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Review Article

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Nonviral Gene Delivery: Principle, Limitations, and Recent Progress

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Abstract. Gene therapy is becoming a promising therapeutic modality for the treatment of genetic and acquired disorders. Nonviral approaches as alternative gene transfer vehicles to the popular viral vectors have received significant attention because of their favorable properties, including lack of immunogenicity, low toxicity, and potential for tissue specificity. Such approaches have been tested in preclinical studies and human clinical trials over the last decade. Although therapeutic benefit has been demonstrated in animal models, gene delivery efficiency of the nonviral approaches remains to be a key obstacle for clinical applications. This review focuses on existing and emerging concepts of chemical and physical methods for delivery of therapeutic nucleic acid molecules *in vivo*. The emphasis is placed on discussion about problems associated with current nonviral methods and recent efforts toward refinement of nonviral approaches.

KEY WORDS: gene delivery; gene therapy; lipoplex; nonviral vectors; polyplex; transfection.

INTRODUCTION

Gene transfer, the technique to introduce new genetic materials to hosts, has become an invaluable experimental tool to study gene function and its regulation, to establish various disease models, to acquire DNA-based immunization, and finally, to explore potential therapeutic applications to various acquired or inherited diseases. Naked DNA molecules do not enter cells efficiently because of their large size and hydrophilic nature due to negatively charged phosphate groups. In addition, they are very susceptible to nuclease-mediated degradation. Therefore, the primary challenge for gene therapy is to develop carriers (commonly called vectors) and physical methods that facilitate gene transfer to targeted cells without degradation of the delivered gene.

Recombinant viruses such as retrovirus, lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus have been widely utilized as vectors for gene transfer (1). Viruses mediate efficient gene transfer through their favorable cell uptake and intracellular trafficking machineries. However, viral vectors have several intrinsic drawbacks including difficulty in production, limited opportunity for repeated administrations due to acute inflammatory response, and delayed humeral or cellular immune responses. Insertional mutagenesis is also a potential issue for some viral vectors that integrate foreign DNA into the genome.

The nonviral gene delivery methods, on the other hand, use synthetic or natural compounds or physical forces to deliver a piece of DNA into a cell. The materials used are generally less toxic and immunogenic than the viral counterparts. In addition, cell or tissue specificity can be achieved by harnessing cell-specific functionality in the design of chemical or biological vectors, while physical procedures can provide spatial precision. Other practical advantages of nonviral approaches include ease of production and the potential for repeat administration. Nonviral methods are generally viewed as less efficacious than the viral methods, and in many cases, the gene expression is short-lived. However, recent developments suggest that gene delivery by some physical methods has reached the efficiency and expression duration that is clinically meaningful. The purpose of this article is to provide an update and concise review in the field of nonviral gene delivery. Particular emphasis will be on the rate-limiting steps that affect the overall transfection and current efforts and strategies to overcome these limitations.

EXTRA- AND INTRACELLULAR BARRIERS FOR GENE DELIVERY

Several anatomical and cellular barriers limit the overall efficiency of gene transfer by nonviral methods (Fig. 1). Anatomical barriers are epithelial, endothelial cell linings and the extracellular matrix surrounding the cells that prevent direct access of macromolecules to the target cells. Professional phagocytes such as Kupffer cells in the liver and residential macrophages in the spleen are largely responsible for the clearance of DNA-loaded colloidal particles administered through blood circulation. In addition, various nucleases existing in blood and extracellular matrix can

