Review Article

Viral and nonviral delivery systems for gene delivery

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Abstract

Gene therapy is the process of introducing foreign genomic materials into host cells to elicit a therapeutic benefit. Although initially the main focus of gene therapy was on special genetic disorders, now diverse diseases with different patterns of inheritance and acquired diseases are targets of gene therapy. There are 2 major categories of gene therapy, including germline gene therapy and somatic gene therapy. Although germline gene therapy may have great potential, because it is currently ethically forbidden, it cannot be used; however, to date human gene therapy has been limited to somatic cells. Although numerous viral and nonviral gene delivery systems have been developed in the last 3 decades, no delivery system has been designed that can be applied in gene therapy of all kinds of cell types *in vitro* and *in vivo* with no limitation and side effects. In this review we explain about the history of gene therapy, all types of gene delivery systems for germline (nuclei, egg cells, embryonic stem cells, pronuclear, microinjection, sperm cells) and somatic cells by viral [retroviral, adenoviral, adeno association, helper-dependent adenoviral systems, hybrid adenoviral systems, herpes simplex, pox virus, lentivirus, *Epstein–Barr virus*)] and nonviral systems (physical: Naked DNA, DNA bombardant, electroporation, hydrodynamic, ultrasound, magnetofection) and (chemical: Cationic lipids, different cationic polymers, lipid polymers). In addition to the above-mentioned, advantages, disadvantages, and practical use of each system are discussed.

Key words: Chemical delivery, gene therapy, non viral delivery systems, physical delivery, viral delivery systems

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INTRODUCTION

Basically gene therapy is an intracellular delivery of genomic materials (transgene) into specific cells to generate a therapeutic effect by correcting an existing abnormality or providing the cells with a new function.[1] Different types of gene delivery systems may be applied in gene therapy to restore a specific gene function or turning off a special gene(s). The ultimate goal of gene therapy is single administration of an appropriate material to replace a defective or missing gene.[2] The first human gene transfer was utilized in 1989 on tumor-infiltrating lymphocytes[3,4] and the first gene therapy was done on ADA gene for treatment of patients with SCID (Severe Combined Immunodeficiency Defect) in 1990.[5] Although initially the main focus of gene therapy was on inherited genetic disorders, now diverse diseases, including autosomal or X-linked recessive single gene disorders (CF(Cystic Fibrosis), ADA (Adenosine Deaminase) –SCID, emphysema, retinitis

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pigmentosa, sickle cell anemia, phenylketonuria, hemophilia, DMD (Duchenne Muscular Dystrophy), some autosomal dominant disorders, even polygenic disorders, different forms of cancers, vascular disease, neurodegenerative disorders, inflammatory conditions, and other acquired diseases are targets of gene therapy. To date, thousands of disorders have been treated by more than hundreds of protocols of gene therapy.[1] There are 2 major categories of gene therapy: Germline gene therapy and somatic gene therapy. Although germline gene therapy may have a great potential, because it is currently ethically forbidden, it cannot be used.[6-8] To date, human gene therapy has been limited to somatic cell alterations and there is a remarkable development in the field. There are different viral and nonviral vectors for gene delivery, but all gene therapy applications depend on the fact that the genetic material needs to be delivered across the cell membrane and ultimately to the cell nucleus. Each of the delivery systems has some advantages and disadvantages, and in this review we explain about all types of gene delivery systems briefly [Figure 1].

DIFFERENT METHODS OF GENE THERAPY

Germline gene therapy

The technology of this type of gene therapy is simple as genetic abnormalities can be corrected by direct manipulation of germline cells with no targeting, and not only achieve a cure for the individual treated, but some gametes could also carry the corrected genotype. Although it almost never has been tested on humans, some different transgenic techniques have been used on other species, which include the following:

(1) Gene delivery to the nuclei taken from somatic cells at metaphase stage.[9,10]

