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Physical methods of gene transfer: Kinetics of gene delivery into cells: A Review

A.K. Das*[1], Parul Gupta[2] and D. Chakraborty

Division of Animal Genetics and Breeding,
Faculty of Veterinary Science and Animal Husbandry, (SKUAST-J), R.S. Pura, Jammu- 181 102, India.

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ABSTRACT

The ability to introduce isolated DNA into cells has tremendous influence on advances of molecular biology. Recently, with the development of attractive strategies for gene therapy, successful gene delivery has gained importance once again and become a major challenge in this field. During the past decades, a wide repertoire of gene transfer techniques has evolved. The intentional introduction of recombinant DNA molecules into a living organism can be achieved in many ways. The array of methods available to move DNA into the nucleus provides the flexibility necessary to transfer genes into cells as physically diverse are Microinjection, Biolistic gene transfer, Electroporation, Sonoporation, Laser irradiation / Photoporation, Magnetofection, Hydroporation and Impalefection.The purpose of this article is to summarise available physical methods of gene transfer, their principles, advantages and limitations.

Key words: Genetic engineering, Gene gun, Gene therapy, Microinjection.

Transporting foreign genes into cells is an important event in molecular biology. This is mainly performed for gene therapy, studies of gene regulation, protein structure and function analyses and production of recombinant proteins. Gene therapy continues to hold great potential for treating many different types of disease and dysfunction. Safe and efficient techniques for gene transfer and expression in vivo are needed to enable gene therapeutic strategies to be effective in patients (Jixiang *et al*., 2011). Gene therapy is a promising strategy for correcting both genetic and acquired diseases (Kohn and Candotti 2009; Kammili *et al*., 2010). The primary challenge for gene therapy is to develop a method that delivers a transgene to selected cells where proper gene expression can be achieved. An ideal gene delivery method needs to meet three major criteria: first it should protect the transgene against degradation by nucleases in intercellular matrices, second it should bring the transgene across the plasma membrane and into the nucleus of target cells, and third it should have no detrimental effects. Viral vectors are able to mediate gene transfer with high efficiency and the possibility of long-term gene expression, and satisfy two out of three criteria. The acute immune response, immunogenicity, and insertion mutagenesis detected in gene therapy have raised serious safety concerns about some commonly used viral vectors. The limitation in the size of the transgene that recombinant viruses can carry is also one of the major limitations in viral based gene delivery. The chemical approaches use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells. Some of them produce toxicity to the cells. Physical or mechanical techniques have the advantage of avoiding the introduction of foreign substances, i.e., chemicals or viruses, into the target cells or tissues and are therefore a cleaner alternative approach. The various types of physical methods of gene delivery are microinjection, gene gun, electroporation, and sonoporation, hydroporation by hydrodynamic delivery, magnetofection, laser irradiation and impalefection, which employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer (Fig. 1).

Microinjection: One of the most widely used direct and most efficient of all transfer methods is **microinjection,** which was first reported about around 30 years ago (Graessmann *et al*., 1974, Celis, 1978).

Glass micropipettes with a fine tip of less than 0.5 μm are used to inject the sample of interest into the cell nucleus or cytoplasm of adherent cells. The microinjection has advantages of transfer efficiencies and survival rates of up to 100%, a huge variety of molecules can be injected, and even injection of entire organelles has been reported (Celis, 1984), and the molecules of interest can be injected at well-defined stages of the cell cycle and cell culture conditions can be modified before, during, or after injection.

Physical methods of gene transfer are done to avoid the complications associated with viral and chemical

*Corresponding author e-mail: achintya137@yahoo.com. [1]ICAR-CIRC, Meerut, [2]KVK-Rajouri, SKUAST-Jammu, India.

