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Advances in Gene Delivery Systems

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Abstract

The transfer of genes into cells, both *in vitro* and *in vivo*, is critical for studying gene function and conducting gene therapy. Methods that utilize viral and nonviral vectors, as well as physical approaches, have been explored. Viral vector-mediated gene transfer employs replication-deficient viruses such as retrovirus, adenovirus, adeno-associated virus and herpes simplex virus. A major advantage of viral vectors is their high gene delivery efficiency. The nonviral vectors developed so far include cationic liposomes, cationic polymers, synthetic peptides and naturally occurring compounds. These nonviral vectors appear to be highly effective in gene delivery to cultured cells *in vitro* but are significantly less effective *in vivo*. Physical methods utilize mechanical pressure, electric shock or hydrodynamic force to transiently permeate the cell membrane to transfer DNA into target cells. They are simpler than viral- and nonviral-based systems and highly effective for localized gene delivery. The past decade has seen significant efforts to establish the most desirable method for safe, effective and target-specific gene delivery, and good progress has been made. The objectives of this review are to (i) explain the rationale for the design of viral, nonviral and physical methods

for gene delivery; (ii) provide a summary on recent advances in gene transfer technology; (iii) discuss advantages and disadvantages of each of the most commonly used gene delivery methods; and (iv) provide future perspectives.

1. Introduction

Gene delivery is an essential first step for studies that require expression of an exogenous gene in a target cell. Because of its large size and anionic property, DNA carrying the therapeutic gene(s) must cross the membrane barrier and reach the nucleus of the target cells where gene expression takes place. For effective gene delivery *in vivo*, it is also necessary to overcome nuclease-mediated degradation and DNA uptake by nontargeted cells after systemic administration of the exogenous DNA.

Currently, three categories of gene delivery methods are available: (i) viral vector based; (ii) nonviral vector based; and (iii) physical methods. Viral vectors are mostly replication deficient and genetically modified viruses. Viral vectors have the advantage of high delivery efficiency to a variety of cells and were employed in the first gene therapy clinical trial in 1990.[1] Nonviral vectors, such as synthetic and natural compounds, are less toxic than the viral vectors, offer flexibility in the size of gene they can deliver and have low immunogenicity. The physical methods employ physical force to facilitate gene transfer into cells. Compared with viral vectors, the nonviral and physical methods developed so far exhibit relatively low delivery efficiency, especially for *in vivo* gene delivery. The following is a brief summary of the recent advances in each of the three gene delivery systems.

2. Virus-Based Gene Delivery Systems

Almost always, virus-based gene delivery is accomplished by using replication-deficient viruses containing the gene of interest, but with the disease-causing sequences deleted from the viral genome.[2] Both RNA and DNA viruses have been utilized.[3] The primary feature of RNA-based viral vectors, such as retroviruses, is that they are capable of long-term transgene expression through gene integration.[4,5] DNA-based viral vectors normally result in transgene expression in episomal form without integration.[6] Major features of the most commonly used viral vectors are summarized in table I.

2.1 Retroviral Vectors

The most commonly used RNA viral vectors are derived from retroviruses. These are enveloped viruses with a diameter of 80–130 nm and a genome size of 8–11 kilobases (kb).[4] The viral genome is encased within the capsid along with integrase and reverse transcriptase. Retroviral vectors are produced simply by replacing replication elements with the gene of interest but retaining all necessary RNA regions, primarily the long terminal repeat (LTR) that plays an important role in packaging, reverse transcription, integration and transcription regulation. The retroviral vectors are produced in packaging cells that provide all essential viral proteins in *trans*. The vectors themselves have all the viral genes deleted and can accept up to 7–10 kb of an exogenous gene sequence.[4,5] A successful gene delivery by retroviral vectors involves interaction of viral envelope proteins with appropriate receptors of the host cells, and fusion of lipid membrane between the viral particles and the host cells. Entry of the viral core into the cell allows the reverse transcriptase to convert the viral genome to a double-stranded DNA provirus, which is then inserted into a host chromosome with the help of integrase.[3,4] To date, retroviral vectors have been used more than any other gene transfer vehicle for gene therapy of severe combined immunodeficiency (SCID) using the *ex vivo* approach.[1,7-9] RNA viral vectors under development include oncoretroviruses encoding structural genes of *gag*, *pol* and *env*, and lentiviruses and spumaviruses, which contain additional viral proteins.[4,5]

2.1.1 Oncoretroviral Vectors

The first generation of retroviral vectors was developed from the murine leukaemia virus. The vectors themselves had all the viral genes deleted and were fully replication deficient. However, a significant overlap existed between the vector and the packaging components, which represented a high risk of recombination to yield replication-competent retroviruses (RCRs). To minimize RCR production, an early improvement was to split the packaging components, placing the *gag, pol* and *env* genes into two separate plasmids.[10] The risk of LTR overlap-based creation of RCRs was reduced by the use of heterologous promoters and polyA signal in the packaging construct.[11] Improvement was made in the titre (number of colony-forming units per mL) by establishing stable packaging cells and by the development of transient expression systems that were capable of producing high titres.[12]

