods for gene and drug delivery *in vitro* and *in vivo*.

Targeted gene/ drug delivery

Physical methods of gene and/or drug transfer must induce reversible alterations in the plasma membrane to allow the direct movement of the therapeutic materials into cells and facilitate access to the inside of the cell [7]. However, cell specificity is an important subject for the selection of the approaches. Specificity can be obtained in two principal ways: a) physical targeting which relies on the spatial focus of physical methods including ultrasound, magnetofection, gene gun, electroporation, hypothermia, hydrodynamic delivery, and photodynamic therapy. Herein, a directed mechanical force, magnetic or electrical field, temperature or light can be used for specific localization; b) special biological and pharmacological characteristics of the target; *e.g.*, tumors [7].

Needle injection

The simplest physical method for gene delivery is by direct injection of DNA through a needle-carrying syringe into tissue. The major application of this strategy is DNA vaccination. The studies showed the expression of transgene after a plasmid DNA injection to skin, cardiac muscle, liver, and solid tumor. The disadvantage of this procedure is low gene delivery, as well as limitation of the transfected cells to the needle injection site. Some efforts have been made to achieve a high level of transgene expression by

optimizing plasmid construct [8]. Microfabricated needles were developed using several materials such as glass, silicon, and metal. They can be used to deliver agents through the skin, into a blood vessel, or into a cell, and can extract fluids by reversing the flow direction. In addition, these systems can be integrated with microelectrodes to determine the cellular response to agents in real time [9]. Delivery to the cornea by microinjection into different corneal tissue was described in several studies [10]. Microinjection to single cells has been extensively used for transduction-challenged cells, production of transgenic animal, and *in vitro* fertilization to transfer DNA, RNA interferences (RNAi), sperms, proteins, peptides and drugs (Figure.1). The advantages of microinjection include the accuracy of delivery dosage and timing, high efficiency of transduction as well as low cytotoxicity. Compared to electroporation, microinjection requires low protein amounts. This is efficient for transferring recombinant proteins and synthesized peptides with high cost. In contrast with chemical transfection (*e.g.*, liposomes) and viral infection, microinjection of the cDNAs into cells is less stressful; therefore, it decreases the cell death observed by these systems. Furthermore, more than one construct can be injected into different groups of cells in one culture. Like any other technique, microinjection has certain limitations: As a single cell technique, microinjection is rarely used to transduce large number of cells and produce over-expressed proteins for *in vitro* experiments including detection and purification [11]. In addition, direct microinjection of proteins is most powerful to deliver cytosolic and nuclear proteins, but not membrane-located proteins, neurotransmitter receptors, or ion channels, although this transfer can be achieved by injecting the cDNAs of these membrane proteins. In dividing cell lines, the injected substances are constantly diluted by cell division; therefore, long incubation after injection of dividing cells is not recommended. Microinjection has been extensively used for primary cultured human neurons, since these cells are difficult to transfect. The researchers have successfully delivered proteins (*e.g.*, recombinant caspases, Bax, Hsp70, neutralizing

antibodies), peptides (e.g., β -amyloid), and various cDNA constructs into human neuronal cytosol [11].

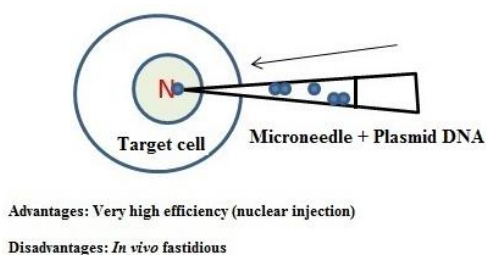


Fig.1. Microinjection: Microinjection strategies utilize microneedles to deliver DNA directly to cell nuclei

Photodynamic therapy

Photodynamic therapy (PDT) contains injection of a tumor photosensitizing agent, which may need metabolic synthesis of a prodrug, followed by activation of the agent using light in a specific wavelength. The preclinical and clinical studies have suggested photodynamic therapy as a useful treatment for some cancers [12-14]. The use of PDT on the CIN treatment has reported since 1990s as a non-destructive treatment [15]. This strategy has been applied to a variety of tissues that are accessible to light, including the retina, bronchial tree, skin, and the gastrointestinal tract [13-16]. Photodynamic therapy is another way to improve nucleic acid delivery to a specific light-exposed site. In this process, amphiphilic photosensitizers co-localize with the nucleic acid in endocytic cellular vesicles and are subsequently activated by light, resulting in the destruction of endocytic membrane structures and releasing co-endocytosed nucleic acids into the cell cytosol [7]. The studies showed that PDT is a common method for treatment of skin cancer. For example, protoporphyrin IX, converted enzymatically from the prodrug 5-aminolevulinic acid (ALA), is used as a photosensitizer in PDT for cancer. Enhancement of ALA penetration in skin can be achieved by other physical methods or addition of chemical penetration enhancers. Some researchers used lipophilic ALA derivatives to develop the transdermal delivery of ALA [17]. The use of nanoparticles such as metallic-, ceramic-, inorganic oxide-, and biodegradable polymer-

based nanomaterials, as carriers of photosensitizers, is a potent approach because they can improve all the requirements for an ideal PDT [18]. Photochemical internalization (PCI) strategy was also utilized to increase the release of endocytosed macromolecules to the cytosol. This method is based on the activation of endocytosed photosensitizers by light to stimulate the release of endocytic vesicle contents before they are transferred to the lysosome. The studies indicated that PCI facilitates endosomal escape of siRNA targeting EGFR [19].