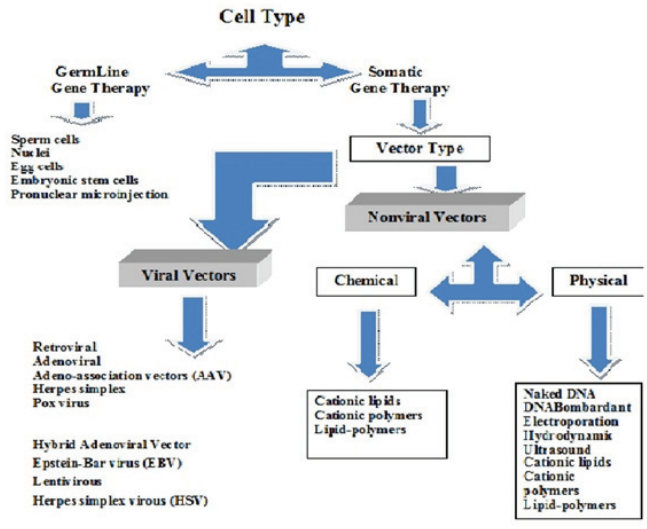

Figure 1: Different gene delivery systems

(2) Ex vivo alteration of egg cells, following *in vitro* fertilization.[11,12]

(3) Manipulation of embryonic stem cells of mouse during *in vitro* culture by different gene delivery systems.[12-14]

(4) Pronuclear microinjection of exogenous DNA solution by a glass needle.[15]

(5) Transgenic delivery into sperm cells by direct or indirect injection to testis or other parts of the genital system.[16,17]

Somatic gene therapy

Somatic gene therapy involves the insertion of genes into diploid cells of an individual where the genetic material is not passed on to its progeny. Somatic cell therapy is viewed as a more conservative, safer approach because it affects only the targeted cells in the patient, and is not passed on to future generations; however, somatic cell therapy is short-lived because the cells of most tissues ultimately die and are replaced by new cells. In addition, transporting the gene to the target cells or tissue is also problematic. Regardless of these difficulties, however, somatic cell gene therapy is appropriate and acceptable for many disorders.

There are 3 types of somatic gene therapy

Ex vivo delivery

In this system the genetic material is explanted from the target tissue or bone marrow, cultivated and manipulated *in vitro*, and then transducted and/or transfected into the target tissue. There are no immunologic problems in this way but only the technique is used in cases where the target cells act as protein secretion resources (like the treatment of ADA or hemophilia) or as a vaccine for cancer treatment, so there are major limitations on the use of *ex vivo* delivery. In addition, at present only a small percentage of reimplanted cells remain viable.[18,19]

In situ delivery

The administration of the genetic material directly into the target tissue is in situ delivery. As most of the current delivery systems need no effective targeting, the way is proper. The system has been utilized in the delivery of CFTR gene by lipid and adenoviral vectors to a specific site in the respiratory tract and is also used in the treatment of different cancers. However, low efficiency of transduction is the main problem of this system, because in cancer therapy one malignant cell can re-establish the tumor again.[20-22]

In vivo delivery

The transfer of genetic material through an appropriate

vector, which can be a viral or nonviral vector, into the target tissue is *in vivo* delivery. This technique is the least advanced strategy at present but potentially it might be the most useful. The problem of this way is insufficient targeting of vectors to the correct tissue sites; however, improvement in targeting and vector development will solve the problem.

DIFFERENT VECTOR SYSTEMS FOR GENE DELIVERY

Viral vectors

One of the successful gene therapy systems available today are viral vectors, such as retrovirus, adenovirus (types 2 and 5), adeno-associated virus, herpes virus, pox virus, human foamy virus (HFV), and lentivirus.[23] All viral vector genomes have been modified by deleting some areas of their genomes so that their replication becomes deranged and it makes them more safe, but the system has some problems, such as their marked immunogenicity that causes induction of inflammatory system leading to degeneration of transducted tissue; and toxin production, including mortality, the insertional mutagenesis; and their limitation in transgenic capacity size.[24,25] During the past few years some viral vectors with specific receptors have been designed that could transfer the transgenes to some other specific cells, which are not their natural target cells (retargeting).[26]