The major advantage of retroviral vectors is that they integrate into the host genome, but this is a double-edged

Table I. Features of virus-based gene delivery systems

Vector	Genetic material	Cloning capacity (kb)	Advantages	Disadvantages
Oncoretrovirus	Single-stranded RNA	8	Highly effective for dividing cells High transduction efficiency	Random integration Low titre
Lentivirus	Single-stranded RNA	8	Highly effective in dividing and nondividing cells High transduction efficiency Sustained gene expression Low immune response	Random integration
Foamy virus	Single-stranded RNA	9.2	Highly effective in dividing cells Forms a stable transduction intermediate in nondividing cells High transduction efficiency Sustained gene expression No expression of viral proteins Low immune response	Random integration
First generation adenovirus	Double-stranded DNA	8–10	Highly effective in dividing and nondividing cells High transduction efficiency	Transient expression Host immune response
Helper-dependent adenovirus	Double-stranded DNA	~37	Highly effective in dividing and nondividing cells Large gene loading capacity Essentially no integration	Transient expression Host immune response
Adeno-associated virus	Single-stranded DNA	4–5	Highly effective in dividing and nondividing cells Nonpathogenic Sustained gene expression No integration Low immune response	Integration may occur Low loading capacity
Herpes simplex virus	Double-stranded DNA	~30	Highly effective in the CNS Large gene loading capacity No integration Sustained gene expression	Transient expression Low transduction efficiency

sword.[13] On the one hand, it allows for the possibility of stable and long-term gene expression that can be passed to the daughter cells but, on the other hand, the possibility of insertion into pre-oncogene or the site for inactivation of tumour suppressor genes renders the risk of subsequent tumour development. Indeed, five of twenty patients who received *ex-vivo* transduced, autologous CD34+ haematopoietic progenitor cells for the treatment of X-linked SCID, developed T-cell leukaemia between 23 and 68 months after treatment.[14] The disease was fatal for one patient and was cured in four others. All five cases featured an uncontrolled proliferation of a specific subtype of T cells caused by the deregulated expression of oncogene (*LMO2*) as a result of the integration of provirus.[15] The enhancer activity of the viral LTR (used to drive therapeutic gene expression) had transactivated the oncogene.[16] Detailed analysis has revealed that the retroviral vectors inserted their sequence into actively transcribed genes in a semi-random manner (including both promoter and gene coding region). On the basis of these observations, the LTR enhancer element in the vectors was removed and the internal promoter added for a safer gene transfer.[17]

Recently, Aiuti et al.[8] reported the clinical outcome of gene therapy for adenosine deaminase deficiency (ADA) SCID. In this trial, ADA-expressing retroviral vectors were transfused into CD34+ bone marrow cells *ex vivo* and eight of ten patients have essentially been treated. Their blood continues to show ADA after the median follow up of 4.0 years (range 1.8–8.0 years), and the patients are no longer in need of enzyme-replacement therapy. No leukaemia developed in any of these patients, suggesting that both vectors and the transgene played the important roles in causing adverse effects in patients.

2.1.2 Lentiviral Vectors

Lentiviral vectors have been the most studied retroviral vectors for gene delivery in recent years. It is molecularly modified lentiviruses that belong to a subclass of retroviruses that have three to six additional accessory viral proteins that regulate viral gene expression and infectivity, in addition to three essential *gag, pol* and *env* gene products.[18] These viral proteins facilitate an active transport of the pre-integration complex through the nucleopore; thus, lentiviral vectors do not require the breakdown of the nuclear membrane[19] and are able

to transduce nondividing cells.[20,21] Lentiviruses favour transgene integration near active transcription sites, while oncoretroviruses preferentially integrate at the transcription start site.[11,22] Without any accessory genes, current lentiviral vectors are considered reasonably safe, because the replication of the vectors is highly inhibited and the possibility of recombination is reduced.[22,23] Recent work showed successful gene transfer by lentiviral vectors into quiescent T and B lymphocytes for immunotherapy of several genetic dysfunctions of the haematopoietic system.[24-27]

Results from an ongoing lentivirus-based gene therapy trial in France for β-thalassaemia showed that one patient had not required blood transfusions for the past 16 months.[28] Another successful use of lentiviral vector in a clinical trial was reported for the treatment of X-linked adrenoleukodystrophy due to a deficiency of the *ABCD1* gene.[29] In this trial, progressive cerebral demyelination in two patients was successfully blocked for 14–16 months after lentiviral delivery of wild-type *ABCD1* to CD34+ cells *ex vivo*.[29] These results encourage more clinical investigation into the use of lentiviral vectors for human gene therapy.