Jet injection

The jet injection method uses mechanical compression to force fluid (DNA in solution) through a small cavity, producing a high pressure flow that can penetrate in different tissues. Previously, a combination of plasmids encoding a marker gene and tumor necrosis factor gene (TNF- α) were jet-injected into subcutaneous Lewis lung carcinoma tumors. After five injections, high gene expression was observed within tumor tissues with a penetration depth of 5-10 mm, deeper than that in gene gun delivery [7, 20]. The capacity of various intradermal (id) DNA immunization delivered by needle or Biojector, with or without electroporation (EP) was studied to stimulate immune responses in mice model. The study showed that a high dose of DNA injected by needle plus EP or Biojector alone (100 μ g) stimulated the levels of immune responses similar to those in lower dose of DNA (10 μ g). The combination of Biojector-injection with EP showed significantly stronger immune responses after immunization with the high dose DNA as compared to the lower dose. Indeed, the combination of both methods could overcome dose limitations observed for DNA encoded antigens. In addition, the humoral responses were significantly enhanced by Biojector, while cellular responses were particularly increased by EP [21]. In general, Jet-injection has become an applicable technology among other non-viral delivery systems, such as electroporation or particle bombardment. This method delivers a drug or vaccine intradermally, subcutaneously, or intramuscularly via high pressure produced by either a carbon-dioxide-filled or nitrogen-filled cartridge or a spring. Furthermore, the

researchers showed that the repeated jet-injections into one target tissue can be performed easily [22, 23]. Up to now, vaccines administered via jet injection could induce immune responses against typhoid, cholera, BCG, tetanus-diphtheria for adults, whole cell diphtheria-tetanus-pertussis (DTP), measles, meningococcal A and C, smallpox, yellow fever, hepatitis A, hepatitis B, influenza, plague, polio, and tetanus [24].

Ballistic DNA injection (particle bombardment)

This method of gene delivery was first developed for gene transfer into plants in 1987. After that, it has been modified to transfer genes into mammalian cells both *in vitro* and *in vivo*. The principle of this method is to transport DNA-coated gold particles against cells and force intracellular DNA transfer. The accelerating force for DNA containing particles can be a high-voltage electronic or helium pressure discharge. Ballistic DNA injection has been successfully used to transfer genes into various cell lines [8]. *In vivo* applications have extensively focused on the skin, muscle, liver or other organs that can be exposed after surgical procedure. Unfortunately, genes delivered by this method are expressed transiently, and there is considerable cell damage occurring at the centre of the discharge site. Among different methods, the gene gun-based delivery is more appropriate for gene delivery to skin due to the shallow penetration of DNA. This method has been used in vaccination against the influenza virus and in gene therapy for treatment of ovarian cancer [8].

Gene Gun

Particle bombardment through a gene gun is an effective and rapid tool to deliver exogenous materials into living tissue [25, 26]. DNA is deposited on the surface of gold (/ tungsten, or silver) particles, which are then accelerated by pressurized helium gas and expelled onto cells or a tissue [4] (Figure.2). Recently, it has been known as a method of labeling neurons in a variety of preparations [25, 26]. This technology led to efficient *in vitro* transfection, even in the cells which are difficult to transfect [27]. Diolistics is ideal for loading cells with optical nano-sensors linked to fluorescent probes. Further development of nano-sensors, combined with the gene gun to deliver them, will enable

the monitoring and quantification of an even greater range of cell metabolites in different cells [26]. Furthermore, this system is expected to have important applications as an effective tool for DNA-based vaccines. Further improvements including chemical modification of the surface of gold particles allow higher capacity and better stability for DNA coating, and also enhancement of the expelling force for exact control of DNA deposition into cells in various tissues [4]. Gas pressure, particle size, and the amount of the particles and DNA loading are critical factors that determine penetration efficiency to the tissues [27, 28]. The low pressure gene gun can deliver plasmid DNA at lower pressure [29]. Gene gun-based gene transfer has been broadly tested for intramuscular, intradermal and intratumor genetic immunization. It was demonstrated that this approach is able to produce more immune response with lower doses comparing to needle injection in large animal models and in clinical trials [28]. Furthermore, intradermal DNA vaccination via a gene gun represents one of the most efficient methods for delivering DNA directly into dendritic cells (DCs) [30]. When DNA vaccines are administered by gene gun, the majority of the plasmid is taken up by keratinocytes as well as antigen processing cells (APCs). It appears that DCs (Langerhans cells and bone marrow-derived DCs) transfected by gene gun play a key role in the stimulation of immunity. These transfected cells rapidly migrate to the draining lymph nodes where cross-presentation of antigen to CD8⁺ T cells occurs. These migratory transfected DCs alone are responsible for immunologic memory. The role of the non-migratory transfected keratinocytes expressing antigen is minimal [31]. However, some data showed that genetic immunization of the skin with gene gun is capable of eliciting immune responses independent of Langerhans cells (LCs) [32]. Gene gun avoids several problems associated with other non-viral methods. The fact that DNA is delivered directly to the cytoplasm without going through the endocytosis pathway results in less degradation (*i.e.*, cell receptor independent) [33, 34]. This also prevents the non-specific immunostimulatory effect observed with