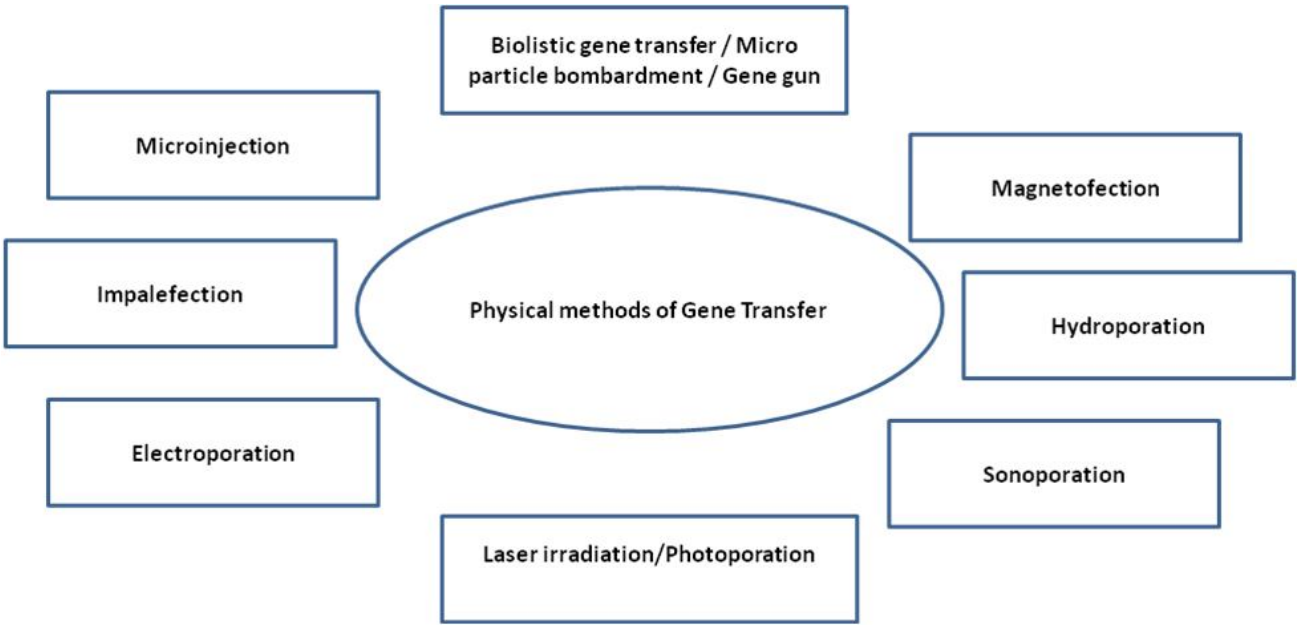

FIG 1: Different physical methods of gene transfer

strategies. In particular, the use of biolistic methods of gene transfer due to its wide spread applicability and low toxicity. Biolistic gene transfer has been used for many years primarily for the study and production of transgenic plants (Helios, 2010).

Microinjection has some disadvantages like it is technically demanding. It requires a lengthy training period until reproducible results are obtained on a routine basis. A further drawback of classical microinjection methodologies is that only a few cells (100-200) can be injected in one experiment. There is also a limitation to the cell types that can be used for microinjection. Cultures that grow in suspension and adherent cells that have only small volume nuclei or cytoplasm are more difficult to use.

Biolistic gene transfer / micro particle bombardment / gene gun: Recently, micro particle bombardment has become increasingly popular as a transfection method, because of a reduced dependency on target cell characteristics. This technology resulted in efficient in vitro transfection, even in the cells which are difficult to transfect. This method will be useful in the design of gene gun device, and bring further improvements to the in vitro and in vivo transfection studies including gene therapy and vaccination (Uchida *et al.*, 2009). Some cells, tissues and intracellular organelles are impermeable to foreign DNA, especially plant cells. Biolistic, including particle bombardment, is a commonly used method for genetic transformation of plants and other organisms. To resolve this problem in gene transfer, the gene gun was made by Klein at Cornell University in 1987 (Klein *et al.*, 1987; Kikkert *et al.*, 2005). On the gene gun technique, Klein and Sanford, published papers, obtained patents and formed a company called biolistics (Klein *et al.*, 1987). The gene gun is part of the gene transfer method called the biolistic (also known as biobalistic or particle bombardment) method. In this method, DNA or RNA adheres to biological inert particles (such as gold or tungsten). By this method, DNA-particle complex is put on the top location of target tissue in a vacuum condition and accelerated by powerful shot to the tissue, then DNA will be effectively introduce into the target cells. Uncoated metal particles could also be shot through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cells. The efficiency of the gene gun transfer could be depended on the following factors: cell type, cell growth condition, culture medium, gene gun ammunition type, gene gun settings and the experimental experiences, etc.