2.1.3 Foamy Viral Vectors

Recently developed foamy viral vectors are modified foamy viruses (FVs), which are the largest retroviruses with a genome size over 13 kb and are capable of packaging 9.2 kb of nucleic sequences.[30-32] FVs infect dividing cells, can also form a stable transduction intermediate in nondividing cells, do not require entry of the core for reverse transcription and have no preference for integrating into the genome.[33] Recent work demonstrated the therapeutic potential of FV vectors in haematopoietic stem cell gene therapy.[34,35] FV vector-based delivery of the Fanconi anemia complementation group C gene in a murine Fanconi anaemia model showed functional correction of haematopoietic stem cells.[35] Bauer et al.[34] reported the successful gene therapy to the canine leukocyte adhesion deficiency (CLAD) model by administrating the FV vectors expressing canine CD18. Four of the five dogs treated showed complete reversal of the CLAD phenotype, which was sustained for more than 2 years. Clinical trials of FV vector-based human gene therapy have not been reported.

2.2 DNA Viral Vectors

2.2.1 Adenoviral Vectors

The most studied DNA viral vectors are modified adenoviruses that were discovered in 1953 as the causative agents of the common cold.[36] In 1977, Graham et al.[37] developed a cell line enabling the first production of recombinant adenoviruses in a helper-free environment. Since that time, adenoviral vectors have received much attention as gene transfer agents.[6] There are over 100 adenoviral serotypes identified that can infect and replicate in a wide range of organs, including 51 classified into six subgroups (A–F).[38,39] Of these, serotypes 2 and 5 in subgroup B are the most characterized viruses.

Adenovirions are icosahedral in shape, 70–90 nm in diameter and not enveloped. The viral genome is large, consisting of a double-stranded DNA molecule of 36–38 kb in size. Viral DNA replication and transcription is complex, and the viral replication and assembly occur only in the nucleus of the infected cells. There are nine major complex transcription units divided into early (E1A, E1B, E2–E4) and late (L1–L5) transcripts flanked by inverted terminal repeat (ITR). As the E1A region encodes the principal protein that activates the expression of other adenoviral transcription units' genes, and E1 proteins play essential roles in viral replication,[40] the *E1* gene was replaced with the transgene in the first generation of adenoviral vectors.[41,42] Combining the E1 and nonessential region of E3, the cloning capacity of adenoviral vectors can be increased up to 8.3 kb.[43]

Adenoviral infection is a highly complex process and is independent of cell cycle. Viral fibre proteins bind to the Coxsackievirus and adenovirus receptor of the target cells to initiate the infection.[39,44] Internalization occurs via receptor-mediated endocytosis followed by release from the endosome.[45] Once inside the cytoplasm, the viral capsid undergoes disassembly as it migrates to the nuclear pore. Nuclear entry of the viral DNA is completed upon capsid dissociation, leading to an active transcription and replication within the nucleus episomally.

In principle, recombinant adenoviral vectors are prepared from two components: a viral DNA vector and a packaging cell line. The first generation of adenoviral DNA vector is a plasmid DNA that contains a portion of the viral genome with E1 and E3 regions deleted and the desired gene sequence cloned into the space of the deletion in the genome. The adenoviral vector is produced using either *in vitro* homologous recombination or ligation. Both the adenoviral vector and the plasmid carrying the necessary viral genes are co-transfected into the packaging cell line. These DNA species are then left to undergo homologous recombination within the cells resulting in vector production. The 293 cells that constitutively express the *E1* gene are utilized for viral vector production as a packaging cell. Generally, approximately 10^{13}–10^{14} viral particles can be produced by fifty 150 mm cell culture plates (about a billion 293 cells).[46]

Although adenoviral vectors are of human origin, numerous other animals have been used to demonstrate efficient gene

transfer, including mice, rats, pigs, rabbits and nonhuman primates. Many types of cells can be transduced depending on the route of administration. Direct injection into the peritoneum, kidney, pancreas, cerebral spinal fluid, skeletal muscle, brain, cardiac muscle, coronary artery and many other tissues, results in transgene expression. However, intravenous injection into rodents results primarily in transgene expression in the liver and spleen. Typically, transgene expression that results from the first generation of adenoviral vectors is transient regardless of the route of administration and the type of cells.[47] Expression usually peaks in 1–7 days and declines rapidly to an undetectable level by 2–4 weeks. Both cellular and humoral immune response against viral proteins is observed and is believed to be responsible for the short duration of transgene expression.[48,49]

Viral DNA included in the vectors is believed to be responsible for transient gene expression. Methods have been examined to eliminate all adenoviral genes and the helper-dependent or 'gutless' adenoviral vectors have been developed that carry no viral sequences except for 100 bp of ITR, which is required for replication, and the *cis*-acting packaging signal. Those removed viral proteins are supplied from the replication incompetent (helper) viruses, *in trans*, in helper cell lines, such as HEK293 cells.[50,51] Through such an improvement, helper-dependent adenoviral vectors have a large cloning capacity up to 37 kb and have an improved safety profile, which results in sustained transgene expression *in vivo*. The persistent gene expression has been shown in mice and baboons for more than 1 year with little decline.[52,53] It is clear from these studies that the toxicology of the helper-dependent adenoviral vectors is significantly less than that of the first generation of adenoviral vectors. While the gutless vectors exhibit much improved properties for gene delivery, the remaining challenge for further development of gutless vectors is to improve vector production and the immunogenicity intrinsic to viral proteins.