endocytosis dependent methods such as liposomes or polymers [34]. Comparing to other non-viral gene delivery methods, gene expression after particle bombardment is typically short term, but can be longer depending on the tissue (e.g., greater than 60 days in muscle). This is not a limiting factor because gene gun are typically applied in situations where transient to near-term expression is necessary [34]. The use of this technology was reported to transfect tumors with granulocyte/macrophage-colony-stimulating factor (GM-CSF) plasmid DNA. This technique was simple to induce high levels of transgene expression [35]. A comparison of *in vivo* transfection efficiency by intramuscular injection and gene gun bombardment of plasmid DNA encoding EGFP gene revealed that the gene gun is more efficient in producing fluorescent fibres. In addition, administration of plasmid DNA showed that the gene gun generated high immune responses (~100-fold) compared to intramuscular injection per unit transfected DNA [36]. A major reason for the difference in efficiency between the two methods is likely a difference in the plasmid delivery mechanism. Intramuscular injection delivers the plasmid to the extracellular space and the plasmid may subsequently be taken up by cells using an unknown mechanism. It has been estimated that around 99% of the extracellularly delivered plasmid DNA is broken down by DNases and/or removed by liquid flow. In contrast, the gene gun technology delivers the plasmid DNA directly into the cytoplasm, thus bypassing the extracellular DNA degradation [36]. In the large animal model (e.g., pig), the combination of gene gun delivery and a plasmid DNA that targets APCs by expressing a CTLA4-ovalbumin (OVA) fusion antigen, resulted in the enhancement of ovalbumin specific antibody responses [37]. Gene gun has the potential to be a safe and effective method of gene delivery; however, this technology has some significant drawbacks. Current designs of gene guns only penetrate superficially into the target tissue, limiting their use to dermal tissues. To overcome this limitation, a modified gene gun was developed to use higher pressures resulting in deeper penetration into the target tissue. This new gene gun was used to deliver a modified

human papilloma virus [HPV] E7 gene as a tumor rejection antigen to mice. All of the treated animals developed protective immunity against HPV-positive tumors suggesting the potency of gene gun transfection in cells [34]. *In vivo* gene transfer into the beating heart is an attractive strategy for cardiovascular diseases [38, 39]. Gene gun-mediated transfer of the Epstein-Barr virus (EBV)-based episomal vector into rat heart resulted in long time gene expression [38]. RNA interference (RNAi) technology has developed into a powerful method for specific post-transcriptional gene silencing *in vitro* and may be applied to diminish the expression of immunosuppressive factors *in vivo* as a complement to traditional DNA vaccines. One approach leading to gene silencing is by transfection of DNA encoding short hairpin RNA (shRNA), targeting the gene of interest. The studies indicated that co-administration of DNA encoding shRNA targeting luciferase significantly reduced luciferase expression in mice intradermally injected luciferase DNA [40]. Furthermore, mice vaccinated with E7-expressing DNA co-administered with DNA encoding shRNA targeting FasL significantly enhanced the cellular immune responses as well as potent therapeutic antitumor effects against E7-expressing tumors. Thus, intradermal administration of DNA encoding shRNA by gene gun represented a plausible approach to silence genes *in vivo* and a potentially useful tool to enhance DNA vaccine potency [40]. Gene gun bombardment has been used for successful mRNA transfection in several cell types and tissues. When mRNA encoding α -1 antitrypsin was delivered in mice, a strong antibody response was observed, indicating the possibility of using this technique as a vaccination strategy. In addition, this approach was used to deliver mRNA encoding human epidermal growth factor (hEGF) suggesting increased wound healing. On the other hand, mRNA encoding GFP delivered by a gene gun resulted in expression up to one week as compared to 30 days when the plasmid DNA analogue was delivered [41]. For many years, it was generally accepted that mRNA is unstable to be efficiently used for gene therapy purposes. But, several research groups proved the

feasibility of mRNA-mediated transfection with appropriate efficiency and duration of protein expression, suggesting its major advantages over the use of plasmid DNA. For example, mRNA does not need to cross the nuclear barrier to exert its biological activity as well as the lack of CpG motifs, which reduces its immunogenicity [41]. Ballistic transfer with the gene gun can be used to transfer cDNA coated gold particles to the epithelium. The results indicated that the use of gene gun to transduce the endothelium was shown to severely damage [10]. In addition, gene gun immunization has been used for humans in a number of clinical trials (e.g., a malaria DNA vaccine) [32]. These trials reported that particle-mediated DNA immunization of humans is safe, well tolerated and results in strong immune responses to the plasmid-encoded antigens. In the case of hepatitis DNA vaccines, gene gun immunization not only induced protective levels of antibody, but also immunized individuals who had not responses to the recombinant protein vaccine (licensed vaccine) [42].

Electroporation

The use of an electric field to alter the cell permeability was known since 1960s. However, the first *in vitro* and *in vivo* attempts to use electroporation (EP) in gene transfer were demonstrated in 1982 and 1991, respectively [28].