Briefly for gene gun practice, the target cells or tissues on the polycarbonate membranes could be positioned in a Biolistic PDS-1000/HE Particle Delivery System (Bio-Rad Laboratories GmbH, München, Germany). Biolistic parameters are 15 in. Hg of chamber vacuum, target distance of 3 cm (stage 1), 900 psi to 1800 psi particle acceleration pressure, and 1.0 mm diameter gold microcarriers (Bio-Rad, USA). Gold microcarriers are prepared, and circular plasmid DNA is precipitated onto the gold using methods recommended by Bio-Rad with the following: 0.6 mg of gold particles carrying ~5 mg of plasmid DNA is used per bombardment. This technique involves accelerating DNA-

coated particles (micro projectiles) directly into intact tissues or cells. It was initially designed to transform plants; however, several other types of organisms have been successfully transformed. Advantages of this method are almost any kinds of cells or tissues can be treated. Device operation is easy. A large number of samples can be treated within a short time by technicians. The introduction of multiple plasmids (co-transformation) is routinely accomplished. Small amount of plasmid DNA is required. Transient gene expression can be examined within a few days. It is conveniently used for evaluating transient expression of different gene constructs in intact tissues. Disadvantages of this method are transformation efficiency is low compared with *Agrobacterium*-mediated or protoplast transformation. Consumable items are expensive in some models and it causes damage to cells or tissue.

Electroporation: The most popular physical genetic transformation method is electroporation. This is due to its quickness, low cost, and simplicity even when it has a low efficiency, requires laborious protocols for regeneration after genetic transformation, and can only be applied to protoplasts (Rivera, *et al.*, 2012, 2014; Nakamura, 2013). Pulse electrical fields can be used to introduce DNA into cells of animal, plant and bacteria. Factors that influence efficiency of transfection by electroporation: applied electric field strength, electric pulse length, temperature, DNA conformation, DNA concentration, and ionic composition of transfection medium, etc. Electroporation is the application of controlled, pulsed electric fields to biological system. When an electroporation pulse is delivered, the result is the formation of temporal pores. The pores formed are of the order of 40-120nm. Before the pores reseal, the target molecules enters into the cells. Upon resealing of the pores, the molecules become incorporated within the cell. Electroporation of cell membranes is used as a tool in injecting drugs and DNA into the cell (Tsong, 1991).

The molecular events underlying electroporation determine the kinetics of opening and closing of membrane pores. The plasma membrane of a cell partitions the molecular contents of the cytoplasm from its external environment. Since the phospholipids bilayer of the plasma membrane has a hydrophobic exterior and a hydrophobic interior any polar molecules, including DNA and protein, are unable to freely pass through the membrane However, the lipid matrix can be disrupted by a strong external electric field leading to an increase in transmembrane conductivity and diffusive permeability. These effects are the result of formation of aqueous pores in the membrane. Electroporation occurs as a result of the reorientation of lipid molecules of the bilayer membrane to form hydrophilic pores in the membrane. Changes in pore radius are effected by surface tension forces on the pore wall, diffusion of water molecules into and out of the pore and an electric field induced force of expansion. The relaxation of the external pulse result in the reorientation of the lipid molecules to close the membrane pores within a few seconds. A very interesting method based on electroporation is Nucleofection, developed in 1998 and introduced to the research market in 2001 (Freeley, 2013; Trompeter, 2003). It has been successful in cancer studies and tissue engineering. Nucleofection is a patented commercial electroporation system developed by Amaxa, and owned by Lonza (Rivera, *et al.*, 2014).

Steps of the electroporation transfection:

*Harvest cells in the mid- to late-logarithmic phase of growth.
*Centrifuge at 500 g (2000 rpm) for 5 min at 4 ^{o}C.
*Resuspend cells in growth medium at concentration of 1 X 10 cells/ml.
*Add 20 μg plasmid DNA in 40 μl cells.
*Electric transfect by 300 V / 1050 μF for 1-2 min.
*Transfer the electroporated cells to culture dish and culture the cells.
*Assay DNA, RNA or protein and continuously culture the cells to get positive cell lines.