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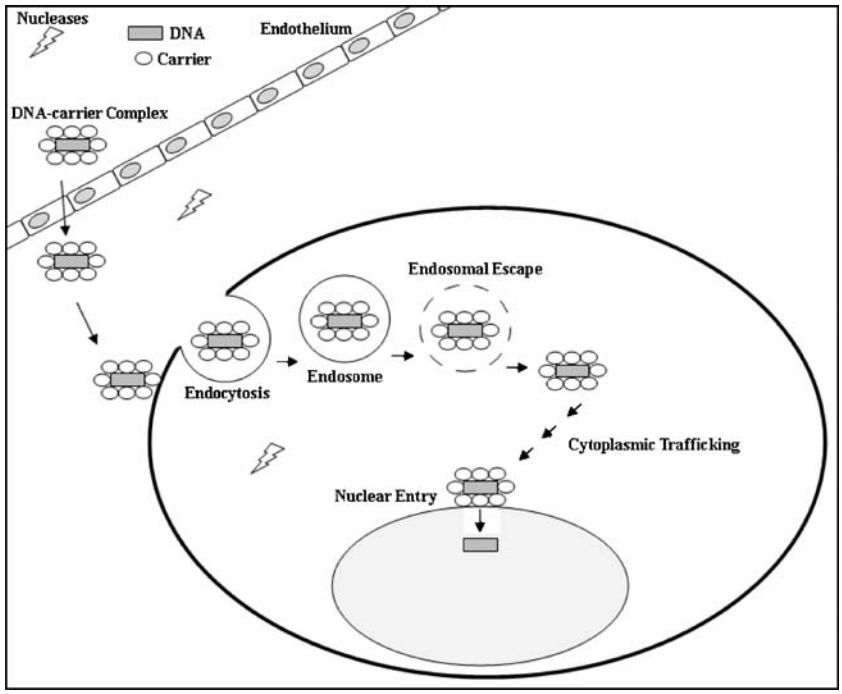

Fig. 1. Schematic representation of barriers limiting nonviral gene transfer *in vivo*

rapidly degrade free and unprotected nucleic acids following systemic administration. Crossing plasma membrane is considered the most critical limiting step for an efficient DNA transfection. Nucleic acids typically cannot pass through cell membrane unless their entry is facilitated by creating transient holes by physical meanings (2), or through various active cell uptake mechanisms such as endocytosis, pinocytosis, or phagocytosis (3).

Upon being taken up via endocytosis, macromolecules captured within the endosomes usually transform into digestive lysosomes unless some escape mechanisms are used to intercept this maturation process. Two escape mechanisms have been explored. The first involves the use of membrane active or fusogenic molecules such as fusion peptides (4) or lipid components with acid-sensitive bond and large hydrophobic portion of the molecules to rupture the endosome membrane (5). The other mechanism acts on building up osmotic pressure within the endosomal compartment that eventually triggers the swelling or burst of endosomal vesicles (6). Weak amine compounds such as chloroquine and cationic polymers (polyethyleneimines (PEI) and partially degraded polyamidoamine dendrimers) absorb protons and slow down the acidification process that is essential for endosome–lysosome transition (7). Consequently, the influx of chloride counter ions builds up osmotic pressure inside the endosomes. For polyester-based carriers such as poly(lactic-co-glycolic acid), the breakdown products by hydrolysis can also build up the osmotic pressure inside the endosome which leads to the release of the contents trapped therein. Several other attempts have been used to increase the rate of endosome release, among which are codelivery of inactivated viral particles or recombinant viral capsule proteins that possess endosomolytic activities (8), and the use of photochemically generated free radicals to cause membrane damage (9).

Upon their release from endosomes, DNA molecules in their free form or as complexes must travel through cytoplasmic space filled with viscous protein solution and a network of cytoskeleton matrix toward the nucleus where transcription takes place. Observations made by direct intracellular microinjection of naked DNA prove that the movement by diffusion is slow and inefficient, and the resulting levels of gene expression are very weak (10). The nuclear envelope represents an important barrier for the entry of DNA. This double-membrane envelope is interrupted by large protein structures called nuclear pore complexes (NPC) which regulate transport through nuclear envelope. NPC have diameters of ~9 nm, which allow free diffusion of ions and molecules of small to medium sizes, such as proteins of up to 40–60 kDa, or nucleic acids of up to ~300 bp, but restrict larger macromolecules passing through freely (11). For resting cells, nuclear uptake of large proteins is an active transport process mediated through sequence-specific recognition of nuclear localization signal peptide (NLS) sequence in their structures by importin proteins (12). Protein-NLS/importin complexes dock at the NPC to allow nuclear entry of DNA. The entry is achieved indirectly through the NLS sequences of transcription factors that are associated with the DNA molecules. For replicating cells, most DNA molecules enter the nuclei through the process of dissolution and reorganization of the nuclear envelope during mitosis (13). Finally, the unpacking of DNA–carrier complexes could constitute yet another rate-limiting step after transfection. Cationic lipids dissociate from DNA through lipid mixing and exchange with host cell lipids at the cytoplasm entry step, while DNA complexes formed with cationic polymers, such as PEI, remain stable after endosome escape. An interesting concept has been reported recently, under which the intracellular trafficking of DNA-loaded nanoparticles is coupled with microtubule-directed transport mechanism (14). The polymer–DNA complexes disintegrate later in nucleus (15).