Retroviral vectors

Retroviral vectors are one of the most frequently employed forms of gene delivery in somatic and germline gene therapies. Retroviruses in contrast to adenoviral and lentiviral vectors, can transfect dividing cells because they can pass through the nuclear pores of mitotic cells; this character of retroviruses make them proper candidates for in situ treatment.[27,28] In addition, all of the viral genes have been removed, creating approximately 8 kb of space for transgenic incorporation. Retroviruses are useful for *ex vivo* delivery of somatic cells because of their ability to linearly integrate into host cell genome; for example, they have been used for human gene therapy of X-SCID successfully but incidence of leukemia in some patients occurred because of integration of retroviruses to the LMO2 gene and inappropriate activation of it.[29-34] Retroviral vectors also have been applied for familial hyperlipidemia gene therapy and tumor vaccination. However, the main limitations of retroviral vectors are their low efficiency *in vivo*, immunogenic problems, the inability to transduce the nondividing cells and the risk of insertion, which could possibly cause oncogene activation or tumor-suppressor gene inactivation.[27-34]

Adenoviral vectors

Adenoviral vectors have been isolated from a large number of different species, and more than 100 different serotypes have been reported. Most adults have been exposed to the adenovirus serotypes most commonly used in gene therapy (types 2 and 5). Adenoviruses type 2 and 5 can be utilized for transferring both dividing and nondividing cells and have low host specificity so can be used for gene delivery into large range of tissues.[35] Adenoviruses are able to deliver large DNA particles (up to 38 kb),[36] but in contrast to retroviruses, as they would not integrate into the host genome, their gene expression is too short term. Natural and acute immunologic responses against adenoviruses have made their clinical application limited to a few tissues, such as liver, lung (especially for CF(Cystic Fibrosis) treatment), or localized cancer gene therapy. Although the risk of serious disease following natural adenovirus infection is rare and the viral genome would not integrate into the host genome, gene therapy by adenoviral vectors has caused serious bad side effects and even death of some patients.[37-40] Recently, in addition to safety of these vectors, several essential genes have been deleted so that viral replication can only occur under control and also most of the viral genome is deleted to obtain sufficient space for 38 kb of transgene particles, this kind of adenoviruses are called “gutless” or “pseudo” adenoviruses.

Adeno-associated vectors

Adeno-associated vectors (AAV) are like adenoviral vectors in their features but because of having some deficiency in their replication and pathogenicity, are safer than adenoviral vectors.[41] In human, AAVs are not associated with any disease. Another special character of AAV is their ability to integrate into a specific site on chromosome 19 with no noticeable effects cause long-term expression *in vivo*. The major disadvantages of these vectors are complicated process of vector production and the limited transgene capacity of the particles (up to 4.8 kb). AAVs have been used in the treatment of some diseases, such as CF, hemophilia B, Leber congenital amaurosis, and AAT (Alpha-1 antitrypsine) deficiency.[41-44]

Helper-dependent adenoviral vector

Helper-dependent adenoviral vector (HdAd), called also as “gutless” or “gutted” vector, are last generation of adenovirus vectors.[35] The disadvantages of the first-generation AdV, such as a packaging capacity limitation (8 kb), immunogenicity, and toxicity, could be overcome, with the development of high-capacity “gutless” Advs (HC-AdV). In this helper-dependent vector system, one vector (the helper) contains all the viral genes required for replication but has a

conditional gene defect in the packaging domain. The second vector contains only the ends of the viral genome, therapeutic gene sequences, and the normal packaging recognition signal, which allows selectively packaged release from cells.[46] Therefore, this helper-dependent system reduces toxicity but helps prolonged gene expression of up to 32 kb of foreign DNA in host cells. Nowadays, gutless adenovirus is administered in different organs, such as muscle, liver, and central nervous system.[45-51]

Hybrid adenoviral vectors

Hybrid adenoviral vectors are made of the high transduction efficiency of a gene-deleted adenoviral vector and the long-term genome-integrating potential of adeno-associated and retroviruses viruses. Such hybrid systems show stable transduction and limited integration sites.[52,53] Among integrating vectors, those derived from retroviruses are most common. One of the family of Retroviridae are called spuma retroviruses or foamy viruses (FVs). FVs are a group of apparently nonpathogenic nonhuman retroviruses, which have been developed only recently.[54,55] The potential advantages of FV vectors include a broad range of hosts, the largest packaging capacity of any retrovirus, and the ability to persist in quiescent cells. Because of these features, FVs have the unique potential to safely and efficiently deliver several genes into a number of different types of cells.[56,57]