This method has the advantages of Electroporation is effective with nearly all cells and species types (Nickoloff, 1995). A large majority of cells take in the target DNA or molecule. In a study on electro transformation of E. coli, 80% of the cells received the foreign DNA (Miller and Nickoloff, 1995). The amount of DNA required is smaller than for other methods (Withers 1995). The procedure may be performed in vivo (Weaver, 1995). Disadvantages are if the pulses are of the wrong length or intensity, some pores may become too large or fail to close causing cell damage or rupture (Weaver, 1995). The transport of material into and out of the cell during the time of electropermeability is relatively nonspecific. This may result in an ion imbalance that could later lead to improper cell function and cell death (Weaver, 1995).

Sonoporation: Sonoporation is the use of ultrasound assisted by encapsulated microbubbles (EMB) that could make cell membranes temporarily open and deliver macromolecules into cells. Ultrasound increases the transfection efficiency of animal cells, in vitro tissues and protoplasts with spatial and temporal specificity. However, it has been reported that ultrasound can damage the cell, completely breaking its membrane (Liu, 2006). Its application in DNA delivery takes advantage of the remarkable ability of ultrasound to produce cavitation activity. Cavitation is the formation and/or activity of gas-filled bubbles in a medium exposed to ultrasound.

There are two types of cavitation, inertial and non inertial. As the pressure wave passes through the media, gas bubbles of any size will expand at low pressure and contract at high pressure. If the resulting oscillation in bubble size is fairly stable (repeatable over many cycles), the cavitation is called stable or non-inertial cavitation. Such oscillation creates a circulating fluid flow called microstreaming around the bubble (Elder 1958) facilitating the entrance of DNA into a cell (Wu *et al.*, 20025, Ross *et al*., 2002). EMB may also oscillate violently and collapse, experiencing inertial cavitation. In either case, cell membranes open for a short time, allowing foreign molecules or DNA to enter the cells with velocities and shear rates proportional to the amplitude of the oscillation.

Advantages:

*Sonoporation can, in theory, deliver DNA or RNA to any type of cell including bacteria fungi, plants and mammalian cells.

*It does not require ion-free media, and therefore can be applied to cells growing in natural media or human body fluids.

*It is a non-invasive method, which does not require direct physical contact.

*It can be used in vivo also.

*One of the advantages of sonoporation is its site specificity (ultrasound can be easily focused into a desired volume)

*Parameters of ultrasound is easy to manipulate

Disadvantage: Transfection efficiency of sonoporation used *in vitro* and *in vivo* (Greenleaf *et al*., 1998; Lawrie *et al.*, 2000; Lu *et al*., 2003) was found to be relatively low.

Laser irradiation/Photoporation: Lasers were shown to be efficient for introduction of foreign DNA into cultured cells (Kurata *et al.*, 1986). The cells upon laser irradiation undergo a change in the permeability of the plasma membrane or form pores in the membrane at the site of contact. It was also reported that hole upon a cultured cell perforated with a finely focused laser beam was found to repair itself within a short period of time (Shirahata *et al.*, 2001). These wavelengths were all used to create pores in the plasma membrane or to change the permeability of the plasma membrane through a variety of effects such as heating, absorption, photochemical effects, or the creation of reactive oxygen species. Several studies reported cell transfection with either Neodymium: yttrium–aluminium–garnet laser (Nd: YAG), Argon ion laser, Femtosecond laser, Holmium: YAG etc.

Advantages:

*Laser irradiation offers the advantage of targeted transfection, which is not possible with chemical, viral, or electroporation methods, which treat all cells in the sample population. The poration of individual cells or groups of cells can be visualized under a microscope, using the same objective for imaging and laser delivery. As a result, cells of interest in a mixed population can be identified and targeted for treatment, but without the need for micromanipulators or microinjection.

*This method also offers the possibility of directly porating not only the cell plasma membrane but the nuclear membrane too. This is important in transfecting slow-growing, non-dividing cells, or primary cell lines such as neurons.

*It does not appear to damage the cells extensively.