PERSISTENCE OF GENE EXPRESSION

Generally, the duration of gene expression from plasmid DNA delivered by cationic lipids or polymers is biphasic in nature, with a dramatic, yet transient expression that only lasts for a few days, followed by a prolonged gene expression at a lower level. Several reasons may contribute to the initial rapid decline. The plasmid-based transgene in most cases stays in the nucleus as an episomal DNA molecules without substantial chance to integrate into the host genome. Such molecules in dividing cells do not replicate and will eventually be diluted away as the population of replicating cells grows. Another possibility could be that the recipient cells are killed due to injuries occurred during the transfection process or that transfected cells undergo programmed cell death due to exposure of the transfection agent, or the degradation products of DNA (16). Inflammation response after transfection may also contribute to shortened duration of transgene expression *in vivo*, either by promoting the clearance of injured cells or through promoter downregulation (17). Immune response to cells expressing foreign proteins may also lead to the elimination of transfected cells. On the other hand, plasmids being physically intact and persistent in the nuclei for a few months to 1 or 2 years have been reported in animals after direct DNA injection in skeletal muscles (18), or through hydrodynamic injection to the liver (19), suggesting that long-term expression from simple plasmids in resting cell populations is possible under certain circumstances. In these cases, promoter shutdown has been suggested as the major cause of a transient nature of gene expression. Certain ubiquitous strong viral promoter sequences such as the cytomegalovirus immediate early promoter/enhancer sequence are silenced over time (20). This could be due to the status of the key transcriptional factors that are most active after cell injury or under an inflammatory response, but not very active at the resting state. Possible gene silencing due to DNA methylation in the promoter region has also been proposed (21). The use of tissue-specific promoters in addition to native or viral scaffold/matrix attachment region has resulted in sustained gene expression (22). The plasmids that provided long-term expression typically have large sizes in comparison to the regular plasmids (23). However, delivery of linearized short DNA fragments has been shown to be beneficial in some studies (24). It was shown that these sequences can be ligated by the host DNA repair mechanisms into large-sized oligomers. Unmethylated CpG sequences, a characteristic of DNA of bacteria origin, could stimulate innate immune response. Plasmids deplete of these CpG sequences (25) or minicircles that contain only the functional part of the plasmid with less CpG contents have been shown to prolong the expression duration (26).

In addition to episomal form of expressive DNA, stable transfection by using DNA integration into genomic DNA has been explored to prolong the duration of gene expression. Retroviral integrase (27), sleeping beauty (28), or phage-derived recombinase (29) have been studied for the use of site-specific integration to the host chromosomes. Favorable low insertion mutation rate has been demonstrated using these systems when compared to retroviral integrases.

PHYSICAL NONVIRAL GENE DELIVERY METHODS

Over the past decade, many physical methods have been investigated for gene transfer. These methods facilitate the transfer of genes from extracellular to nucleus by creating transient membrane holes/defects using physical forces such as local or rapid systemic injection, particle impact, electric pulse, ultrasound, or laser irradiation.

Needle and Jet Injection

Localized injection of naked DNA was first demonstrated intramuscularly in 1990 (30) and afterward in several other tissues, including liver, skin, and brain. DNA uptake is mostly localized in the area where needle track is, suggesting that physical damage induced by needle insertion is responsible in part for the uptake of DNA. Different agents such as transferrin, water-immiscible solvents, nonionic polymers, surfactants, or nuclease inhibitors have been tested to enhance the overall gene expression by this procedure (31–34).