Adenoviral vectors have been extensively studied for cancer gene therapy. Recently, efforts have been made in the development of oncolytic adenoviral vectors that are capable of replicating in tumour cells. Replication of adenoviral vectors in cancer cells amplifies the initial viral inoculum, resulting in the destruction of the infected tumour cells. H101 (Shanghai Sunway Biotech, Shanghai, China), the E1b55K-deleted oncolytic adenoviruses, have been approved as a drug in China for the treatment of cancer.[54] A response rate of 79% (41/52) in patients injected with H101 while maintaining chemotherapy has been reported in a phase III trial.[54] In the US, there are several clinical trials involving oncolytic adenovirus such as ONYX-015 (Onyx Pharmaceuticals, Emeryville, CA, USA).[55]

2.2.2 Adeno-Associated Viral Vectors

Adeno-associated virus (AAV) vectors are derived from a nonpathogenic parvovirus, a satellite virus of human adenovirus and the herpes simplex virus (HSV).[56,57] There are 12 human serotypes and more than 100 serotypes of AAVs from nonhuman primates identified with different targeting capacities.[58] Among these serotypes, the AAV serotype 2 (AAV2) was the first clone identified and used to deliver transgene.[59] The small icosahedra virion is approximately 18–26 nm in diameter and contains a single strand DNA of 4–5 kb. The virion contains either the sense or antisense strand of the DNA and appears to have no strand preference. There are two genes in viral genome DNA, *rep* and *cap*, which encode seven major transcription units, *Rep40, Rep52, Rep68, Rep78, VP1, VP2* and *VP3*, respectively. The viral genome has a palindromic sequence at each end, referred to as the ITR. These ITRs are important in the site-specific integration of AAV DNA into a specific site in chromosome 19,[60] although AAV exist primarily in episomal form. The ability of the wild-type AAV to selectively integrate into chromosome 19 made it an attractive candidate as a vector for the treatment of genetic diseases. Rep proteins are required for replication and packaging, and VP proteins are structural proteins forming viral capsid. The viral particle enters the cell through receptor-mediated endocytosis followed by migration and release of its genome into the nucleus. It is worth noting that each serotype of AAV has its unique approach for infecting the host cells. Single-stranded DNA is converted to double-stranded vector genome from which the transgene is expressed.

Construction of AAV vectors consists of the recombinant AAV vector plasmid DNA replacing the viral *rep* and *cap* genes with transgene sequences, and a non-rescueable AAV helper plasmid that encodes for the AAV capsid proteins. The AAV vector is produced by a plasmid-based system analogous to retroviral plasmid vectors but, in this case, the gene of interest is bracketed by ITRs. Also required is either a wild-type adenovirus or an HSV, and a cell line for viral propagation.[59,61] Unlike the vector systems described in this section, the cell line does not need to contain any portion of the AAV genome since all required genomic elements are provided by the two plasmids. Cells are first infected with the wild-type adenovirus or HSV, and then both recombinant AAV vector plasmid DNA and the helper plasmid are co-transfected into the cells. The cells produce mature recombinant AAV vectors as well as wild-type adenovirus or HSV. The wild-type adenovirus or HSV is removed by either density gradient centrifugation or heat inactivation. Further improvement to reduce the risk of production of a wild-type virus was made by establishing the helper

virus-free method. In this system, the transfection of mini-adenovirus helper plasmid together with vector and packaging plasmid into the adenovirusE1-expressing HEK293 cells is achieved using a standard procedure of transfection.[62,63]

The target-organ preference depends on the infectivity of wild serotype to those organs. AAV1, 2 and 5, preferentially transduce into neuronal and muscular cells and AAV8 prefer hepatocytes.[58] As AAV vectors do not contain the *rep* gene, the majority of the AAV genome stays in episomal form in the host cell nucleus. The toxicity of AAV vectors is much lower than other viral vectors.

Phase I studies were performed using AAV2 vectors for gene therapy of haemophilia B, initially through intramuscular injection and later by intraportal injection to target liver cells.[64,65] Factor IX protein was detected in two cases and sustained for 4–9 weeks.[66] The transient gene expression is likely caused by the activation of the memory CD8+ T cells against the AAV capsid protein, rather than Factor IX protein.[66] Additional efforts are needed to reduce the immune response and extend its packaging capacity of the AAV imposed by the small size of the AAV genome (4681 nt), and inefficient delivery of genes that are larger than its genome.