Disadvantages:

*The transfection rate is low.

*As a consequence of the high impulses which increases transfection, the mortality rate also increases significantly.

*This method is limited for clinical use, as the electric energy is difficult to focus and highly disruptive. However, lasers might be a better choice for the gene delivery to local application. (Sagi *et al*., 2003)

Magnetofection: Magnetofection is the method of transfection in which nucleic acids or other vectors are associated with magnetic nanoparticles coated with cationic molecules. The resulting molecular complexes are then targeted to and endocyted by cells, supported by an appropriate magnetic field. The magnetic force accelerates the nanoparticle transport and enables rapid process times with significantly improved transfection rates. Membrane architecture and structure stay intact in contrast to other physical transfection methods that damage, create hole or electroshock the cell membranes. The magnetic nanoparticles are made of iron oxide, which is completely biodegradable and not toxic at the recommended doses.

Advantages:

*The vector dose required in this method is quite low.

*The incubation times required to achieve high transfection is short

*There is a possibility of gene delivery to otherwise non-permissive hard-to-transfect cells, primary cells and non dividing or slowly dividing cells.

*The method is inexpensive.

*Magnetofection has been successfully tested on a broad range of cells and cell lines.

*Combining magnetic nanoparticles to gene vectors of any kind results in a dramatic increase of uptake of these vectors and high transfection or delivery efficiency. These advantages make magnetofection an ideal tool for *ex vivo* gene therapy approaches. For *in vivo* gene- and nucleic acid-based

therapies, magnetofection may become a good choice where local treatment is required.

Hydroporation: In case of hydroporation, hydrodynamic delivery of DNA causes transient pores to open in the cell membrane, and allow entry of DNA into the cytoplasm. It closes within 10 minutes of injection. Rapid tail vein injection of a large volume of plasmid DNA solution into a mouse results in high level of transgene expression in the liver (Zhang *et al*., 1999). Gene transfer efficiency of this hydrodynamics-based procedure is determined by the combined effect of a large volume and high injection speed.

Advantages: It is the simplest and convenient method of *in-vivo* gene transfer. The efficiency of hydroporation is also high.

Disadvantages: Besides liver its applications to other tissues have not been fully explored in the past. Recently hydroporation is also used in muscle and kidney, this method needs to be developed to be used in other tissue.

Impalefection: Integrating nanotechnology with biology and medicine is of major interest among the researchers. Impalefection is a method of gene delivery using nanomaterials, such as carbon nanofibres, nanotubes and nanowires (McKnight *et al.*, 2004). Needle-like nanostructures, vertically aligned carbon nanofibers (VACNF) are synthesized perpendicular to the surface of a substrate. Plasmid DNA containing the gene is attached to the nanostructure surface. A chip with arrays of these needles is then pressed against cells or tissue. The tips could penetrate cell membranes and the DNA was simultaneously released into many cells at the same time.

Advantges:

*VACNF arrays provides the ability to track specific gene delivery in discrete single cells without constant observation under the microscope

*DNA immobilized on VACNFs may minimize the potential for incorporation of foreign genes into the chromosomes of manipulated cells. (McKnight *et al*., 2004)

*Only one single DNA molecule was enough for expression. (McKnight *et al*., 2003)

CONCLUSION

Physical methods for gene transfer include biolistics, jet injection, ultrasound and so forth. These methods have been developed quickly because such methods can directly penetrate genes into cells by stimulations of electric impulses, fine needle puncture or high-pressure gas, which may bypass some of the side effects linked to viral or biochemical approaches, such as limitation of the gene length that can be carried by the physical vectors. Physical methods mediate the direct penetration into the cytosol of both small and large nucleic acid molecules, as well as any other non-permeable molecule. Moreover, these physical systems are effective for single or multiple target cells at an intended location and carry little risk of dispersion of transfection reagents. However, they also present several drawbacks. On one hand, it is difficult for the genes to be transported to the nucleus because of little access in passing through the membrane or enzymatic digestion of the naked DNA or RNA, which results in the low transfection efficiency and limits its clinical application. On the other hand, they present damage to cells, difficulty in large-scale manipulation, labor-intensive protocols and/or the necessity of costly instruments.

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