From application standpoint, this procedure is particularly attractive because of its simplicity and lack of safety concerns. Direct injection of therapeutic genes into muscle or skin has been a very useful tool for evaluation of various aspects of DNA-based vaccination. Intramuscular injection of plasmid DNAs coding for interleukin-12 and carcinoembryonic antigen gene was able to improve the antitumor immunity (35). A phase I/II clinical trial examining the therapeutic effect of direct myocardial injection of VEGF-2 gene to patients suffering a severe ischemic heart disease has generated some positive results including improved exercise capacity (36). As of March 2009, more than 18.1% (n=278) of clinical trials in gene therapy were conducted using this method (http://www.wiley.co.uk/genetherapy/clinical/).

The jet injection was first described in 1947 as a needle-free drug delivery method in contrast to the conventional needle injection (37). Jet injection is accomplished through a high-speed, ultrafine stream of DNA solution driven by a pressurized gas, usually CO_2. The injection generates pores on membranes of target cells and allows intracellular gene transfer. The penetration power in this method can be controlled by the gas pressure depending on the mechanical properties of the target tissues. The standard procedure for jet injection includes loading of DNA solution (usually 3–5 μl), choosing the strength of the pressure (usually 1–3 bars), aiming the injector directly to the target tissue, and finally, pulling the trigger. Several jet injectors varying in their capacities of injection volume and the modes of repetitive injection have been used for gene transfer. A direct comparison between needle injection and various types of jet injectors showed that the levels of gene expression by jet injection are 50-folds higher than conventional needle injection (38). The models with low-volume injectors are more suitable than those with high-volume injectors in carrying out multiple injections (39).

The jet injection gene transfer is well tolerated by the target tissues, and no serious side effects have been reported. However, localized pain, edema, and bleeding at the injection site have been reported, particularly when old models of injectors were used (40).

The jet injection-based gene transfer is ideal for DNA-based vaccine development and for topical immunization purpose. In addition, this method has been used to directly transfect skin cancer cells to facilitate conventional chemotherapy. Stein *et al.* have demonstrated that after *in vivo* intratumoral transfer of short hairpin RNA expression vector against multidrug resistance gene 1 (MDR1), a complete reversal of the phenotype of MDR1 and subsequently enhanced the efficacy of chemotherapy for tumor growth inhibition were achieved (41). Furthermore, jet injection has shown its potential for *in vivo* gene delivery studies and is currently under investigation in phase I clinical trial for treatment of skin metastases of breast cancer and malignant melanoma (42).

Hydrodynamic Gene Transfer

The hydrodynamic procedure was reported in 1999 (43). When rapid injection of large volume of DNA solution into a mouse via the tail vein was performed, efficient transfection in liver, lung, kidney, and heart was achieved. The hydrodynamic method employs the high pressure as a driving force for gene transfer. The injection of large DNA volume, 8–12% of body weight in short time (3–5 s), leads to a reversible permeability change in the endothelial lining and the generation of transient pores in hepatocyte membranes allowing the DNA molecules to diffuse internally (44). Up to 30–40% of the hepatocytes can be efficiently transfected (45). Currently, this method is considered to be the most efficient nonviral gene transfer method for *in vivo* gene delivery in rodents. Using this method, it was possible to provide levels of transgene expression close to average levels of physiological gene expression. By using catheter-assisted perfusion, efficient gene transfer can also be achieved in kidney, muscle, or a specific lobe in the liver (46). The simplicity and safety of the hydrodynamic gene delivery allows a wide range of use of this technique for *in vivo* transfection of hepatocytes to study promoter function, gene function, and therapeutic effects of liver-generated secreted proteins in established disease models (47,48).

Until recently, the idea of employing this procedure for *in vivo* human gene delivery was ruled out basically due to the fact that proportionally large injection volume should be used that is beyond an acceptable level for patients. Several modifications have been made to make this procedure less invasive. For instance, inserting a balloon catheter into a vasculature in the targeted tissue followed by injection with lower injection speed and volume has resulted in efficient gene transfer in large animals (49). This suggests that clinical application of the hydrodynamic gene transfer is feasible, particularly after the recent development of computer-controlled injection device (50).

