**GENE TRANSFER TECHNIQUES: PHYSICAL OR MECHANICAL METHODS**

1. **Gene transfer techniques**

It has been discussed earlier that due to amphipathic nature of the phospholipid bilayer of the plasma membrane, polar molecules such as DNA and protein are unable to freely pass through the membrane. Various physical or mechanical methods are employed to overcome this and aid in gene transfer as listed below-

1. Electroporation

2. Microinjection

3. Particle Bombardment

4. Liposome

5. viral mediated delivery

1. Electroporation

 • Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane.

• This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells.

• It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells.

• It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.

The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

Procedure

The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram***.***

•When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.

• Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.

• This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.

• When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.

• The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent.

flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

Applications

Electroporation is widely used in many areas of molecular biology and in medical field. Some applications of electroporation include:

• **DNA transfection or transformation**

Electroporation is mainly used in DNA transfection/transformation which involves introduction of foreign DNA into the host cell (animal, bacterial or plant cell).

• **Direct transfer of plasmids between cells**

It involves the incubation of bacterial cells containing a plasmid with another strain lacking plasmids but containing some other desirable features. The voltage of electroporation creates pores, allowing the transfer of plasmids from one cell to another. This type of transfer may also be performed between species. As a result, a large number of plasmids may be grown in rapidly dividing bacterial colonies and transferred to yeast cells by electroporation.

• **Gene transfer to a wide range of tissues**

Electroporation can be performed in vivo for more efficient gene transfer in a wide range of tissues like skin, muscle, lung, kidney, liver, artery, brain, cornea etc. It avoids the vector-specific immune-responses that are achieved with recombinant viral vectors and thus are promising in clinical applications.

 Advantages

• It is highly versatile and effective for nearly all cell types and species.

• It is highly efficient method as majority of cells take in the target DNA molecule.

• It can be performed at a small scale and only a small amount of DNA is required as compared to other methods.

Disadvantages

• Cell damage is one of the limitations of this method caused by irregular intensity pulses resulting in too large pores which fail to close after membrane discharge.

• Another limitation is the non-specific transport which may result in an ion imbalance causing improper cell function and cell death.

 **2. Microinjection**

• DNA microinjection was first proposed by Dr. Marshall A. Barber in the early of nineteenth century.

• This method is widely used for gene transfection in mammals.

• It involves delivery of foreign DNA into a living cell (e.g. a cell, egg, oocyte, embryos of animals) through a fine glass micropipette. The introduced DNA may lead to the over or under expression of certain genes.

• It is used to identify the characteristic function of dominant genes.

Procedure

• The delivery of foreign DNA is done under a powerful microscope using a glass micropipette tip of 0.5 mm diameter.

• Cells to be microinjected are placed in a container. A holding pipette is placed in the field of view of the microscope that sucks and holds a target cell at the tip. The tip of micropipette is injected through the membrane of the cell to deliver the contents of the needle into the cytoplasm and then the empty needle is taken out.

Advantages

• No requirement of a marker gene.

• Introduction of the target gene directly into a single cell.

• Easy identification of transformed cells upon injection of dye along with the DNA.

• No requirement of selection of the transformed cells using antibiotic resistance or herbicide resistance markers.

• It can be used for creating transgenic organisms, particularly mammals.

**3. BIOLISTICS / Particle bombardment/gene gun method**

• Prof Sanford and colleagues at Cornell University (USA) developed the original bombardment concept in 1987 and coined the term “biolistics” (short for “biological ballistics”) for both the process and the device.

• Also termed as particle bombardment, particle gun, micro projectile bombardment and particle acceleration.

• It employs high-velocity micro projectiles to deliver substances into cells and tissues.

Uses

• This method is commonly employed for genetic transformation of plants and many organisms.

• This method is applicable for the plants having less regeneration capacity and those which fail to show sufficient response to *Agrobacterium*- mediated gene transfer in rice, corn, wheat, chickpea, sorghum and pigeon-pea.

Apparatus

The biolistic gun employs the principle of conservation of momentum and uses the passage of helium gas through the cylinder with arrange of velocities required for optimal transformation of various cell types. It consists of a bombardment chamber which is connected to an outlet for vacuum creation. The bombardment chamber consists of a plastic rupture disk below which macro carrier is loaded with micro carriers. These micro carriers consist of gold or tungsten micro pellets coated with DNA for transformation.

