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Review Article

Genetics Section

# Non Viral Vectors in Gene Therapy- An Overview

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## ABSTRACT

Non-viral vectors are simple in theory but complex in practice. Apart from intra cellular and extracellular barriers, number of other challenges also needs to be overcome in order to increase the effectiveness of non-viral gene transfer. These barriers are categorized as production, formulation and storage. No one-size-fits-all solution to gene delivery, which is why in spite of various developments in liposome, polymer formulation and optimization, new compounds are constantly being proposed and investigated. In this review, we will see in detail about various types of non-viral vectors highlighting promising development and recent advances that had improved the non-viral gene transfer efficiency of translating from "Bench to bedside".


**Keywords:** Biological vectors, Engineered vectors, Gene transfer, Transfection

## INTRODUCTION

Gene therapy is defined as the procedure used to treat or improve the health condition of the patient by modifying the patient's cells genetically [1]. It provides an unique approach to treat both inherited and acquired diseases by delivering a therapeutic gene material and its associated regulatory elements into the nucleus; in order to correct the loss of function caused by mutation or to express the deficient gene product at physiologic levels [2]. It is well documented that almost all human diseases occur due to defect in either a single gene or set of genes due to mutation.

Gene therapy is considered as an alternative for enzyme /protein replacement therapy. The disadvantages like in vivo clearance and manufacturing cost faced by the replacement therapy makes gene therapy a potential alternative for various rare genetic disorders.

In spite of various methods or types of gene therapy, the therapy starts with the identification of mutant gene which is responsible for the cause of the disease. The next step is cloning the identical healthy gene. This is called therapeutic gene or transgene. The therapeutic gene is tailored to the need i.e. to augment or suppress or repair. Once the therapeutic gene is produced it is loaded in a vehicle called vector. The function of the vector is to deliver the therapeutic gene to the patient target cell. After the vector reaches the target cell, it delivers the genetic material to the nucleus. In the nucleus the genetic material gets integrated into DNA and corrects the defective or mutated gene. The most critical step in achieving gene therapy is choosing the vectors. Sequential key steps in gene therapy are shown in [Table/Fig-1].

Vectors are vehicles that ferry the genetic material into a wide variety of cells, tissues and whole organs. The optimal vector and delivery system depends on the target cells and its characteristics, duration of expression and the size of the genetic material to be incorporated in the vector [3,4].The present vectors used for gene therapy are broadly classified as Viral vectors, Non-viral vectors and engineered vectors. The non-viral vectors are Naked DNA, particle based and chemical based. They are administered by direct administration (plasmid DNA/Naked DNA)/ chemical /physical. Most of cardiovascular clinical trials use non-viral vectors as a mode of gene transfer.

## RATIONALE FOR USING NON-VIRAL VECTORS

The efficiency of transfecting host cells is relatively high with viral vectors compared to non-viral methods. The main drawbacks of using virus vectors are its immunogenicity and cytotoxicity. The first related fatality of gene therapy clinical trial was related to the inflammatory reaction to the viral vector (Adenovirus). Additional cause of concern over using viral gene transfer vehicle is the phenomenon known as insertional mutagenesis i.e. ectopic chromosomal integration of viral DNA disrupts the expression of tumour suppression gene or activates oncogene leading to the malignant transformation of cells. Due to its demonstrated reduced pathogenicity, low cost and ease of production, non-viral vectors have important safety advantage over viral approaches. The major advantage of using non-viral vectors is its bio-safety. However the application of non-viral gene transfer have been ignored for a long time in past because of their poor efficiency of delivery thereby low transient expression of their transgenes [2]. Non-viral vectors have drawn significant attention due to its less immunotoxicity. Use of non-viral vectors in clinical trials increased from 2004 to 2013 while that of viral vector saw significant decrease. Advances in efficiency, specificity, gene expression duration and safety led to an increased number of non-viral vector products entering clinical trials.

Unfortunately none of the currently available non-viral vectors fulfills ideal vector properties. This has led to research focus on suitable ideal vector delivery system.