Gene Gun

Gene gun delivery, also called ballistic DNA transfer or DNA-coated particle bombardment, was first used in 1987 for gene transfer in plants (51). This method depends on the impact of heavy metal particles on target tissues and delivery of coated DNA on particles in passing. The particles are accelerated to sufficient velocity by highly pressurized inert gas, usually helium. Macroparticles made of gold, tungsten, or silver have been used for gene delivery through gene gun. Gas pressure, particle size, and dosing frequency are critical factors that determine penetration efficiency to the tissues, the degree of tissue injury, and overall gene transfer levels (52). Gene gun-based gene transfer has been extensively tested for intramuscular, intradermal, and intratumor genetic immunization. It was demonstrated that this approach is able to produce more immune response with lower doses comparing to needle injection in large animal models and in clinical human trials. Goudy *et al.* have shown that vaccination in an animal model, at late preclinical stage of type 1 diabetes, with glutamic acid decarboxylase gene has induced type 2 immunity that consequently resulted in blocking β cell autoimmunity (53). As of March 2009, about 0.3% (n=5) of clinical trials in gene therapy were conducted using gene gun (http://www.wiley.co.uk/genetherapy/clinical/).

Electroporation

The use of an electric field to alter the cell permeability was known since 1960s. However, the first *in vitro* and *in vivo* attempts to utilize electroporation in gene transfer were demonstrated in 1982 (54) and 1991 (55), respectively. *In vivo* electroporation depends on electric pulses to drive gene transfer. These pulses generated transient pores in cell membranes followed by intracellular electrophoretic DNA movement.

Typically, *in vivo* electroporation is conducted by first injecting DNA to the target tissue followed by electric pulses, with varied voltage, pulse duration, and number of cycles, from two electrodes applied. *In vivo* electroporation technique is generally safe, efficient, and can produce good reproducibility compared to other nonviral methods. When parameters are optimized, this method can generate transfection efficiency equal to that achieved by viral vectors (56). In addition to local injection and electroporation, Sakai *et al.* have reported that *in vivo* electroporation can be performed in localized manner after a systemic injection of plasmid DNA. They demonstrated that a localized electroporation at particular lobe of rat liver after systemic injection of plasmid DNA resulted in a widespread transfection in hepatocytes in the treated lobe, but not in the surrounding lobes (57). One of the encouraging applications of electroporation was reported recently by Marti *et al.* who, using the *in vivo* electroporation, demonstrated an enhanced wound healing after transfection of the affected area with keratinocyte growth factor-1 (58). Despite the progress made recently, limitation of the *in vivo* electroporation-mediated gene transfer to solid tissues is the accessibility of the electrodes to the internal organs. More basic research and technological developments are likely to speed its clinical applications.

Sonoporation

The first indication that ultrasound might enhance the transdermal penetration of drugs was demonstrated in 1954 (59). Sonoporation, as the term suggested, is a technique that uses ultrasound waves to create plasma membrane defects by acoustic cavitation. With each ultrasonic cycle, a fraction of the energy of the propagating wave is absorbed by the tissue

resulting in local heating which affects the structure of cell membranes. Tissue absorption to ultrasound waves depends on tissue type and ultrasound frequency and intensity. Most gene delivery studies use the therapeutic ultrasound at frequency of 1–3 MHz with intensity of 0.5–2.5 W/cm^2 (60).