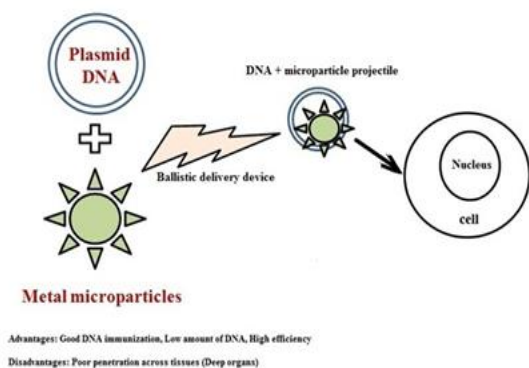


Fig.2. Ballistic gene delivery (Gene gun): Plasmid DNA is mixed with gold or tungsten particles ranging in size from nanometers to microns

Electroporation is a method that uses short pulses with high voltage to carry DNA across

the cell membrane. This shock is thought to cause temporary formation of pores in the cell membrane, allowing DNA molecules to pass through [43, 44]. Electroporation is generally efficient and works across a broad range of cell types. However, a high rate of cell death following electroporation has limited its use, including clinical applications [10]. Electroporation method employs high field strength, square-wave electric pulses to allow the penetration of therapeutics molecules across cell membrane (Figure.3). While this method does not involve any biochemical agents and is thus considered a safe choice, electroporation can cause severe cell damage and induce immunogenic reactions. Electroporation was successfully used to deliver IL-10 gene to mice corneas. *In vivo* gene transfer to the endothelium and stromal keratocytes has been reported in rats. However, high voltage (> 500V) can be hazardous [10]. Electroporation can induce long-lasting gene expression and can be used in various tissues [2]. However, it is necessary to optimize factors such as dose of DNA, electrode shape and number, electrical field strength and duration. The optimized conditions were applied for expression of hepatitis B surface antigen, erythropoietin and IL-12 in cells. For example, potent immune responses against hepatitis B surface antigen and HIV gag protein were obtained by electroporation of muscle after intramuscular injection of plasmid DNA. Comparing with local injection of DNA to the liver, systemic injection has the advantage of delivering genes more equally to the liver [2]. The *in vivo* electroporation was evaluated for the enhancement of immune responses induced by DNA plasmids encoding the pre-erythrocytic *Plasmodium yoelii* antigens PyCSP and PyHEP17 administered intramuscularly and intradermally to mice. Immunization with 5 µg of DNA via EP was equivalent to 50 µg of DNA via conventional needle, thus reducing by 10-fold the required dose to produce the same immune effects [45]. 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 [46]. 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-based vaccines [47-50]. Electroporation is a versatile method that has been broadly examined in many types of tissues *in vivo*. The level of reporter gene expression was 2 to 3 times higher than that with plasmid DNA alone. Long-term expression over one year was observed after a single electroporation treatment. The amount of DNA and how well the injected plasmid DNA distributes within the treated tissue prior to electroporation appear to have an important effect on transfection efficacy [4]. It was also reported that age of the recipient animals affects the transfection efficiency in mice. Treatment of muscle with hyaluronidase prior to injection of plasmid DNA to relax the surrounding extracellular matrix significantly enhanced transfection, likely due to improved distribution of plasmid DNA in the tissue. Alternatively, plasmid DNA administration through the portal vein followed by localized electroporation on rat liver resulted in widespread transfection in hepatocytes in the treated lobe but not in the surrounding lobes. This result suggests the possibility that cells can be transferred with plasmid DNA via blood circulation and then electroporation is used to a selected area to achieve localized gene transfer. A short time interval between DNA injection and electroporation is critical to minimize DNA degradation by extracellular nucleases [4, 28]. Several major drawbacks exist for *in vivo* use of electroporation including a) it has a limited effective range of ~1 cm between the electrodes, which makes it difficult to transfect cells in a large area of tissues; b) a surgical procedure is required to

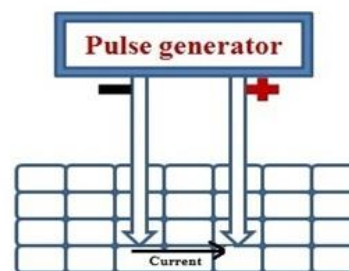
place the electrodes deep into the internal organs; c) high voltage used to tissues can result in irreversible tissue damage as a result of thermal heating. Ca^{2+} influx due to disruption of cell membranes may induce tissue damage because of Ca^{2+} -mediated protease activation. The possibility that the high voltage applied to cells could affect the stability of genomic DNA, is an additional safety concern. However, some of these concerns may be solved by optimizing the design of electrodes, their spatial arrangement, the field strength, and the duration and frequency of electric pulses [4]. *In vivo* electroporation technique is generally safe, efficient, and can produce good reproducibility compared to other non-viral delivery systems. When parameters are optimized, this method can generate transfection efficiency equal to that achieved by viral vectors [28]. Electroporation efficiently works on cells that are suspended in solution, but also works on cells in solid tissue where electrodes can be applied [8, 41]. *In vivo* electroporation induces a low level of inflammation at the injection site, facilitating DNA uptake by parenchyma cells and antigen-presenting cells [8]. 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, [51, 52]; b) Intratumoral VCL-IM01 (encoding IL-2) [ID: NCT00223899; phase I clinical trials in patients with metastatic melanoma]; c) Xenogeneic tyrosinase DNA vaccine [ID: NCT00471133, phase I clinical trials in patients with melanoma]; d) VGX-3100 [ID: NCT00685412, phase I clinical trials for HPV infections], and e) IM injection prostate-specific membrane antigen (PSMA)/ pDOM fusion gene [ID: UK-112, phase I/II clinical trials for prostate cancer [44, 53-56]. The limitation of the current