### Technical challenges and limitations to successful Non-Viral Gene transfer

The major technical limitations or critical steps in attaining a successful gene therapy are categorized into [5-8]: efficiency of vector transport and unloading into target cells, perseverance, activity, immune response, regulatory issues and ethical concerns and commercialization. These different stages pose a big challenge to gene therapy to be efficiently treating the disease. The cost of gene therapy creates an image that it is meant for the affluent. This was clearly evident with the first commercialized gene therapy Aliopogene tiparvovec for Lipoprotein Lipase deficiency in November 2013. The


**[Table/Fig-1]:** Schematic illustration of key steps in gene therapy

| Delivery methods | Key Mechanism | Tissue on which it is effective | Advantage | Disadvantage |
|---|---|---|---|---|
| Naked plasma/ Plasmid DNA[p DNA] –Direct delivery | Endocytosis | Muscle, skin, liver, cardiac muscle and solid tumour. | Safety. Simplicity. | Low transfection efficiency. |
| Gene Gun | High pressure helium stream | Ovarian cancer cell line[invitro and in vivo preclinical model] | Flexibility. Low cytotoxicity. Good efficiency. | Shallow penetration |
| Electroporation | Enhancement of cell membrane permeability | Skin, muscle. | Good efficiency. Repeatable. | Tissue damage. Accessibility of electrodes to internal organ are limited. |
| Ultrasound + micro bubble | Enhancement of cell membrane permeability | Brain, cornea, kidney, peritoneal cavity, muscle, heart, vascular cells. | Safety. Flexibility. | Low efficiency. |
| Magnetofection | Pinocytosis and endocytosis | primary cells and cells difficult to transfect by other methods[only invitro] | Flexibility. Low cytotoxicity. | Transient transfection. |
| Inorganic molecules | Endocytosis | Invitro | Easy Production Storage stability. Surface functionalization. | Low efficiency |
| Type of vector | Key mechanism | Target tissue | Advantage | Disadvantage |
| Lipoplexes[Lipid based] | Endocytosis, DNA condensation, | Airway epithelial cells, endothelial cells, hepatocytes, muscle cells. | Safety Low cytotoxicity | Low to medium efficiency Some results immunogenicity. |
| Polyplexes and Dendrimers | Endocytosis, DNA condensation, protein sponge effect | Lung, oral cavity. | Low immunogenicity Fair efficiency | Complement activation Low efficiency Cytotoxicity. |

**[Table/Fig-2]:** Overview of different non-viral vector delivery methods

estimated treatment cost for LPLD gene therapy is about 1.6million/ patient [9]. This tends to be the major hurdle in commercializing the gene therapy if proven successful.

## Types of Nucleic acids

Non-viral vectors are generally used to transfer following types of nucleic acids [10,11].

- Small DNA (Oligodeoxynucleotides) or related molecules synthesized chemically.
- Large DNA molecules (Plasmid DNA:p DNA)
- RNA(Ribozymes, Si RNA, m RNA)

## Various delivery Systems:

**I. Physical Methods:** Gene therapy researchers are more attracted towards physical means of transferring gene material as it is simpler. These methods employ physical force to counteract the membrane barrier of the cells thus facilitating intracellular delivery of the genetic material.

**1. Needle:** The genetic material of interest is administered through a needle carrying syringe into tissue or systemic injection from a vessel. Without any carrier it is the simplest and safest method of gene transfer. Attractive candidate tissues are muscle, skin, liver, cardiac muscle and solid tumours. However, the efficiency is low due to rapid degradation by nucleases in serum and cleared by mononuclear phagocyte system [6,12,13].