2.2.3 Herpes Simplex Viral Vectors

HSV is a human neurotropic virus with an overall diameter of 180–200 nm. Its genome is a double-stranded DNA molecule of about 150 kb in length, encoding over 80 proteins.[67] The virus itself is transmitted by direct contact and replicates in the skin or mucosal membranes before infecting cells of the CNS.[67] It exhibits both lytic and latent activity. The lytic cycle results in viral replication and cell death. The latent infection allows for the virus to be maintained in the host for an extended period of time. The virus enters cells by fusion triggered by binding of viral glycoprotein *gB* and *gD* to the heparin sulfate moiety of the cell surface protein.[68] DNA enters the cell and circularizes.

A similar strategy to that of producing adenoviral vectors has been used to generate HSV vectors.[68] A few of the immediate early protein genes were removed to generate viral vectors unable to replicate except in a complementing cell line. Because of its large genome size, the recombinant HSV vectors should be capable of containing an inserted gene up to 30 kb.[67,69] Since HSV is a neurotropic virus, many studies have shown persistent gene expression in neuronal cells, and clinical trials for gene therapy of brain tumours have been performed using HSV vectors.[70,71] Recent studies revealed that the HSV vector-mediated gene transfer resulted in blockade pain transmission or reversal of the chronic pain state *in vivo*.[72,73]

2.3 Other Types of Viral Vectors

In addition to the more commonly used viral vectors described in the previous sections, many other viruses have been considered as a potential carrier for gene delivery. While most of them have not been used in clinical studies, there is an abundance of developmental and preclinical data demonstrating the various merits of these systems. Several members of the alphavirus genus, including the Semliki Forest virus, Sindbis virus and Venezuelan equine encephalitis, have been developed into gene delivery vectors. While their use has not been widespread, and problems related to virus-induced toxicity have been seen, their prominent neurotropism has made them attractive for use in gene delivery to cells in the CNS. In addition, efforts to blend properties of different viral vectors to obtain new and more desirable vectors are seen.[3] One active area is the combination of AAV and adenoviruses. A double-stranded AAV genome inserted into an adenoviral capsid to produce a combination of AAV and adenoviral ITR, has been shown to transduce and integrate in cells.[74] A different approach involves the placement of an AAV vector into gutless adenoviruses and engineering the vector to transiently express Rep protein in an attempt to get site-specific integration and to avoid insertional mutagenesis.[75] Other hybrid viral vectors include HSV/AAV and HSV/Epstein-Barr virus.[76] Interestingly, several groups have attempted to coat the viral vectors with polymers or lipids.[77] The primary objective of the coating is to hide the virus from the host immune system upon *in vivo* administration, which in turn prevents degradation and increases vector circulation time and, subsequently, tissue transduction. Another function of viral encapsulation is to retarget viral vectors by ablating its native tropism and then re-targeting the virus by conjugating targeting ligands directly to the polymers.

3. Nonviral Vector-Based Gene Delivery

Another strategy for gene delivery is based on the use of natural or synthetic compounds (also called nonviral vectors) in which complexes of DNA, proteins, polymers or lipids are formed in particles capable of efficiently transferring genes into cells. Delivery of genetic material using nonviral vectors preceded the development of viral-based vectors. The nonviral approach for gene delivery can be traced back to the work of Avery, MacLeod and McCarthy who showed a change of cellular phenotype following exogenous DNA exposure.[78] The first nonviral technique to gain wide acceptance was calcium phosphate-mediated transfection.[79] This system has undergone little change since being developed in the early 1970s.

Table II. Features of nonviral gene delivery systems

Method	Critical features for gene delivery	Advantages	Disadvantages
Cationic lipid	Cationic charge	High efficiency *in vitro*, easy to prepare	Low efficiency *in vivo*, may provoke an acute immune response
Cationic polymer	Cationic charge	Highly effective *in vitro*, easy to prepare	Toxic to cells, low efficiency *in vivo*, may provoke an acute immune response
Lipid-polymer hybrid system	Cationic charge	Highly effective *in vitro*	Low activity *in vivo*
Needle injection	Mechanic	Simple	Low efficiency, expression limited to needle track
Gene gun	Pressure	Good efficiency	Gene transfer limited to targeted area, needs surgical procedure for internal organs
Electroporation	Electric pulse	High efficiency	Tissue damage, gene transfer limited to targeted area, needs surgical procedure for internal organs
Sonoporation	Ultrasound	Site specific	Low efficiency, tissue damage
Laser assisted	Laser beam	Site specific	Low efficiency, tissue damage
Magnetofection	Magnetic field	Site specific	Low efficiency
Hydrodynamic delivery	Hydrodynamic pressure	Simple, high efficiency, site specific	Needs catheter insertion in large animals

Since then, the nonviral vectors earned their candidacy for gene therapy and various clinical trials are ongoing.[80,81] The major characteristics of the nonviral approach for gene delivery are summarized in table II.