Herpes simplex virus

Herpes simplex virus (HSV) is one of the recent viruses candidate in gene delivery. HSV systems include the development of the so-called disabled infectious single copy (DISC) viruses, which comprise a glycoprotein H defective mutant HSV genome. When the defective HSV propagated in complementing cells' viral particles are generated, they can infect in subsequent cells permanently replicating their own genome but not producing more infectious particles.[58] Herpes vectors can deliver up to 150 kb transgenic DNA and because of its neuronotropic features, it has the greatest potential for gene delivery to nervous system,[59] tumors, and cancer cells.[60-64]

Lentiviruses

Lentiviruses are a subclass of retroviruses. They have recently been used as gene delivery vectors due to their ability to naturally integrate with nondividing cells, which is the unique feature of lentiviruses as compared with other retroviruses, which can infect only the dividing cells. Lentiviral vectors can deliver 8 kb of sequence. Because lentiviruses have strong tropism for neural stem cells, extensively used for *ex vivo* gene transfer in central nervous system with no significant immune responses and no unwanted side effects. Lentiviral vectors have the advantages of high-efficiency infection of dividing and nondividing cells, long-term stable expression of a transgene, low immunogenicity, and the ability to accommodate larger transgenes.[65-67]

There are numerous examples of effective long-term treatment of animal models of neurologic disorders, such as motor neuron diseases, Parkinson, Alzheimer, Huntington's disease, lysosomal storage diseases, and spinal injury.[68-73]

Poxvirus vectors

Poxvirus vectors are members of the Poxviridae family that are widely used for high-level cytoplasmatic expression of transgenes. The high stable insertion capacity (more than 25 KB) of this virus is the most advantageous feature of it for gene delivery. The insertion of the transgene sequences is somewhat different from the other vector systems and utilizes homologous recombination or *in vitro* ligation for construction of recombinant vaccinia virus vectors.[74-76] Poxviruses have been used for cancer therapy in various studies, such as prostate cancer, colorectal cancer, breast cancer, and lung cancer.[77,78] Recombinant vaccinia virus vectors were also used for expression of *E6* and *E7* genes of human papilloma virus types 16 and 18 in cervical cancer patients to induce tumor regression.[79]

There are some problems in utilizing poxviruses for gene delivery because of their complex structure and biology, so further studies are required to improve their safety and to reduce the risk of cytopathic effects.

Epstein–Barr virus

Epstein–Barr virus as a herpes virus can be used for the expression of large DNA fragments in target cells. Because *Epstein–Barr virus* (EBV) establishes itself in the host nucleus in a latent state as extrachromosomal circular plasmid, this virus is suitable for long-term retention in the target cell.[80-82] Because of the natural B-cell tropism of the virus, EBV-derived vectors, such as B-cell lymphoma, have been tested for immune therapy of cancer.[83]

However, other types of viruses are under investigation to date and recently, many more different virus vector systems are being developed. These are derived from vaccinia virus, human cytomegalovirus, EBV, but as mentioned earlier, problems, such as their mutagen and carcinogen properties and long-term maintenance, are major limitations in utilizing the viral vectors in gene therapy.

NONVIRAL DELIVERY SYSTEMS

Nonviral systems comprise all the physical and chemical systems except viral systems and generally include either chemical methods, such as cationic liposomes and polymers, or physical methods, such as gene gun, electroporation, particle bombardment, ultrasound utilization, and magnetofection. Efficiency of this system is less than viral systems in gene transduction, but their cost-effectiveness, availability, and more importantly less induction of immune system and no limitation in size of transgenic DNA compared with viral system have made them more effective for gene delivery than nonviral delivery systems to date.[84,85]

Physical methods of nonviral gene delivery

Physical methods applied for *in vitro* and *in vivo* gene delivery are based on making transient penetration in cell membrane by mechanical, electrical, ultrasonic, hydrodynamic, or laser-based energy so that DNA entrance into the targeted cells is facilitated.

Naked DNA

Naked DNA alone is able to transfer a gene (2–19 kb) into skin, thymus, cardiac muscle, and especially skeletal muscle and liver cells when directly injected,[86,87] also it has been applied directly.[87] Long-term expression has been observed in skeletal muscle following injection for more than 19 months. Single injection yields transgenic expression in less than 1% of total myofibers of the muscle but multiple injection would improve it. Although naked DNA injection is a safe and simple method, its efficiency for gene delivery is low so it is only proper for some applications, such as DNA vaccination.