**2. Ballistic DNA:** Particle bombardment, micro projectile gene transfer or gene gun are the other terms used for ballistic DNA. This method was first used as gene transfer technique to plants. The

| Barriers | Functional components | Strategies -rationale | Molecules studied |
|---|---|---|---|
| Extracellular stability | Carrier molecules | DNA condensation- protects from nucleases | Protamine, lipids, gelation |
| | Hydrophobic moiety | Steric stability achieved by surface charge shielding | PEG ylation |
| Internalization | Targeting ligands | Receptor mediated endocytosis | Transferrin, EGF, antibodies, RGD |
| Intracellular trafficking | Endosomal disruptive agent | Escape from endosomes and unpacking by proton sponge effect | PEI, DOPE |
| Nuclear entry | Nuclear localization signal | Nuclear entry | Tat, ReV. |

**[Table/Fig-3]:** Strategies to improve gene transfer efficacy of non viral vectors

method is based on the principle of delivering DNA coated heavy metal particles by crossing target tissue at a certain speed. The sufficient speed is achieved by high voltage electronic discharge, spark discharge or helium pressure discharge. Gas pressure, particle size, dose frequency are the critical parameters in determining the efficiency of gene transfer. Gold, tungsten and silver are used as metal particles and they are typically 1 μm diameter. The major advantage of gene gun is precise delivery of DNA doses. It is most commonly used in gene therapy research in ovarian cancer [6,12,14].

**3. Electroporation:** The other terms used for electroporation are gene electro injection, gene electro transfer, electrically mediated gene therapy, electro gene transfer. Applying an electric field that is greater than the membrane capacitance will cause charges of opposite polarity to line up on either side of cell membrane thus forming a potential difference at a specific point on the cell surface. As a result membrane breakdown form a pore and allows the molecule to pass. Pore formation occurs in approximately 10 nanoseconds. The pore of the membrane can be reversible based on the field strength and pulse duration. If it is reversible cells remain viable, otherwise cell death results. Irreversible electroporation is used in cancer treatment to destroy cancer cells. The permeability of the membrane to the gene transfer is controlled by the amplitude and duration of pulse. Currently used field strength are either high field strength [>700V/cm] or low field strength {<700V/cm} with short pulses (microseconds) or long pulses (milliseconds). Target tissue determines this combination of variables. Generally cancer cells require low field strength with long pulse, whereas muscle cells need short pulse with high field strength. Electroporation has emerged as a reliable physical method for delivering plasmid DNA. The therapy can be delivered by intradermally, intramuscularly or as intratumoural [6,12,14-16].

**4. Sonoporation:** Sonoporation is a noninvasive site specific technique which utilizes ultrasound wave to temporarily permeablize the cell membrane to allow cellular uptake of DNA. Genetic material of interest is incorporated within micro bubble and administered into systemic circulation. This is followed by external application of ultrasound. The ultrasound wave's cavitate the micro bubble within the microcirculation of target tissue, produces bio effects that result in deposition of targeted transfection of therapeutic gene. Micro bubbles are composed of gas filled core [air/nitrogen/inert gas] with high molecular weight such as per fluorocarbon or sulfur hexafluoride. The outer shell consists of biocompatible compounds like lipids, proteins or synthetic biopolymers. Micro bubbles resemble red blood cells in circulation (mean diameter of 2-4 μm). Sonoporation technique is generally used in brain, cornea, kidney, peritoneal cavity, and muscle and heart tissues. The gene products are paired with micro bubbles in any of following three ways [17].

1. Generating micro bubbles in conjunction with gene product [DNA is incorporated into the shell/lumen].
2. Charge coupling (coincubation with DNA and attached to shell by electrostatic interaction).
3. Non coupled (co administering micro bubble and genetic material).

**5. Photoporation:** This physical method utilizes single laser pulse to generate transient pores on a cell membrane to allow DNA to enter into the cell. Focal point and pulse frequency of the laser controls the efficiency. It is claimed that the level of transgene expression is identical to that of electroporation. This technique lacks documented evidence [18].

**6. Magnetofection:** It is based on the hypothesis of magnetically targeted drug delivery. The technique is based on coupling therapeutic gene to magnetic nanoparticle. This complex is introduced in the cell culture. The field gradient produced by rare, earth electromagnets which are placed below cell culture increases sedimentation of complex and increases transfection speed. In case of in vivo, the therapeutic gene-magnetic particle complex is administered intravenously. Using strong high gradient external magnets, the complex is captured and held at the target. The genetic material is released by enzymatic cleavage of cross linking molecule, charge interaction or degradation of the matrix. This technique is mostly used in invitro research for transfecting primary cells and cells that are difficult to transfect by other means [6,19,20].