procedure is that the number of cells transfected is relatively small and that surgery is required to reach internal organs [8]. Recent patents have been focused on the use of genetic immunomodulators, such as 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 increase immune responses against cancer. Electroporation-based DNA delivery technology could efficiently enhance cellular uptake of these DNA vaccines [57]. Electroporation with mRNA has been performed in dendritic cells, because of their possible use in vaccination strategies. Loading DCs with mRNA encoding different tumor associated antigens (TAAs) proved to be efficient, transfecting up to 50-75% of treated cells. Electroporation of DCs with mRNA is a safe and relatively easy method. It has already been tested in clinical trials (e.g. transfection of mRNA encoding prostate specific antigen (PSA)) [41]. Altogether, this technology has been successfully used to enhance the skin permeability of molecules with differing lipophilicity and size (i.e., small molecules, proteins, peptides and oligonucleotides) including biopharmaceuticals with molecular weights greater than 7 kDa [58]. Drug delivery by electroporation has been used for cancer treatment since 1991 as shown in eleven studies of electrochemotherapy (ECT) of malignant cutaneous or subcutaneous lesions, e.g., metastases from melanoma, breast or head-and neck cancer. The treatments were well tolerated in patients [59].

Ultrasound

This system can directly deliver plasmid DNA and siRNA into cytosol without endocytosis pathway. Therefore, these genes are able to escape from degradation in lysosome and result in enhancing the efficiency of gene

expression.



Advantages: Good DNA immunization, Low amount of DNA, High efficiency
Disadvantages: Toxicity, Invasiveness (electrodes)

Fig.3. Electroporation: Electroporation strategies use a current across cells or tissues to make cell membranes more permeable to exogenous DNA

In addition, it is expected that ultrasound-mediated gene delivery using nano/microbubbles would be a system to establish non-invasive and tissue specific gene expression because ultrasound can transdermally expose to target tissues and organs [60]. Ultrasound can increase the permeability of cell membrane to macromolecules such as plasmid DNA (Figure.4). Indeed, enhancement of gene expression was observed by irradiating ultrasonic wave to the tissue after injection of DNA. Since the use of ultrasound is flexible and safe, its use in gene delivery has a great advantage in clinical use. Recently, it was reported that combination of microbubble with ultrasound could further increase the gene expression level. Microbubbles, or ultrasound contrast agents, decrease the threshold for cavitation by ultrasound energy. In most cases, perfluoropropane filled albumin microbubbles or Optison were used as microbubbles. It was modified with plasmid DNA before injection, followed by irradiation of ultrasound. At present, this technique is used for gene delivery to vascular cells, muscle and fetal mouse [2, 61]. The transfection efficiency of this system is determined by several factors, including the frequency, the output strength of the ultrasound, the duration of ultrasound treatment, and the amount of plasmid DNA. The efficiency can be enhanced by conditions that make membranes more fluidic. Unlike electroporation, which moves DNA along the electric field, ultrasound creates membrane pores and facilitates

intracellular gene transfer through passive diffusion of DNA across the membrane pores. Consequently, the size and local concentration of plasmid DNA play an important role in determining the transfection efficiency. Efforts to reduce DNA size for gene transfer through proper formulation could result in further improvement. Interestingly, significant enhancement has been reported *in vitro* and *in vivo* when complexes of DNA and cationic lipids have been used. So far, the major problem for ultrasound-facilitated gene delivery is low gene delivery efficiency [4]. The effects of ultrasound not only enhanced the vaccine delivery but also stimulated activation of APCs in the epidermis of animals [43]. The results suggested that high-intensity ultrasound could be useful for the treatment of small cancerous tumors such as low-grade prostate carcinoma [62]. The researchers indicated that complexation with the PEG-introduced cationized dextran combined with US irradiation is a promising way to target the plasmid DNA to the tumor for gene expression. Fluorescent microscopic studies revealed that the localization of plasmid DNA and the gene expression were observed in the tumor tissue injected with the PEG introduced cationized dextran-plasmid DNA complex plus the subsequent US irradiation [63]. The use of ultrasound for the delivery of drugs through the skin is commonly known as sonophoresis or phonophoresis [64]. The first indication that ultrasound might enhance the transdermal penetration of drugs was demonstrated in 1954. Sonoporation, is a technique that uses ultrasound waves to create plasma membrane defects by acoustic cavitation. With each ultrasonic cycle, a fraction of the energy of the propagating wave is absorbed by the tissue resulting in local heating which affects the structure of cell membranes. Tissue absorption to ultrasound waves depends on tissue type and ultrasound frequency and intensity. A major improvement in ultrasound-based gene transfer was the combination of ultrasound irradiation with microbubbles. The size of microbubbles (~ 1-6 μm), is important for the efficient transfection and for not being eliminated by the reticular endothelial system (RES) [28]. Modification of microbubbles through lipid or polymer coating resulted in