3.1 Liposome-Based Nonviral Vectors

The most effective nonviral vectors developed so far are made of liposomes. Liposomes were first described in 1965 as a model of cellular membrane,[82] and were quickly applied to the delivery of substances to cells. They are microscopic particles consisting of one or more concentric lipid bilayers enclosing an aqueous compartment and are formed spontaneously when a film of lipids is hydrated in an aqueous solution. Many different types of liposomes have been developed in the past and liposomes composed of cationic lipids are found to be most active in gene delivery.[83] These lipids are made of mono- or multicationic head groups and a hydrophobic anchor (alphatic chains or cholesterol moiety) bridged together by a linker.[84] Depending on the chemical structure and the conditions for liposome preparation, these cationic lipids can be used to make cationic liposomes with or without additional lipids (helper lipids). A variety of positively charged lipid formulations are now commercially available and many others are under development (for reviews see Liu et al.[85] and Montier et al.[86]).

Cationic liposomes and DNA interact spontaneously to form complexes (lipoplexes) with 100% DNA loading efficiency. In other words, all of the DNA molecules are complexed with the liposomes, provided enough cationic liposomes are available. It is believed that the negative charges of the DNA interact with the positively charged groups of the liposomes. The lipid to DNA ratio, and overall lipid concentrations used in forming these complexes, are extremely important for efficient gene delivery and vary with applications.[87]

Liposomes offer several advantages in delivering genes to cells as follows: (i) being synthetic, they are relatively cheap to make and do not cause disease; (ii) they offer a degree of protection to the DNA from nuclease-mediated degradation; (iii) they can carry large pieces of DNA, potentially as large as a chromosome; and (iv) can be targeted to specific cells or tissues. In addition, liposomes overcome problems inherent with viral vectors with regard to immunogenicity and replication-competent virus contamination. The ability to synthesize a wide variety of lipids has resulted in a highly adaptable and flexible system capable of gene delivery both *in vitro* and *in vivo*. As liposome technology is further developed, it should be possible to produce reagents with improved *in vivo* gene delivery into specific tissues. Successful delivery of DNA and RNA to a variety of cell types has been reported, including tumour, airway epithelial cells, endothelial cells, hepatocytes, muscle cells and others by intratissue or intravenous injection into animals.[88-90]

Various liposome-based vectors have been utilized in a number of clinical trials for cancer treatment. For example, Stopeck et al.[91,92] reported the results of phase I and II clinical trials assessing the safety and efficacy of Allovectin-7® (Vical, San Diego, CA, USA), a plasmid DNA carrying *HLAB7* and

β_2-microglobulin genes complexed with DMRIE/DOPE liposomes. Currently, this immunotherapeutic is in phase III development for patients with stage III or IV melanoma. The remaining challenge for liposome-based gene delivery is to enhance their *in vivo* delivery efficiency.

3.2 Polymer-Based Nonviral Vectors

Cationic polymers constitute another category of nonviral vectors that have been used for gene delivery. A significant number of polymers, such as polyethylenimine,[93-95] polyamidoamine,[96-98] polyallylamine,[99] chitosan,[100] dendrimers,[101] cationic proteins[102] and peptides,[103] have been studied for *in vivo* and *in vitro* gene delivery. These cationic polymers condense DNA into small particles and prevent DNA from degradation. The cellular uptake is via receptor-mediated endocytosis. Similar to cationic lipids, polymers are also easy to make and flexible for additional modifications. Combining 'stealth' properties of polyethylene glycol for prolonged half-life in blood with cationic polymer for DNA condensation has resulted in a very attractive property of DNA/polymer complexes (polyplexes) for gene delivery to tumours.[104] In fact, polyplexes are being investigated in phase I and phase II clinical trials for the treatment of cystic fibrosis and ocular degenerative diseases.[105]

Hybrids of lipids with polymers have been explored to improve gene delivery.[106] In this system, DNA is pre-condensed with either poly-L-lysine,[107] protamine,[108] histone or several synthetic polypeptides,[109] followed by lipid wrapping using either cationic liposomes, anionic liposomes or amphiphilic polymers.[110] Additional efforts are needed to prove that the lipid/polymer hybrid system is superior to that of lipoplexes or polyplexes for *in vivo* gene delivery.[111]

4. Gene Delivery Using Physical Approaches

Gene delivery using physical principles, commonly called physical methods, has attracted increasing attention in recent years. It usually employs a physical force to overcome the membrane barrier of the cells and facilitates intracellular gene transfer. An obvious advantage of physical methods is the simplicity. A fragment of DNA or plasmid containing transgene, and the regulatory elements for its expression, is directly delivered into cells without involving any substances that could be cytotoxic or immunogenic as commonly seen in viral or nonviral vectors.