**7. Hydroporation:** It is also called as hydrodynamic gene transfer. The technique uses hydrodynamic pressure to penetrate the cell membrane. Hydrodynamic pressure is created by injecting large volume of DNA solution in a fraction of time. This creates increased permeability of capillary endothelium and forms pores in plasma membrane encircling parenchyma cells. The therapeutic gene of interest can reach the cell through these pores and these membrane pores are closed later thus keeping the genetic material inside the cell. This technique is most commonly used for gene therapy research in hepatic cells [12,21].

**8. Mechanical Massage:** It is based on the hypothesis that mechanical massage of liver generates transient membrane defects for a few minutes, which facilitates plasmid DNA to enter into hepatic cells by diffusion. But it was highly debatable and studies are scarce in using mechanical massage as a mode of gene transfer [15].

**II. Chemical Carriers:** Chemical vectors are broadly classified into inorganic particles, lipid based, polymer based and peptide based. They are generally categorized as [15,22].

1. Those forming condensed complex with therapeutic gene to protect them from nucleases and other blood components.
2. Those designed to target specific cells.
3. Those designed to increase the delivery of genetic material to cytosol or nucleus.
4. Those designed to disintegrate from DNA/RNA in the cytosol.
5. Those designed for sustained or controlled release of therapeutic gene in tissue.

Chemical non-viral nucleic acid delivery systems are generally DNA/Cationic lipid (Lipoplexes), DNA/cationic polymer (Polyplexes) and DNA/cationic Polymer/cationic Lipid (Lipopolyplexes)[10].

**A. Inorganic particles [6]:** They are generally nanoparticles that can be engineered by varying in size, shape and porosity in order to escape from reticulo endothelial system or to protect an entrapped molecule from degradation. Calcium sulphate, silica, gold, magnetic compounds, quantum dots. Carbon nanotubes, fullerenes, supra molecular system are most studied in this category.

**1.Calcium phosphate:** Calcium phosphate particles were the first ones to be used in this system. It is biocompatible and biodegradable. Calcium plays a vital role in endocytosis and has the advantage of being readily absorbed and it poses high binding affinity. However calcium phosphate nano crystals grow with time reducing its capacity to store. This is later overcome by adding magnesium.

**2.Silica:** It is the major component of widely used materials like sand and glass by humans. Its relative ease of functionalizing makes it attractive to use as gene delivery vehicle. The most commonly used silica as gene delivery agent is obtained by functionalizing nanoparticles with amino silicanes due to its low toxicity. Its decreased delivery efficiency in the presence of serum containing media due to the interaction between serum proteins is a major limiting factor.

**3.Gold:** The properties like ease of preparation, unlimited surface characterization and inert nature attracted researchers towards gold nanoparticles. Gold nanoparticles have strong absorption of light near infra-red region. The near infra-red light can penetrate deeply into tissues. Modifying the surface of gold with DNA can be used to transfect the cell by using photo thermal effect. Thermal denaturation induced by photo thermal effect helps to control the release of gene. Studies had proved that transfection efficiency with gold is comparable to lipoplexes comparatively with lower toxicity in vitro. However, major concern is its high chemical stability, so it is not easily dissolved in cell resulting in accumulation in cell which may harm the cell growth.

Magnetic nanoparticles (supermagnetic iron oxide mostly magnetite), fullerenes (soluble carbon molecules), carbon nanotubes (cylindrical fullerenes), quantum dots (semi conduction nanomaterial) and supramolecular systems all claimed some promising result in invitro and animal models. Surface of these inorganic nanoparticles can be coated to facilitate DNA binding. The hypothesis is that small particle size can efficiently by pass most of the physiological and cellular barriers and produce higher transfection efficiency. Still studies require on long-term safety, surface functionalization effect of type, size, and shape on transfection efficiency to accelerate their clinical application.

