Review Article

PHYSICAL METHODS IN GENE DELIVERY
Muhammad Ali Sheraz¹, Sofia Ahmed¹ and Iqbal Ahmad²

ABSTRACT
The types of gene delivery systems are not restricted to viral vectors but can also be the simple administration of the gene itself. The use of viral vectors for gene delivery has some risks regarding the safety of the patient whereas non-viral vectors are considered to be more safe but so far are less effective in vivo. Non-viral gene delivery system includes both physical and chemical approaches that are gaining popularity for a variety of gene-mediated therapies in humans. From the beginning of naked DNA induction directly into the cells, a number of other physical techniques have also been introduced. So far these methods have employed pressure, large volumes, electricity, ultrasound, laser, manganic force, etc. for the transfer and expression of gene to the target site and some of them are found to be quite efficient. This review highlights the major physical methods used for the delivery of genes in living organisms.

Keywords. Gene therapy, non-viral vectors, physical gene delivery systems, gene transfer, gene expression.

INTRODUCTION
Gene therapy is currently the most researched field and will be the therapeutic strategy for diseases in the near future. For an effective therapy, it is necessary that the genes should be delivered effectively with no harm to the patient¹. In order to achieve this, both viral and non-viral approaches have been investigated. Both types of approaches have some advantages as well as some shortcomings over one another. Viral vectors to some extent are unable to meet the needs of the pharmaceutical community as they are not reproducible in large-scale proportions in cost-effective ways. On the other hand, non-viral vectors are attractive to the pharmaceutical industry as alternatives to viral vectors because of compound stability and easy chemical modification. Furthermore, the low cost and consistent standard of production (compared to growth of viruses in bioreactors followed by purification), higher biosafety and high flexibility make these compounds very attractive²-⁴. The biosafety of non-viral vectors is higher due to less immunogenicity as compared to viruses and also, they will not recombine with wild-type viruses and will very rarely insert into the host genome (unless integration sequences are incorporated into the delivered plasmid). In spite of these advantages, non-viral vectors are generally considered less efficient in delivering DNA and expressing proteins as compared to their viral counterparts, particularly when used in vivo².

NON-VIRAL DELIVERY SYSTEMS FOR GENE THERAPY
A number of techniques have been employed for the delivery of genes. On the basis of those methodologies, gene delivery methods can be classified into physical, chemical and biological systems. Both physical and chemical delivery systems employ non-viral approaches while biological systems include the use of viral vectors⁵. Popularity of physical and chemical systems have also increased in the recent years due to the side effects of viral vectors. Gene transfer in a non-viral system must correctly be addressed as transfection whereas in biological system it should be referred to as transduction⁶.

PHYSICAL METHODS
A number of physical methods have been investigated for gene transfer. These methods facilitate the transfer of genes from extracellular to nucleus by using physical forces which are discussed as follows:
1. Naked DNA Transfer by Needle
The gene can be administered as a circular double-stranded DNA that is propagated in bacteria and than the therapeutic DNA is directly injected into the target cells without any additional help from either a chemical agent or a physical force\textsuperscript{7,8,9}. This is termed naked DNA and it refers to solutions of DNA in saline 10 or hyper\textsuperscript{11} / hypotonic\textsuperscript{12} solutions or other physiological solutions such as transferrin\textsuperscript{13}, water-immiscible solvents\textsuperscript{14,15} surfactants\textsuperscript{6}, non-ionic polymers\textsuperscript{17}, etc. Selection of appropriate solution is an important aspect for optimal DNA transfections\textsuperscript{18}. This technique was first applied in 1990 by Wolff et al. by intramuscularly injecting the naked DNA in skeletal muscles, which leads to gene expression\textsuperscript{19}. Afterwards, some other tissues like brain\textsuperscript{20-22}, liver\textsuperscript{23-26}, lungs\textsuperscript{27,28}, skin\textsuperscript{29,30}, etc. were also injected with the naked DNA, which also resulted in gene expression. Delivery of naked DNA to cells elicits minimal immune response as no anti-DNA antibodies are generated\textsuperscript{8}. Thus, this lack of immunogenicity makes it a good prospect for gene therapy. Therefore, gene transfer with naked DNA is attractive to many researchers because of its simplicity and lack of toxicity\textsuperscript{7,9}, but there are certain limitations with this approach such as:\n\begin{itemize}
  \item[i.] It requires large amounts of DNA.
  \item[ii.] The naked DNA is unprotected against nuclease degradation.
  \item[iii.] The DNA does not have target specificity.
  \item[iv.] It can be used only with certain tissues since no target ligands are attached to the DNA.
\end{itemize}

Due to these reasons the genes or DNA sequences are not appropriately transfected as naked molecules. In order to enhance the transfer efficiencies of naked DNA, some modifications in the methodologies have been made. These advancements have resulted in improved targeted delivery and gene expression of naked DNA such as inclusion of nuclease inhibitor. Nuclease inhibitors have been shown to enhance naked DNA mediated gene transfer in cultured cells\textsuperscript{31}, muscle\textsuperscript{32} and lungs\textsuperscript{33}. Similarly, transfer efficiencies are also increased by delivering naked DNA in combination with other physical methods (for e.g. gene gun, electroporation, sonoporation, etc.) which are discussed below.