enhanced transfection efficiency. However, enhancement of fluidity of the cell membrane by feeding cells with long-chain unsaturated fatty acids, which facilitates its flexibility and minimizes cellular resistance to sonication, was also suggested to improve the effect of sonoporation. The major advantage for sonoporation is its safety, noninvasiveness, and being able to reach internal organs without surgical procedure. Recently, ultrasound has been shown to enhance the permeability of blood-brain barrier. Interestingly, targeted gene delivery can be achieved through sonoporation using non-targeted microbubbles or through microbubbles equipped with site specific ligands, such as antibodies or biotin-streptavidin that helps in transferring of microbubbles to certain tissue or organ [28]. Sonoporation mediated gene delivery has been demonstrated in the cornea, brain, CNS, bone, peritoneal cavity, kidney, pancreas, liver, embryonic tissue, dental pulp, muscle and heart. More recent studies in mouse liver showed that inclusion of gas-filled microbubbles enhanced gene delivery efficiency. Similar results were also obtained in tumor, vascular tissue and skeletal muscles [8]. A combination of ultrasound therapy with topical drug therapy was done to achieve therapeutic drug concentrations at selected sites in the skin. In this technique, the drug was mixed with a coupling agent usually a gel but sometimes a cream or ointment was used which transfers ultrasonic energy from the device to the skin through this coupling agent. Application of low frequency ultrasound (20-100 KHZ) enhances skin permeability more effectively than high-frequency ultrasound (1-16 MHZ). The mechanism of transdermal skin permeation involved disruption of the stratum corneum lipids, thus allowing the drug to pass through the skin. A corresponding reduction in skin resistance was observed due to cavitation, microstreaming and heat generation [58]. Generally, ultrasound is very effective in permeabilizing the skin especially at low frequencies. This strategy has been shown to enhance the delivery of vaccines into skin. The studies indicated that the immune response generated by ultrasonically delivered vaccine was about 10-fold higher than that in SC injection per unit dose of the vaccine [24].

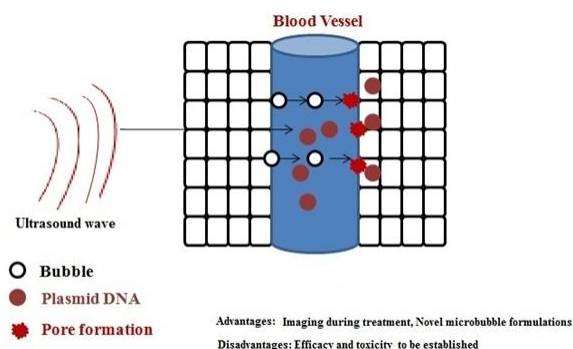


Fig.4. Sonoporation: Ultrasonic frequencies are used to induce the cavitation of microbubbles for creating pores in cells, which allows cells to be temporarily more permeable to plasmid DNA

Hydrodynamic injection (Hydroporation)

Hydrodynamic injection, a rapid injection of a large volume of naked DNA solution (5 mg plasmid DNA injected in 5-8 s in 1.6 ml saline solution for a 20 g mouse) via the tail vein, can induce potent gene transfer in internal organs, especially the liver. The naked plasmid DNA is taken up by receptor-mediated pathway in hepatocytes [2]. Certain DNA receptors have been found in various tissues; however, their function has not been elucidated. It has been proposed that the injected DNA solution accumulates mainly in the liver because of its flexible structure which can accommodate large volume of solution, and the hydrostatic pressure drives DNA into the liver cells before it is mixed with blood. Furthermore, breaking of the endothelial barrier by the pressure has been proposed as the major mechanism responsible for the highly efficient expression in the liver [2]. Hydrodynamics-based transfection (HBT) of hepatocytes has been reported to produce suitable transfection efficiency in mice⁶⁵. Hydrodynamic gene delivery is a simple method that introduces naked plasmid DNA into cells in highly perfused internal organs with an impressive efficiency. The gene delivery efficiency is determined by the anatomic structure of the organ, the injection volume, and the speed of injection. Electron microscopy shows the existence of transient membrane defects in hepatocytes shortly after the hydrodynamic treatment, which could be the mechanism for plasmid DNA to enter the hepatocytes. Approximately 30- 40% of the

hepatocytes are transfected by a single hydrodynamic injection of less than 50 μg of plasmid DNA. Various substances of different molecular weight and chemical structure including small dye molecules, proteins, oligonucleotides, small interfering RNA, and linear or circular DNA fragments as large as 175 kb have been delivered by this method. The non-specific nature of hydrodynamic delivery suggests that this method can be applied to intracellular delivery of any water-soluble compounds, small colloidal particles, or viral particles. Hydrodynamic delivery allows direct transfer of substances into cytoplasm without endocytosis [4]. This method has been used to express proteins of therapeutic value such as hemophilia factors, α -1 antitrypsin, cytokines, hepatic growth factors and erythropoietin in mouse and rat models. Depending on the plasmid construct and the regulatory elements driving expression of the transgene, the level of gene expression in some cases has exceeded the physiological level. The fact that a bacterial artificial chromosome containing an entire chromosomal transcription unit and replication origin (>150 kb) can be delivered successfully to the liver using this method, opens up many applications for gene therapy in liver-associated genetic diseases [4]. The real challenge for gene transfer by the hydrodynamic method is how to translate this simple and effective procedure to one that is applicable to humans. Rat liver can be transfected similarly through tail vein injection using an injection volume equivalent to 8-9% of body weight. If the same ratio is extrapolated to humans, one would have to inject up to 7.5 L of saline at a high rate, which is obviously many times over the maximal volume that a person can tolerate. However, successful liver transfection has been achieved using balloon catheter-based and occlusion-assisted infusion to specific lobes in rabbit and swine models, indicating that with modification, hydrodynamic gene delivery can become a clinically relevant procedure [4]. Hydrodynamic *i.v.* injection involves the rapid *i.v.* injection of siRNA in large volumes of physiological buffer to achieve effective localization of duplex siRNA mainly in the liver, although distribution to the kidney, lung, and pancreas has been reported. Effective gene silencing in the liver of