4.1 Gene Delivery by Needle Injection

The simplest physical method for gene delivery is by direct injection of DNA through a needle-carrying syringe into tissue. Following the initial report of successful gene transfer to muscle cells in mice by Wolff et al.,[112] tissues that exhibit transgene expression following a plasmid DNA injection have been expanded to skin,[113] cardiac muscle,[114] liver[115] and solid tumour.[116] The major application of this gene delivery method is DNA vaccination.[117] The shortfall of this procedure is low gene delivery efficiency, and the transfected cells are limited to the needle track. Some efforts have been made to achieve a higher level of transgene expression by optimizing plasmid construct.[118]

4.2 Ballistic DNA Injection

This method of gene delivery is also known as particle bombardment, microprojectile gene transfer or gene gun, and was first developed for gene transfer into plants in 1987.[119] Since its initial introduction, it has been modified to transfer genes into mammalian cells both *in vitro* and *in vivo*.[120] The principle of this method is to propel DNA-coated gold particles against cells and force intracellular DNA transfer. The accelerating force for DNA-containing particles can be a high-voltage electronic discharge, spark discharge or helium pressure discharge. Ballistic DNA injection has been successfully used to transfer genes into a wide variety of cell lines. *In vivo* applications have predominantly focused on the liver, skin, muscle or other organs that can be surgically exposed.[121] Ballistic DNA injection also offers the capacity to deliver precise DNA dosages. Unfortunately, genes delivered by this method are expressed transiently, and there is considerable cell damage occurring at the centre of the discharge site. The gun-based gene delivery method is more appropriate for gene delivery to skin for vaccination[122-125] and immune therapy[126] because of the shallow penetration of DNA. This method has been utilized in vaccination against the influenza virus[127] and in gene therapy for treatment of ovarian cancer.[128]

4.3 Electroporation

Electroporation was first utilized for gene transfer to mammalian cells by Neumann et al.[129] Gene delivery is achieved by generating pores on a cell membrane through electric pulses. Electroporation works best on cells that are suspended in solution, but also works on cells in solid tissue where electrodes can be applied.[130] Gene delivery efficiency is determined by the pulse intensity, duration and frequency.[131] *In vivo* electroporation creates transient permeability of the cell membrane and induces a low level of inflammation at the injection site, facilitating DNA uptake by parenchyma cells and antigen-presenting cells.[132] Thus, this method has been

clinically tested for DNA-based vaccination and for cancer treatment.[132-135] Currently, clinical trials are ongoing in the treatment of melanoma, prostate cancer, papilloma virus infection, hepatitis C virus infection and HIV infection.[135] The limitation of the current procedure is that the number of cells transfected is relatively small and that surgery is required to reach internal organs.

4.4 Sonoporation

The sonoporation gene delivery technique was developed in the 1990s.[136-138] It utilizes ultrasound to temporarily permeabilize the cell membrane to allow cellular uptake of DNA. Unlike other nonviral methods, sonoporation combines gene delivery with the possibility of restricting the effect to the area where ultrasound is applied. Gene delivery efficiency appears to be controlled by pulse intensity, frequency and duration.[139] Sonoporation-mediated gene delivery has been demonstrated in the cornea,[140] brain,[141] CNS,[142-144] bone,[145] peritoneal cavity,[146] kidney,[147] pancreas,[148] liver,[149,150] embryonic tissue,[151] dental pulp,[152] muscle[153,154] and heart.[155,156] More recent studies in mouse liver showed that inclusion of gas-filled microbubbles enhanced gene delivery efficiency.[149,150] Similar results were also obtained in tumour,[157-159] vascular tissue[160] and skeletal muscles.[161] Compared with other nonviral approaches, sonoporation-mediated gene delivery remains in its infancy. Additional efforts are needed to improve its efficiency, especially for *in vivo* applications.

4.5 Laser-Assisted Method (Photoporation)

The photoporation method, first reported by Zeira et al.,[162] employs a single laser pulse as the physical force to generate transient pores on a cell membrane to allow DNA to enter. Gene delivery efficiency appears to be controlled by the size of the focal point and pulse frequency of the laser. The level of transgene expression reported was similar to that of electroporation. In recent years, several advances have been made to improve gene delivery efficiency, one of which involved the use of carbon black nanoparticles to generate photoacoustic force upon laser stimulation.[163] More study is needed before this highly sophisticated procedure becomes a practical technique for gene delivery, not only *in vitro* but also *in vivo*.