2. Gene Transfer by Jet Injection
Jet injection is a needle-free delivery of the substance as compared to conventional needle injection\textsuperscript{34}. This method was first used for the delivery of insulin\textsuperscript{35}. Now this technique has been employed for the transfer of naked DNA to various tissues\textsuperscript{36}. A direct comparison between needle injection and various types of jet injector showed that the levels of gene expression by jet injection are 50-folds higher than conventional needle injection\textsuperscript{7,37}.

In jet injection, ultrafine DNA solution is loaded, required pressure is set and the injector is directly aimed at the target site or tissue and then triggered\textsuperscript{36}. The DNA solution are prepared in simple solutions of water or buffers etc. The force or pressure is developed either through spring-forced systems or by application of pressurized air or gas i.e. usually CO\textsubscript{2}\textsuperscript{7,36}. It is the result of high-speed pressure due to which DNA penetrates the skin and underlying tissues, thus resulting inefficient transfection of the gene\textsuperscript{38}. These strong forces have almost negligible alterations in DNA integrity\textsuperscript{39}. Depending on the type of jet injector, volumes of 3-10 mL are usually ejected at a time\textsuperscript{7,36}.

Some new injectors can be used for multiple ejections as they have the single filling capacity of up to 200 mL (e.g. Swiss-Injector, EMS Medical, Nyon, Switzerland)\textsuperscript{36}. It is reported that the models with low-volume injectors are more suitable as compared to those with high-volume injectors in carrying out multiple injection\textsuperscript{39-42}.

The jet injection gene transfer is well tolerated by the target tissues, and no serious side effects have been reported. However, localized pain, edema and bleeding at the injection site have been reported, particularly when old models of injectors were used\textsuperscript{7,43}.

3. Hydrodynamic Gene Transfer
It is defined as the gene delivery by the rapid infusion of large volumes of naked DNA into blood vessels\textsuperscript{7,9,10,44}. Hydrodynamic delivery allows direct
transfer of substances into cytoplasm without endocytosis. This method also employs high pressure for delivery of genes. Rapid and large amount are two key terms for hydrodynamic gene transfer. If solution is not transferred rapidly or if large volume is not administered, reduction in gene expression is reported. So far this method has proved very effective in rodents for in vivo gene transfer but due to high volumes of DNA solutions (~4-9 liters) it is not recommended for human use. Due to the non-specific nature of hydrodynamic delivery system, it can be applied to intracellular delivery of water-soluble compounds, small colloidal particles (molecular assembly), viral particles, etc.

4. Gene Gun Method

Gene gun delivery is also known as ballistic DNA transfer or bioballistics or DNA-coated particle bombardment method. It was first used in 1987 for transfer in plants. In this method, the coated DNA is shot or delivered by a pressurized blast from the cylinder of gene gun. The momentum allows the coated particles to penetrate a few millimeters deep into a tissue and release DNA into the cytoplasm of the target cells or tissue. Gas pressure, particle size and dosing frequency are critical factors that determine penetration efficiency to the tissues, the degree of tissue injury, and overall gene transfer levels. The coating of DNA molecules is done with microparticles of gold or tungsten or silver in order to increase its density. The particles are accelerated to sufficient velocity by highly pressurized inert gas (propellant) such as compressed nitrogen or helium. This method has limited penetration into the deeper parts of the targeted tissue. However, this method has been found effective in delivering genes to the skin, mucosa, or surgically exposed tissue within a confined area. Due to these reasons they are obviously not suitable for systemic application. Some other drawbacks include the need of very stable DNA and the high development costs. Further, it is reported that this method produces more immune response with lower doses compared to needle injection in large animal models and in clinical human trials.

5. Electroporation (Electroporation Gene Delivery)

It is the technique that uses electric field to increase the cell permeability thus resulting in increased DNA uptake by the target cells. Therefore, this technique is also known as electroporation. The use of electric field to increase cell permeability is in use since 1960s. However, the first in vitro attempt to utilize electroporation in gene transfer was demonstrated in 1982 whereas its first in vivo use was reported in 1991. In this method naked DNA is delivered by needle into the target tissue, which is then stimulated by the application of an electric field for few minutes of varying voltage, pulse duration and number of cycles. This method of gene delivery has been used to target a wide variety of tissues but is particularly useful for delivery to more superficial tissues such as skin and muscle. Gene transfer by electroporation has showed good reproducibility and less variation in efficiency across species as compared to other non-viral method such as direct DNA injection.