The apparatus is placed in Laminar flow while working to maintain sterile conditions. The target cells/tissue is placed in the apparatus and a stopping screen is placed between the target cells and micro carrier assembly. The passage of high pressure helium ruptures the plastic rupture disk propelling the macro carrier and micro carriers. The stopping screen prevents the passage of macro projectiles but allows the DNA coated micro pellets to pass through it thereby, delivering DNA into the target cells.

Advantages

• Simple and convenient method involving coating DNA or RNA on to gold microcarrier, loading sample cartridges, pointing the nozzle and firing the device.

• No need to obtain protoplast as the intact cell wall can be penetrated.

• Manipulation of genome of sub-cellular organelles can be done.

• Eliminates the use of potentially harmful viruses or toxic chemical treatment as gene delivery vehicle.

• This device offers to place DNA or RNA exactly where it is needed into any organism.

Disadvantages

• The transformation efficiency may be lower than *Agrobacterium- mediated transformation*.

• Specialized equipment is needed. Moreover the device and consumables are costly.

• Associated cell damage can occur.

• The target tissue should have regeneration capacity.

• Random integration is also a concern.

• Chances of multiple copy insertions could cause gene silencing.

**4. Lipofection**

• Lipofection is a method of transformation first described in 1965 as a model of cellular membranes using liposomes.

• Liposomes are artificial phospholipid vesicles used for the delivery of a variety of molecules into the cells. They may be multi-lamellar or unilamellar vesicles with a size range of0.1 to 10 micrometer or 20-25 nanometers respectively.

• They can be preloaded with DNA by two common methods- membrane-membrane fusion and endocytosis thus forming DNA- liposome complex. This complex fuses with the protoplasts to release the contents into the cell. Animal cells, plant cells, bacteria, yeast protoplasts are susceptible to lipofection method.

• Liposomes can be classified as either cationic liposome or pH-sensitive.

Cationic liposomes

• Cationic liposomes are positively charged liposomes which associate with the negatively charged DNA molecules by electrostatic interactions forming a stable complex.

Neutral liposomes are generally used as DNA carriers and helpers of cationic liposomes due to their non-toxic nature and high stability in serum. A positively charged lipid is often mixed with a neutral co-lipid, also called helper lipid to enhance the efficiency of gene transfer by stabilizing the liposome complex (lipoplex). Dioleoylphosphatidyl ethanolamine (DOPE) or dioleoylphosphatidyl choline (DOPC) are some commonly used neutral co-lipids.

• The negatively charged DNA molecule interacts with the positively charged groups of the DOPE or DOPC. DOPE is more efficient and useful than DOPC due to the ability of its inverted hexagonal phase to disrupt the membrane integrity.

• The overall net positive charge allows the close association of the lipoplex with the negatively charged cell membrane followed by uptake into the cell and then into nucleus.

• The lipid: DNA ratio and overall lipid concentration used in the formation of these complexes is particularly required for efficient gene transfer which varies with application.

**Negatively charged liposomes**

• Generally pH-sensitive or negatively-charged liposomes are not efficient for gene transfer. They do not form a complex with it due to repulsive electrostatic interactions between the phosphate backbone of DNA and negatively charged groups of the lipids. Some of the DNA molecules get entrapped within the aqueous interior of these liposomes.

• However, formation of lipoplex, a complex between DNA and anionic lipidscan occur by using divalent cations (e.g. Ca2+, Mg2+, Mn2+, and Ba2+) which

canneutralize the mutual electrostatic repulsion. These anionic lipoplexes comprise anionic lipids, divalent cations, and plasmid DNA which are physiologically safe components.

• They are termed as **pH sensitive** due to destabilization at low pH.

The efficiency of both *in vivo* and *in vitro* gene delivery using cationic liposomes is higher thanthat of pH sensitive liposomes. But the cationic liposomes get inactivated and unstable in the presence of serum and exhibit cytotoxicity. Due to reduced toxicity and interference from serum proteins, pH-sensitive liposomes are considered as potential gene delivery vehicles than the cationic liposomes.

**Liposome Action.**

In addition, liposomes can be directed to cells using monoclonal antibodies which recognize and bind to the specific surface antigens of cells along with the liposomes. Liposomes can be prevented from destruction by the cell’s lysosomes by pre- treating the cells with chemicals such as chloroquine, cytochalasin B, colchicine etc. Liposome mediated transfer into the nucleusis still not completely understood.