## B. Synthetic/natural biodegradable:

**i. Cationic lipids:** Hundreds of lipids have been developed for gene transfer. All of them share the common structures of positively charged hydrophilic head and hydrophobic tail with linker structure that connects both. The positively charged head group binds with negatively charged phosphate group in nucleic acids and form uniquely compacted structure called lipoplexes. Transfection efficiency depends on overall geometric shape, number of charged group per molecules, nature of lipid anchor and linker bondage. Lipoplexes due to their positive charge electrostatically interact with negatively charged glycoproteins and proteoglycans of cell membrane which may facilitate cellular uptake of nucleic acids. The positively charged lipids surrounding the genetic material help it to protect against intracellular and extracellular nucleases. However the problem lies with surface charge, this reduces the half-life of lipoplexes circulation in blood limiting its utility not beyond vascular endothelial cells. Neutral polymer like polyethylene glycol (PEG) is used as surface shielding to overcome the excessive charge and to prolong the half-life. Though considered to be of low toxicity, lipoplexes become cytotoxic beyond 3:1 ratio of lipid: DNA [6,12,15,20,23-25].

**ii. Lipid Nano Emulsions:** Lipid emulsion is a dispersion of one immiscible liquid in another stabilized by emulsifying agent. They are particles of around 200nm comprises of oil, water and surfactant. Lipid nano emulsion is considered to be superior to liposomes in scaling up, and stability, thus making it more serum resistant. The data shows they are less toxic than liposomes [6].

**iii. Solid lipid nanoparticles:** Solid lipid particles are made from lipid are solid at both room temperature and body temperature. It has advantages of both cationic lipids and lipid nano emulsions. It is shown that cationic solid lipid nanoparticle can effectively protect

nucleic acid from nuclease degradation. It is currently the choice of delivery system for SiRNA [6].

**iv. Peptide based:** Peptide based vectors are considered advantageous over other non-viral vectors in tight compact and protecting DNA, target specific cell receptor, disrupting endosomal membrane and delivering genetic cargo into nucleus. Cationic peptides are rich in basic residues like lysine and/or arginine. Attaching peptide ligands to polyplex or lipoplexes enables vector to achieve specific target. Short peptide sequence taken from viral protein enables the vector to provide nuclear localization signal that assist transport of genetic material into nucleus. Due to these advantages peptides are frequently used to functionalize cationic lipoplexes or polyplexes [6].

### v. Polymer based vectors [26]:

Cationic polymers mix with DNA to form nanosized complex called polyplexes. Polyplexes are more stable than lipoplexes. Polymers are categorized into natural and synthetic polymers.

Natural- proteins, peptides, polysaccharides.

Synthetic- Polyethylene mine (PEI), Dendrimers, and Polyphosphoesters.

**a. Polyethylenimine (PEI):** PEI is considered as a gold standard for in vivo and invitro gene transfer. Cationic polymers have high density amine groups which exert protein sponge effect that ultimately stops the acidification of endosomal pH. This leads to the influx of chloride within the compartment and increases the osmotic pressure, leading to the swelling and rupture of endosomal membrane.

**b.Chitosan:** It is a natural polymer based on cationic polysaccharide. One of the most studied non-viral vectors. It is nontoxic even at high concentrations. It is a linear cationic polysaccharide composed of glucosamine. The positive charge of chitosan electrostatically bind with negative charged DNA. On account of its mucoadhesive properties chitosan/DNA polyplexes are widely used in oral and nasal gene therapy. To effectively negotiate intracellular barriers, chitosan is conjugated to folic acid.