mice has been demonstrated using this delivery strategy with both unmodified and chemically modified siRNAs. Although the exact mechanisms of how siRNA is delivered to cells by hydrodynamic injection are unclear, it is possible that the high pressure causes membrane perturbations that facilitate siRNA uptake *in vivo* by some cell types, even though they all might show membrane disruption. However, this delivery method cannot be directly translated to clinical use due to both the large injection volumes and the cellular toxicities caused by the high pressure of the injection [66].

Laser-Assisted Method (Photoporation)

The photoporation method employs a single laser pulse as the physical force to generate transient pores on a cell membrane to allow DNA to enter. Gene delivery efficiency appears to be controlled by the size of the focal point and pulse frequency of the laser (Figure.5). The level of transgene expression was similar to that of electroporation. In recent years, several advances have been made to improve gene delivery efficiency, one of which involved the use of carbon black nanoparticles to generate photoacoustic force upon laser stimulation⁸. Applications of the femtosecond laser are becoming more accepted in corneal refractive surgery and transplantation, due to high precision and safety, compared to conventional laser. The femtosecond laser can be used to create a pocket where in the corneal surface to assist the delivery of therapeutic agents. It was used to deliver the vector to the stroma, therefore it is suggested to be used in chronic stromal herpetic keratitis conditions, if the latency of HSV-1 is in the stroma [10].

Magnetofection

Magnetofection uses a magnetic field to promote transfection. This method employs magnetic nanoparticles made of iron oxide and coated with cationic lipids or polymers to complex with DNA through electrostatic interaction. The magnetic particles are then concentrated on the target cells by the influence of an external magnetic field. Similar to the mechanism of non-viral vector-based gene delivery, the cellular uptake of DNA is accomplished by endocytosis and pinocytosis.

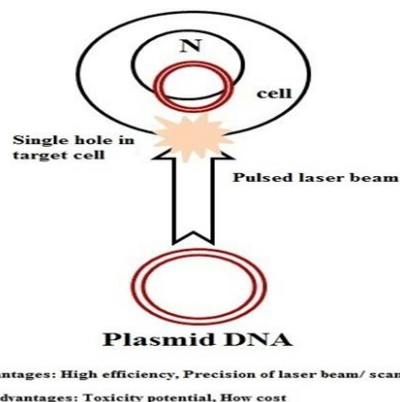


Fig.5. Laser Induced Pore Formation: Pulsed lasers have been shown to perforate cell membranes similar to microinjection strategies, but without the use of a needle.

It is postulated that DNA are released into the cytoplasm depending on the composition of the magnetic nanoparticles. Magnetofection has been successfully applied to a wide range of primary cells and cells that are hard to transfect using other non-viral methods. Recent work using a local injection of the nanoparticles into the gastrointestinal tract and the ear vasculature involve that this method for *in vitro* gene delivery may be applicable to *in vivo* gene delivery [8, 43]. More importantly, the high transduction efficiency observed *in vitro* was reproduced *in vivo* with magnetic field-guided local transfection in the gastrointestinal tract and in blood vessels. Magnetic targeting uses paramagnetic particles as drug carriers, conducts their accumulation in target tissues with local strong magnetic fields, and has been used with some success in the treatment of cancer patients [67]. Magnetic forces have been previously used to enhance delivery of anticancer drugs to tumors, as well as in the context of gene therapy. More recently, magnetofection has been used to transfect primary airway cells *in vitro*, as well as airway epithelium from porcine trachea, *ex vivo*. The proposed mechanism for magnetofection is an increase in the concentration of the vector on the cell surface, thereby increasing the contact time and subsequently gene transfer [68].