4.6 Magnetofection

Magnetofection utilizes a magnetic field to promote transfection. Magnetofection employs magnetic nanoparticles made of iron oxide and coated with cationic lipids or polymers to complex with DNA through electrostatic interaction. The magnetic particles are then concentrated on the target cells by the influence of an external magnetic field. Similar to the mechanism of nonviral vector-based gene delivery, the cellular uptake of DNA is accomplished by endocytosis and pinocytosis.[164] It is postulated that DNA are released into the cytoplasm depending on the composition of the magnetic nanoparticles.[165] Magnetofection has been successfully applied to a wide range of primary cells and cells that are hard to transfect using other nonviral methods.[166] Recent work using a local injection of the nanoparticles into the gastrointestinal track and the ear vasculature[167] imply that this well accepted method for *in vitro* gene delivery may be applicable to *in vivo* gene delivery.

4.7 Hydrodynamic Gene Delivery (Hydroporation)

The hydrodynamic gene delivery method was reported by Liu et al.[168] and Zhang et al.[169] in 1999 and, since then, it has become one of the most commonly used methods for gene delivery to hepatocytes in rodents. Intrahepatic gene delivery is achieved by a rapid injection of a large volume of DNA solution via the tail vein in rodents that results in a transient enlargement of fenestrae, generation of a transient membrane defect on the plasma membrane and gene transfer into hepatocytes.[170] In mice, the optimal condition for gene delivery includes an injection volume equal to 8–10% of bodyweight, an injection time of 5–7 seconds and <20 μg of plasmid DNA per mouse.[168] Because of its simplicity, high efficiency and reproducibility, hydrodynamic gene delivery has been utilized for the delivery of DNA, small interfering RNA, proteins, small compounds and even viral vectors.[171-179] This technique has been widely used for gene therapy studies, gene knockdown, functional analysis of genetic elements and for establishing a disease model in animals.[171,176,180]

In an attempt to apply this simple procedure of gene delivery to the clinic, efforts have been made to reduce the total injection volume. Eastman et al.[181] demonstrated in rabbits that a volume of 15 mL/kg can be safely injected into an isolated rabbit liver. Similar studies have been reported by several groups using pigs as an animal model.[172,182-184] A computer-controlled injection device has been developed for hydrodynamic gene delivery in large animals.[185] Using this system, Suda and colleagues[185] have reported safe and efficient gene delivery in pig liver, kidney and muscle. By combining the computer-controlled injection device with the image-guided catheterization technique, Kamimura et al.[186] has

demonstrated lobe-specific gene delivery to the liver of pigs. Kamimura et al.[187] also assessed the effectiveness of the procedure in gene delivery to skeletal muscle in pigs. Comprehensive assessment of tissue damage has been conducted for hydrodynamic gene delivery to liver and muscles in small and large animals and the results showed no major tissue damage.[186,187] The image-guided, lobe-specific hydrodynamic procedure has great potential to become the method of choice for human gene therapy without using viral or nonviral vectors.

5. Conclusions and Future Perspectives

The three gene delivery systems described in this review are not inclusive, but represent those commonly used in preclinical and clinical research or at an advanced stage of development. Considering the clinical trials conducted so far, 67% employed viral vectors and approximately 24% were performed using nonviral methods.[188] Although the methods summarized in this review have made and will continue to make important contributions in research and gene therapy, a number of different viral and nonviral vectors, some of which have yet to be fully exploited or even discovered, are likely to complement the current armamentarium. Practically, no single gene delivery method is likely to be optimal for all gene delivery needs. For a specific application, however, a 'perfect' method could be developed for gene delivery through noninvasive delivery routes, capable of targeting to the desired cells with high delivery efficiency and the appropriate amount of gene product for a desirable period of time.

Significant progress has been made in each of the three gene delivery systems. However, the success of gene therapy still needs a significant effort made to develop a safe and effective method applicable to human gene therapy. While viral vectors are highly effective and have been used in a few clinical trials, the intrinsic property of viral genome and proteins in stimulating carcinogenesis and an immune response remains the largest hurdle.[189,190] The key to this problem may lie in the effort to find a mechanism capable of integrating transgenes into a selected site in a chromosome and, thus, avoiding the need for repeated administration and insertion mutagenesis. For nonviral vector-based gene delivery, a variety of natural and synthetic compounds has been used in gene delivery studies, but their effectiveness in gene delivery remains orders of magnitude lower than that of viral vectors. As a result, current nonviral vectors are generally considered unacceptable for clinical use. Nevertheless, studies designed to identify factors critical for successful gene delivery *in vitro* and *in vivo* have provided important information to guide the future design of nonviral vectors. The concept of tailor-made gene carriers for gene delivery to specific types of cells may hold the key for future success. Gene delivery using a physical approach is relatively new, but has demonstrated its potential to transfer DNA into cells directly. The techniques described in this review have both positive and negative features, and it is unlikely that a single approach will be ideal for all applications. With the involvement of new technology and computer systems, and improved understanding of biological systems, solving technical problems of current physical methods is highly feasible. It is highly likely that the future of gene delivery for gene therapy lies in the hands of those who are able to integrate the principles of cell biology, engineering and computer sciences.

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