DNA particle bombardant by gene gun

DNA particle bombardant by gene gun is an ideal alternative technique to injection of naked DNA. Gold or tungsten spherical particles (1–3 μm diameter) are coated with plasmid DNA and then accelerated to high speed by pressurized gas to penetrate into target tissue cells.[88] Actually it is a modification of a technique called "biolistic," originally developed for plant transgenesis, but now used for *in vitro* and *in vivo* gene delivery into mammalian cells too,[89,90] such as skin, mucosa, or surgically exposed tissue and especially for DNA-based immunization or vaccination.[91]

Electroporation

Electroporation is temporary destabilization of the cell membrane targeted tissue by insertion of a pair of electrodes into it so that DNA molecules in the surrounding media of the destabilized membrane would be able to penetrate into cytoplasm and nucleoplasm of the cell[92,93] but unfortunately the trangene can integrate only to 0.01% of the treated cells.[94] Electroporation has been used *in vivo* for many types of tissues, such as skin, muscle, lung,[95-97] HPRT gene delivery,[98] and tumor treatment.[99] There are some problems in this method too that the more important are the difficulty in surgical procedure in the placement of electrodes into the internal tissues and that the high voltage applied to tissue might damage the organ and affect genomic DNA stability.[100]

Hydrodynamic

Hydrodynamic is a simple and highly efficient method for direct intracellular delivery of any water-soluble compounds and particles into internal organs.[101] The efficiency of this simple method *in vivo* is higher than any other nonviral system. This method has been successful for gene delivery into rodent liver and expression of hemophilia factors,[102] cytokines,[103] erythropoietin,[104] and hepatic growth factors,[105] in mouse and rat but it has been successful only in small animals and not in human.

Ultrasound

Ultrasound can make some nanomeric pores in membrane to facilitate intracellular delivery of DNA particles into cells of internal organs or tumors, so the size and concentration of plasmid DNA have great role in efficiency of the system.[106,107] The most important limitation of the system is low efficiency of it, especially *in vivo*.

Magnetofection

Magnetofection is a simple and efficient transfection method that has the advantages of the nonviral biochemical (cationic lipids or polymers) and physical (electroporation, gene gun) transfection systems in one system while excluding their inconveniences, such as low efficiency and toxicity. In this method the magnetic fields are used to concentrate particles containing nucleic acid into the target cells.[108,109] In this way, the magnetic force allows a very rapid concentration of the entire applied vector dose onto cells, so that 100% of the cells get in contact with a significant vector dose. Magnetofection has been adapted to all types of nucleic acids (DNA, siRNA, dsRNA, shRNA, mRNA, ODN,…), nonviral transfection systems (transfection reagents) and viruses. It has been successfully tested on a broad range of cell lines, hard-to-transfect and primary cells.[110,111]

Chemical nonviral delivery systems

Chemical systems are more common than physical methods and generally are nanomeric complexes, which include compaction of negatively charged nucleic acid by polycationic nanomeric particles, belonging to cationic liposome/micelle or cationic polymers. The nanomeric complex between a cationic liposome or micelle and

nucleic acids is called lipoplex; but polyplex is the nanomeric complex formed between a cationic polymer and nucleic acids. These nanomeric complexes are generally stable enough to produce their bound nucleic acids from degradation and are competent to enter cells usually by endocytosis.[112] Cationic nonviral delivery systems have several advantages compared to other nonviral systems and especially viral vectors, such as low toxicity and antigenicity because they are made of only biological lipids, long-term expression with less risk of insertional oncogenesis but still low efficiency is the disadvantage of this system as well. Generally cationic lipids are included in 6 subcategories:

(1) Monovalent cationic lipids
(2) Polyvalent cationic lipids
(3) Guanidine containing
(4) Cholesterol derivative compounds
(5) Cationic polymers: Poly(ethylenimine) (PEI)
Poly-l-lysine) (PLL)
Protamine
Other cationic polymers[113]
(6) Lipid–polymer hybrid

Mechanism of gene delivery by cationic particles

The mechanism of gene delivery by cationic systems includes 4 steps:

(1) Nonspecific interaction between cationic particles and cell surface
(2) Endocytosis into endocytosis vesicles (endosomes)
(3) Compaction and release of the DNA particle from endosomes
(4) Translocation of the DNA particle to nucleus by membrane receptors and transgenic expression of it.[114]

For targeting of cationic particles various cell-targeting legends are covalently attached to a lipid anchor (in lipoplexes) or a DNA-binding cationic polymer (in polyplexes),[115] including proteins,[116-118] antibodies,[119,120] small chemical compounds,[121] carbohydrates,[122] peptide ligands,[123] and vitamins,[124] some of these ligands have enhanced the vector efficiency from 10- to 1000-folds. When lipoplex or polyplex particles made association with cell surface, they would enter the cell by endocytosis. It seems more of the lipid particles in early endosomes become trapped in lysosomes and degenerate by nucleases so the interaction of endosome with lysosome is a consensus and lipoplex or polyplex particles should be released before contraction of lysosome to endosome, so fugenic peptides can help it, these peptides originating from viruses can cut off the endosomal membrane to release the genomic DNA leading to increase of genetic translocation efficiency of the liposome.[125]

In this section we focus mainly on the 2 most common cationic particles: Cationic lipids and cationic polymers:

Cationic liposomes

Cationic liposomes are the more important current nonviral polycationic systems, which compact negatively charged nucleic acids lead to the formation of nanomeric complexes. Cationic liposomes have unique characteristics, such as capability to incorporate hydrophilic and hydrophobic drugs, low toxicity, no activation of immune system, and targeted delivery of bioactive compounds to the site of action.[126-129] But the rapid degradation of liposomes due to the reticuloendothelial system and the inability to achieve sustained drug delivery over a prolonged period of time are 2 drawbacks of these delivery systems that have been overcome by modification of the surface of liposomes with hydrophilic polymers, such as polyethylene glycol (PEG)[128] and integration of the pre-encapsulated drug-loaded liposomes within depot polymer-based systems.[130]

All liposomes have 1 or 2 fatty acids and alkyl moieties that are 12–18 carbons in length, in addition to a positively charged polar head group hydrophobic groups, this hydrophobic structure causes the cationic lipids. Since the first monovalent cationic lipid, DOTAP, was synthesized by Felgner *et al.* in 1987,[131] hundreds of new cationic liposome/micelle systems have been reported for gene delivery *in vitro* or *in vivo*. The routine way to prepare a lipoplex is mixing the solution of plasmid DNA and liposome in a proper buffer. The gene delivery efficiency of liposomes is dependent on the size, structure, and even the amount of the liposome, the charge ratio between transgenic DNA and cationic liposome, presence of helper lipid, and the structure and proportion of it and cell type.

As mentioned earlier, cationic systems are mad of either a single synthetic cationic amphiphile (cytofectin), such as DOTAP, DOTMA, DOSPA, DOGS, or more commonly of a combination of a cationic amphiphile and a neutral lipid, such as DOPE and cholesterol, these neutral helper lipids unstabilize the endosomal membrane to facilitate lipid exchange and membrane fusion between lipoplexes and endosomal membrane leading to more gene expression.[132,133] Cationic liposome-mediated delivery of DNA materials is optimal *in vivo* when the mol ratio of cationic liposome to nucleic acid in the lipoplex mixture is such that the positive/negative charge ratio is around 1 or greater[134-136] and *in vitro* the optimal ratio is closer to 1.[137-140] However, multivalent lipids with long and unsaturated hydrocarbon chains are more efficient than monovalent cationic lipids with the same hydrophobic chains.[141]

Cationic liposomes are being used in gene delivery into lung, skeletal muscles, spleen, kidney, liver, testis, heart, and skin cells.[141-148]

For gene transfer *in vivo*, many complexes (in equimolar ratios) are used that the more general ones are Chol/DOPE (1:1), DOTMA/DOPE (1:1), and DOTAP/DOPE (1:1).