**c. Poly (DL- Lactide) (PLA) and Poly ( DL-Lactide- co-glycoside) (PLGA):** They are biodegradable polyesters undergo bulk hydrolysis thus providing sustained delivery. The degradation products are removed by citric acid cycle. PLGA is approved by FDA as vehicle for protein delivery. Less than 10 μm in size, they are easily phagocytosed by antigen presenting cell and inducing immune reaction

**d. Dendrimers:** Dendrimer molecules are symmetrical in size and shape with terminal group functionality. It binds to genetic material when positively charged peripheral groups interact with nucleic acids in physiological pH. due to nanometric size it can interact effectively with cell membranes, organelles, and proteins. The terminal amino group and positive charge density determine the toxicity profile.

**e. Polymethacrylate** Polymethacrylate are vinyl based polymer able to condense polynucleotides into nanometer size particle. But the transfection is limited due to their low ability to interact with membranes.

## MATERIALS

Literatures were searched using electronic databases Medline via Ovid (1946 – May 1st 2014), EMBASE via Ovid (1980- May 1st 2014), Cochrane central register for clinical trials(CENTRAL), gene therapy trials {www://genetherapynet.com}, US national institutes of health trials register {http://clinicaltrials.gov} and the world health organization international clinical trials registry platform{www.who.int/trialsearch} using the MESH terms non-viral vectors, gene transfer vehicle, vectors in gene therapy, gene therapy, non-viral gene transfer. No restrictions were applied on the language or date of publication while searching the electronic databases.

## RESULTS

A comparative overview of different actively used non-viral vectors based on the invitro, in vivo animal and human studies are summarized in the following [Table/Fig 2][27-31].

## DISCUSSION

Delivery efficiency is the major hurdle for almost all of the non-viral vectors. From the result table it is much evident that if the vector shown good efficiency its toxicological profile increases, if toxicological profile is good, efficiency is compromised. The primary rate limiting step in achieving better efficiency (transfection) is contributed to the anatomical barriers (epithelial, endothelial cell linings and extracellular matrix around the cells). Physical methods like electroporation, ultrasound assisted by micro bubbles, and magnetofection shows some promising outcome in overcoming this hurdle still it's problematic to some extent [32].

Liposomes possessed excellent biocompatibility, low immunogenicity, ability to deliver large piece of nucleic acid and ease of handling. Due to positive charge liposomes may undergo nonspecific interaction with negatively charged serum protein, enzymes and result in decrease cell adhesion, hemolysis and low transfection. To overcome this positive charge heterocyclic ring like imidazolium, pyridinum, and polyamine groups were added. But the progress is not great enough and full of difficulties and challenges [22]. PEGylation is one of the popular mechanism considered to reduce the opsonization and aggregation of liposomes in reticuloendothelial system. However the drawback of PEGylated surface is reduced biological activity because of decreased uptake by target cells. Neutral helper lipid is proposed to improve the target uptake of PEGylated liposomes. But all this exhaustive and extensive efforts yield very limited improvements clinically [6].

Cationic polymers shown promise as a predictable safe biodegradable alternative to virus, but the problem is its unpredictable endocytosis. The other major issue is its cytotoxicity. Coating with human serum albumin, dextran, PEG is considered. Theoretically this step demonstrated as less cytotoxic, but in vivo exhibited immune response. Chitosan being ecologically safe and of low toxicity and immunogenicity has been studied for almost two decades. Still the solubility (insoluble under physiologic pH) remains as main limitation. To improve this limitation hydrophobic and hydrophilic modifications are considered this include such as deoxycholic acid modification, thiolation, PEGylation, quarternization. Nevertheless ideal transfection efficiency was not attained due to certain factors acted differently [22].

Traditional non-viral vectors like various lipoplexes and polyplexes (polyethylenimine) showed excellent results in invitro experiments, but their translation to in vivo is not effective and able to confer only transient gene expression. Nevertheless endosomal escape remains a critical bottleneck for non-viral vectors. Finally the last hurdle is not able to replicate in the nucleus and lost during mitosis.

Over the past decade several strategies have been developed to improve the poor outcome of non-viral vectors by focusing; extracellular stability (polynucleotide degradation in extracellular space), internalization (internalization of carrier), intracellular trafficking (endosomal rupture and polynucleotide release), nuclear entry (dissociation of polynucleotide from the carrier and entry of polynucleotide into the nucleus). The overview of these strategies are shown in the [Table/Fig 3] [18].