Tattooing

Tattooing is one of a number of DNA delivery methods which results in an efficient

gene expression in the epidermal and dermal layers of the skin. The tattoo procedure causes many minor mechanical injuries followed by hemorrhage, necrosis, inflammation and regeneration of the skin and thus non-specifically stimulates the immune system. DNA vaccines delivered by tattooing have been shown to induce higher specific humoral and cellular immune responses than intramuscularly injected DNA [69]. In a study, the comparison of HPV 16 L1 DNA immunization protocols using different routes of DNA injection (intradermal tattoo versus intramuscular injection) and molecular adjuvants (cardiotoxin pre-treatment or GM-CSF DNA codelivery) was done. Cardiotoxin pretreatment or GM-CSF DNA codelivery substantially enhanced the efficacy of DNA vaccine delivered intramuscularly by needle injection but had virtually no effect on the tattoo vaccination. The promoting effect of both adjuvants was more prominent after three rather than four immunizations. Tattooing elicited significantly higher L1-specific humoral and cellular immune responses than intramuscularly delivered DNA in combination with adjuvants. Indeed, the route of DNA delivery had a higher effect on the vaccination efficiency than the use of adjuvants (e.g., GM-CSF and cardiotoxin) [69]. The mechanisms involving DNA tattooing to generate better immune response include: a) better uptake of the DNA by non-antigen-presenting cells, b) better uptake of DNA by antigen-presenting cells, c) duration of expression or d) the induced traumata by the tattooing. The advantage of tattoo treatment is the low price of the tattoo device and a standardized method for the use. In particular, the local traumata induced by tattooing might not be considered acceptable in prophylactic vaccination; but, However, DNA vaccination via tattoo seems to be suitable if faster and stronger immune responses have to be achieved [69]. In a study, for determination of the effect of the tattooing process on DNA vaccine stability, the change of DNA topology was evaluated such as critical factors for antigen expression and immune response. It was found that the DNA tattooing had minor effect on DNA structure and activity. In addition, an adenoviral vector-based vaccine against respiratory syncytial virus, and a peptide vaccine

against human papillomavirus were administered by ID tattooing. In the case of the adenoviral vector vaccine, tattooing showed similar results to ID injection. Tattooing of the peptide vaccine with CpG motifs as an adjuvant showed better response than IM vaccination with adjuvant [24].

Transdermal drug delivery system (TDDS)

Transdermal drug delivery system is a novel drug delivery system and its aim to achieve a programmed delivery of the therapeutic products when applied on the skin for the optimal beneficial effects while avoiding the side effects of drugs [70, 71]. Drug selection criteria for transdermal patch contain: a) The dose of drug should be low i.e. < 20 mg/day; b) The drug should have short half life; c) The drug should have Molecular weight < 400 Daltons (high molecular weight fail to penetrate the *stratum corneum*); d) The drug should have partition coefficient between 1.0 and 4; e) Drug should be non-irritating and non-sensitizing to the skin; f) The drug should have low oral bioavailability; g) The drug should have low therapeutic index; j) The drug should have affinity for both lipophilic and hydrophilic phases, and k) The drug should have low melting point (less than 200°C) [72]. The effective transdermal drug delivery can be formulated by considering three factors as drug, skin, and the vehicles. So the factors affecting can be divided into classes as biological factors and physicochemical factors. Biological factors include skin condition, skin age, blood supply, regional skin site, skin metabolism and species differences. Physicochemical factors include skin hydration, temperature and pH, diffusion coefficient, drug concentration, partition coefficient, and molecular size and shape. Electrically-based enhancement techniques have already been studied for drug delivery such as a) Iontophoresis: In iontophoretic delivery devices, drug is placed on the skin under the active electrode, and a current (< 0.5mA) passed between the two electrodes effectively repelling drug away from the active electrode and into the skin; b) Ultrasound: The use of ultrasound with a suitable frequency significantly enhances the transdermal transport of drugs through skin system (phonophoresis or sonophoresis). It is a combination of ultrasound therapy with topical drug therapy to achieve therapeutic drug

concentrations at selected sites in the skin [72]; c) Photomechanical waves: The mechanism of photochemical wave was found to act by producing changes in the lacunar system which results in the formation of transient channels through the *stratum corneum* by permeabilization mechanism; d) Electroporation: In this method, aqueous pores are generated in

the lipid bilayers by the use of short electrical pulses of approx 100-1000 volt/cm. It may combine with Iontophoresis to enhance the permeation of peptide; e) Electro-osmosis: If a charged porous membrane is subjected to a voltage difference, a bulk fluid or volume flow, called electro-osmosis [70].

CONCLUSION

Although, various viral and non-viral delivery systems have been improved in the recent years, all of them have limitations in their clinical application; however, some delivery systems have been determined which can be efficient for gene delivery to specific cells or tissues. It seems that more efforts are needed for developing successful delivery systems. Altogether, key points in improving the current systems include: a) improving extracellular targeting and delivery, b) increasing intracellular delivery and long-time expression, and c) reducing toxicity and side effects on human body. So far, different non-viral approaches have been proposed for efficient drug and gene delivery, such as physical techniques and chemical methods. Physical delivery systems are one of the efficient non-viral methods including electroporation, micro-injection, gene gun, tattooing, laser and ultrasound. Physical methods of gene (and/or drug) transfer, need to combine two effects to deliver the therapeutic material into cells. The physical methods must induce

reversible alterations in the plasma membrane to allow the direct transfer of the molecules of interest into the cell cytosol. They must also bring the nucleic acids in contact with the permeabilized plasma membrane or facilitate access to the inside of the cell. These two effects can be achieved in one or more steps, depending on the methods used. Regarding to previous studies, among different physical approaches, electroporation and then gene gun has been used into clinical therapies, such as deliver drugs to tumors. Delivery of DNA vaccines using physical delivery systems especially 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. However, all these systems need to be optimized to reduce the main side effects for clinical trials

“The authors declare no conflict of interest”

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