Some drawbacks or limitations such as significant tissue damage related to harsh electroporation conditions raise serious safety concerns in healthy tissues. The possibility that the high voltage applied to cells could affect the stability of genomic DNA is an additional safety concern. Electroporation of internalized tissues such as kidney and liver has shown gene expression but major limitations exist due to the invasive nature of the delivery (i.e., surgery is done to apply electric field to the target tissue). In order to overcome this damage or enhance gene expression at weaker electrical impulses, the use of modified DNA formulations is suggested. These formulations include the addition of transfection reagent(s), membrane-permeating agents, tissue matrix modifiers, targeted ligands, or agents modifying electrical conductivity or membrane stability. Some other methods are also reported for the reduction of damage.
in tissues by electrical pulses such as use of specific electrodes, changes in pulses of current, etc. 60-62. These limitations have lead to the development of nanoelectroporation or nanosecond electroportaion. In this, nanosecond electric pulses (10-300 ns) are used at very high magnitudes (10-300 kV/cm) for in vitro transfer of DNA directly to the nucleus 63,64.

Due to the serious tissue damage by electroporation technique, scientists have looked for some alternative methods for effective gene delivery. This search has led to the development of a novel and efficient technique that uses lasers for the expression 1. Laser beam gene transduction (LBGT) may provide a less damaging and less invasive alternative to electroporation 1. Naked DNA is injected into a target tissue in a similar manner as that in electroporation method. This is followed by the direct application of laser (femtosecond laser) beam for short intervals (for e.g. 5 seconds) 65. The results suggest that this method is comparable to electroporatoron for in vivo gene expression. No histological or biochemical evidence of muscle damage has been reported. On the basis of these finding it is suggested that LBGT is a simple, safe, effective and reproducible method for intradermal gene delivery with significant clinical potential 65-67.

7. Gene Transfer by Ultrasound (Sonoporation Gene Delivery)
Sonoporation or ultrasound delivery system employs ultrasound waves or energy to facilitate gene transfer at cellular 68 and tissue levels 69. Transdermal penetration of drugs through ultrasound has previously been reported 70. The major advantage of sonoporation is its safety, noninvasiveness, and the ability to reach internal organs without surgical procedure 7. The efficiency of sonoporation based gene transfer depends on the tissue type; ultrasound frequency and its intensity, presence of contrast agent, DNA concentration, and the duration of exposure 1,7,9.

Both high and low intensity ultrasound have been found to improve the gene expression in a variety of tissues including muscle, carotid artery, heart and tumors 71. Sonoporation has also shown to enhance the permeability of blood-brain barrier 72. Most gene delivery studies utilizing ultrasound have used these waves with an intensity of 0.5-0.5 W/cm² at a frequency of 1-3 MHz 34-73. Unlike electroporation, which moves DNA along the electric field, ultrasound creates membrane pores (cavitation) and facilitates intracellular gene transfer through passive diffusion DNA across the membrane pores 68,74. Contrast agents or microbubbles are air-filled particles / vesicles that rapidly expand and shrink under ultrasound irradiation 7,9,75. Commonly used contrast agents include gas-filled human albumin microspheres / microparticles, polymers, phospholipids, etc. 7,9,76,77. Ultrasonic energy is delivered as a sound wave, which like any other wave may result in the loss of intensity while transmitting though a heterogeneous medium. This loss may occur due to absorption and deflection or refraction within the medium or tissue 34. Absorption of the wave in the tissue varies with the protein, fat, water content and the acoustic impedance (i.e. the total reaction of a medium to the transmission of sound through it, expressed as the ratio of sound pressure to particle velocity at a given point in the medium). Proteins are reported to absorb ultrasound waves better than fat and skin 34. The interfaces formed with media such as air or blood reflect the change of refraction of the wave. So far, the major problem for ultrasound-facilitated gene delivery is low gene delivery efficiency 34.

8. Gene Transfer by Magnetic Force (Magnetofection)
A new innovation in the field of gene delivery systems is Magnetofection 78-81. This novel method uses magnetic fields for the transfection of DNA by bounding gene vectors to magnetic nanoparticles such as iron oxide. In vivo studies have shown that cells incubated in a magnetic field displayed greater gene expression than those not exposed to the magnet. It was also observed that the time required for more efficient transfection was reduced from hours to
minutes as compared to the controls.\textsuperscript{78} Magnetoefection does not necessarily improve the overall performance of any given standard gene transfer method \textit{in vitro}, its major potential lies in the extraordinarily rapid and efficient transfection at low vector doses and the possibility of remotely controlled vector targeting \textit{in vivo}\textsuperscript{80,81}.

CONCLUSION
Generally viral methods are more efficient than non-viral method for the delivery of genes. However, viral vectors present safety issues because of co-introduction of essential genetic elements from the parent viruses, immunogenicity and alterations of host genomic structure. In general, non-viral vectors are less toxic and less immunogenic than viral vectors. However, most non-viral methods are less efficient for gene transfer, especially \textit{in vivo}. Therefore, to develop an \textit{in vivo} gene delivery system with high efficiency and low toxicity, the limitations of one type of vector system should be compensated by introducing the strengths of another system. A number of physical methods have been employed for the delivery of genes. Although physical methods are not as effective as viral vectors in gene expression \textit{in vivo} but it is expected that ample amount of research and development in this area would increase their efficiency in near future.

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