Despite this different approach to overcome the hurdles faced by the vector, the transfection efficiency is still inefficient compared to viral vectors although good results are seen in invitro and preclinical models. Numerous publications reported successful phenotype correction of human disease in mouse model. In any given year in past 3-5 y there were more than 30 publications hyping the successful phenotype correction of mouse/preclinical models with diseases. However, the translation of this success to larger

animals or humans doesn't yield the same result. Various factors are considered to contribute this difference in results. Those include are species specific differences, immune response to vectors or encoded gene product and sheer size difference of preclinical models and humans.

Presently researchers are concentrating more on developing cell penetrating peptides, nano shell, sleeping beauty transposon, conjugated polymers, and biological vectors to be effective in non-viral gene transfer as compare to viral vectors [33-37] Apart from above mentioned SPION (super paramagnetic nano particle), mitochondria targeting strategies {mitochondria leader peptide (MLP), mitochondria targeting sequence (MTS) +DNA, liposome based carrier (Dequalinium) DQAsomes} are also under present review of developing into potential gene transfer agent [38].

## FUTURE PERSPECTIVE

Various researches are concentrating on the new approaches of gene therapy like gene splicing using ribozymes, triple helix forming oligonucleotides, antisense, spliceosome-mediated RNA trans-splicing (SMaRT), Hybrid vectors, zinc finger nucleases and Nano-robotics [39,40]. Nano robotics uses powerful nanocomputers and fast sequenators. The fast sequenators guide the nanorobot inside the cell and this one examines deletes the defective part and stitches like cut and paste [13]. This is able to restore the whole organs. It seems like science fiction may be real in future.

**Possible strategies to improve current non-viral vector system:** Based on the documented evidences and published results we can hypothesize that to be efficient and to produce significant clinical results the vectors either viral or non-viral it should be designed based on its

1. Capacity to efficiently interact with serum components without losing the therapeutic material.
2. Appropriate circulating time in the body and bio distribution.
3. Escape from immune system and macrophages.
4. Targeting ability of the cell.
5. Interaction with surface of cell.
6. Penetration through cell membrane barrier.
7. Intracellular trafficking capacity( release from endosomes and escape from degradation by nucleases).
8. Nuclear import capability.
9. Persistence in nucleus.
10. Maintaining gene expression(time dependent).
11. Passage to succedenous cells(progeny cells).
12. Ability to transcript.

Theoretically, a modest 2 fold enhancement in each of this steps of currently using vectors without any untoward immune reaction or side effects will result in dramatic $2^{12}$ fold increase in level of therapeutic protein in target cells [41]. Thus, improving the transfection efficiency. Finally finding appropriate animal models will help the scientific faculty to carry the research in right direction without causing significance loss in time and money. Microscopy and X-ray diffraction studies have illustrated the high degree of variability in the structure of DNA complexes. Better understanding of internal trafficking and cell architecture will help us to identify the potential hurdles and help researchers to design much efficient non-viral vector. Combinatorial synthesis and high throughput screening of polymer libraries with diverse chemical structure may offer a better idea to identify potential high transfection efficient biodegradable polymer in future.

## SUMMARY AND CONCLUSION

Gene therapy has a potential to treat some of the life threatening orphan diseases. Advances in Genome sequencing and genetic analysis have improved our understanding of human diseases, diagnostic ability but therapeutic benefit remains largely ineffective. Failure of finding an ideal vector remains major hurdle in treating human diseases with gene therapy. Past few years the trend for using non-viral vectors is significantly increasing. Further improvements to increase the transfection efficiency are needed before to see any remarkable clinical results. These achievements might be relied on our understanding of limiting steps and methods to overcome it.

Developing specific intracellular probes may help us to understand the intracellular trafficking in a better way and open new improvements. The strategies that merge non-viral and viral free biological vectors might be beneficial to achieve predictable long lasting, efficient and nontoxic gene delivery system.

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