Liposome-based technology has progressed from the first-generation conventional vesicles to stealth liposomes, targeted liposomes, and more recently stimuli-sensitive liposomes.[149,150] These new generation of liposomes overcome most of the challenges encountered by conventional liposomes, such as the inability to escape from immune system, toxicity due to charged liposomes, and low half-life stability.[151-153]

Cationic polymers

Cationic polymers at first were introduced by Wu *et al.* 1987[184] as PLL, the same year of synthesizing the first cationic lipids, and were further expanded by a second generation, PEI by Behr *et al.* in 1995.[154] To date a variety of linear or branched cationic polymers have been synthesized, including PLL-containing peptides, endosomolytic peptides (histidine-rich peptides), fusogenic peptides, nuclear localization peptides (mono partite NLS(Nuclear localization signal), bipartite NLS, nonclassical NLS), proteosomes.[155] However, PLL is still the most widely studied cationic polymer and has been used in a variety of polymerizations of lysine ranging from 19 to 1116 amino acid residues (3.97–233.2 kDa). While the molecular weight of the polymer increases, the net positive charge of it also increases and are therefore able to bind DNA tighter and form more stable complexes, totally. There is a relationship between the length of the polymer, gene delivery efficiency, and toxicity as the length of the polymer increases, so does its efficiency and its toxicity.[155,156] However, the efficiency of PLL-mediated polyplexes are low when the PLL is used alone so some conjugation agents are used to facilitate cellular uptake *in vitro* (as EGF(fibroblast growth factor) or transferring) or endosomal escape *in vivo* (as fusogenic peptides or defective viruses). Also the attachment of PEG to the polymer can prevent plasma protein binding and increase circulation of half-life of the complex.[157,158] Different homogenous PLL-conjugated peptides have been developed that have low toxicity, higher efficiency, and site-specific attachment of ligands used for cell targeting.[159-162] The optimal peptide sequence contains 18 lysines followed by a tryptophan and alkylated cysteine (AlkCWK18). A variety of branched forms of cationic peptides with a lysine as branching point have been explored.[162]

PEI is the most important cationic polymer next to PLL. PEI is one of the most positively charged dense polymers, synthesized in linear (LPEI) or branched (BPEI) form, which have high transfection activity *in vitro* and moderate activity *in vivo* but the linear forms have low toxicity and high efficiency than branched forms.[163] As PLL, conjugation of some agents, such as galactose, anti-CD3 antibodies and RGD motif-containing peptides can facilitate PEI polyplex cellular uptake.[164-166] Two advantages of PEI is that it forms toroidal polyplex particles, which are stable to aggregation in physiological buffer conditions, PEI also has a strong buffering capacity at almost any pH because of the great number of primary, secondary, and tertiary amino groups.[167] One disadvantage of PEI is its nonbiodegradable nature[168] and its serious toxicity *in vivo* (in contrast to cationic liposome/micelle). There are conflicting associations between the gene delivery efficiency and PEI toxicity, such as PLL, the most active PEI is 25 k for BPEI and 22 k for LPEI.[169] Unfortunately, due to this property there are some limitations in the application of PEI in nonviral vector *in vivo* delivery. More biodegradable cationic polymers, such as aminoesters have been explored that have less toxicity than PEI and PLL.[170] However, as mentioned earlier, there are a variety of new cationic polymer groups but each of them have some advantages and disadvantages.[155] The notable factors for *in vivo* application are toxicity and transfection efficiency.

Lipid–polymer systems

Lipid–polymer systems are 3-part systems in which DNA is first precondensed with polycations and then coated with either cationic liposomes, anionic liposomes, or amphiphilic polymers with or without helper lipids.[171-174]

CONCLUSION

Although numerous viral and nonviral gene delivery systems have been developed in the last 3 decades, all of them have some disadvantages that have made some limitations in their clinical application and yet no delivery system has been designed that can be applied in gene therapy of all kinds of cell types *in vitro* and *in vivo* with no limitation and side effects; however, some delivery systems has been explored, which can be efficient for gene delivery to specific cells or tissues. So it seems that the process of developing successful delivery systems, especially nonviral systems, for use in *in vivo* is still in its adolescence and more efforts are needed. Totally, key steps effective in improving the currently available systems include the following: (1) improving extracellular targeting and delivery, (2) enhancing intracellular delivery and long-time

expression, and (3) reducing toxicity and side effects on human body. However, clinical successes in 2009–2011 have bolstered new optimism in the promise of gene therapy. These include successful treatment of patients with the retinal disease Leber congenital amaurosis,[175-178] X-linked SCID,[179] ADA–SCID,[180] adrenoleukodystrophy,[180] and Parkinson's disease.[181]

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