**Advantages**

• Economic

• Efficient delivery of nucleic acids to cells in a culture dish.

• Delivery of the nucleic acids with minimal toxicity.

• Protection of nucleic acids from degradation.

• Measurable changes due to transfected nucleic acids in sequential processes.

• Easy to use, requirement of minimal steps and adaptable to high-throughput systems.

**Disadvantages**

• It is not applicable to all cell types.

• It fails for the transfection of some cell lines with lipids.

**Transduction**

This method involves the introduction of genes into host cell’s genome using viruses as carriers. The viruses are used in gene transfer due to following features-

• Efficiency of viruses to deliver their nucleic acid into cells

• High level of replication and gene expression.

The foreign gene is packaged into the virus particles to enter the host cell. The entry of virus particle containing the candidate gene sequences into the cell and then to the nuclear genome is a receptor- mediated process. The vector genome undergoes complex processes ending up with ds-DNA depending on the vector that can persist as an episome or integrate into the host genome followed by the expression of the candidate gene.

**5.Viral vectors as therapeutic agents**

Viruses have paved a way into clinical field in order to treat cancer, inherited and infectious diseases. They can be used as vectors to deliver a therapeutic gene into the infected cells. They can be genetically engineered to carry therapeutic gene without having the ability to replicate or cause disease.

**Strategy for engineering a virus into a vector**:

The strategy for engineering a virus into a vector requires the following-

 • **Helper virus**

It contains all the viral genes essential for replication but lack the sequence coding for packaging domain (ψ) making it less probable to be packaged into a virion. It can be delivered as helper virus or can stably integrate into the host chromosomal DNA of packaging cell. Some vectors also possess the helper DNA lacking additional transfer functions to increase safety.

• **Vector DNA**

It contains non-coding *cis*-acting viral elements, therapeutic gene sequences (up to 28–32 kb) and the normal packaging recognition signal allowing the selective packaging and release from cells. Some vectors comprise relatively inactivated viral genes as a wide type infection due to lack of other viral genes.

then synthesize multiple copies of the vector genome (DNA or RNA, depending upon the type of vector). These structural proteins recognize the vector (psi plus) but not the helper (psi negative) nucleic acid resulting in the packaging of the vector genome into viral particles.

Transgene may be incorporated into viral vectors either by addition to the whole genome or by replacing one or more viral genes which can be generally achieved by ligation or homologous recombination.

• If the transgene is added to the genome or replaces one or more non-essential genes for the infection cycle in the expression host, the vector is described as **replication-competent or helper-independent**, as it can propagate independently *e.g.* helper independent adenoviral vectors.

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|  **Retrovirus**  | Actively dividing cells  | Receptor-binding, membrane fusion  | Integrated  | Long term (years)  | SCID, Hyperlipedemia, solid tumors  |
| **Lentivirus**  | Dividing and non-dividing cells  | Receptor-binding, membrane fusion  | Episomal  | Stable  | Hematopoetic cells, muscles, neuron, hepatocytes  |
| **Adeno virus**  | Most cells  | CAR (Coxsackie and Adenovirus Receptor)-mediated endocytosis endosomal escape  | Episomal  | Transient (short term for weeks)  | CNS, hepatocytes, pancreas |

• However, if the transgene replaces an essential viral gene, this renders the vector **replication-defective or helper-dependent**, so that missing functions must be supplied *intrans.* This can be accomplished by co-introducing a helper virus or transfecting the cells with a helper plasmid, each of which carry the missing genes *e.g.* helper dependent retroviruses

• An alternative to the co-introduction of helpers is to use a complementary cell line, which is transformed with the appropriate genes called as ‘packaging lines’. The vectors from which all viral coding sequences have been deleted and depend on a helper virus which can provide viral gene products *in trans* for packaging and vector DNA replication are known as `*gutless vectors’*. **Advantages**

• High capacity for foreign DNA

• The vector has no intrinsic cytotoxic effects.

**Viral vectors**

Various kinds of viruses can be used as viral vectors, but five classes of viral vector are used in human gene therapy-

1. Adenovirus

2. Adeno- associated virus (AAV)

3. Herpes virus

4. Retrovirus

5. Lentivirus