A major improvement in ultrasound-based gene transfer was the combination of ultrasound irradiation with contrast agents or microbubbles (61). Microbubbles are air-filled vesicles stabilized by surface active molecules such as albumin, polymers, or phospholipids. Upon absorption of ultrasound waves, microbubbles cavitate, oscillate, break up, and release local shock waves in the form of high-velocity microjet that can disrupt the nearby cell membranes. This promotes the transient pore formation which, consequently, facilitates local DNA transfer. One of the most commonly used contrast agent is Optison (Molecular Biosystems, San Diego, CA, USA) which consists of gas-filled human albumin microspheres. The size of microbubbles, usually 1–6 μm in diameter, is critical for the efficient transfection and for not being removed by the reticular endothelial system. Smaller-sized nanobubbles have been also employed but were found to require higher frequency ultrasound exposure to be rendered effective. Modification of microbubbles through lipid or polymer coating resulted in enhanced transfection efficiency (62). In general, the efficiency of sonoporation-based gene transfer is dependent on the frequency and intensity of ultrasound irradiation, the presence of contrast agent, DNA concentration, and the duration of exposure. However, enhancement of fluidity of the cell membrane by feeding cells with long-chain unsaturated fatty acids, which facilitates its flexibility and minimizes cellular resistance to sonication, was also suggested to improve the effectiveness of sonoporation (63). The major advantage for sonoporation is its safety, noninvasiveness, and being able to reach internal organs without surgical procedure. Recently, ultrasound has been shown to be able to enhance the permeability of blood–brain barrier (64). Interestingly, targeted gene delivery can be achieved through focused sonoporation using nontargeted microbubbles or through microbubbles equipped with site-specific ligands, such as antibodies or biotin–streptavidin that helps in transferring of microbubbles to certain tissue or organ. Tsunoda *et al.* demonstrated successful *in vivo* gene transfer to injured myocardial tissues using sonoporation after an intraventricular injection (65). Sheyn *et al.* have found the use of ultrasound-mediated gene therapy to facilitate bone tissue regeneration and subsequently bone formation upon transfer of recombinant human bone morphogenetic protein-9 (66).

CHEMICAL-BASED NONVIRAL VECTORS

Chemical vectors such as cationic lipids and cationic polymers form condensed complexes with negatively charged DNA through electrostatic interactions. The complexes protect DNA and facilitate cell uptake and intracellular delivery. Cationic lipids and cationic polymers as gene delivery tools have been well studied, and the subject has been covered by many review articles in great details (67). Below is a brief summary of chemical vectors employed for gene transfer.

Cationic Lipids

Felgner and colleagues pioneered cationic lipid-based gene transfer in 1987 (68). Cationic liposome-mediated gene transfer or lipofection represents the most extensively investigated and commonly used nonviral gene delivery method. Currently, hundreds of lipids have been developed and tested for gene transfer. They share the common structure of positively charged hydrophilic head and hydrophobic tail that are connected via a linker structure. The most commonly seen hydrophilic head groups are primary, secondary, tertiary amines, or quaternary ammonium salts. However, guanidino, imidazole, pyridinium, phosphorus, and arsenic groups have also been developed. The positively charged head group is necessary for binding with negatively charged phosphate groups in nucleic acids. The hydrophobic tails are usually made of two types of hydrophobic moieties, aliphatic chains, cholesterol, or other types of steroid rings. Most of the linkage between the hydrophilic and hydrophobic moiety are ether, ester, carbamate, or amide bonds that can affect the rate of biodegradability. Table I summarizes some cationic lipids used in gene transfer. Transfection efficiency of cationic lipids varies dramatically depending on the structure of cationic lipids (the overall geometric shape, the number of charged groups per molecules, the nature of lipid anchor, and linker bondages), the charge ratio used to form DNA–lipid complexes, and the properties of the colipid (69). When mixed with the negatively charged DNA, the positive-charged liposomes spontaneously form uniquely compacted structures called lipoplexes. In lipoplex structure, DNA molecules are surrounded with positively charged lipids which grant them protection against extracellular or intracellular nucleases. Furthermore, lipoplexes, due to their positive charges, tend to electrostatically interact with the negatively charged molecules of the cell membrane (glycoproteins and proteoglycans) that may facilitate their cellular uptake.

The most commonly used colipids are cholesterol and dioleoylphosphatidylethanolamine (DOPE). The contribution of these "helper" lipids on cationic liposome-mediated gene transfer to a large extent depends on the structure of cationic lipid. Some lipids absolutely require DOPE for an appreciate level of transfection, while some cationic lipids, particularly those with double fatty chains capable of forming bilayer or micellar structures, have transfection activity without any helper lipids. For the latter one, the presence of DOPE in most cases reduces the charge ratio of lipid to DNA needed to reach the maximal transfection *in vitro* in the absence of serum. Most cationic lipids are more or less toxic to cells, and the presence of DOPE could lead to reduced charge ratio, thus less toxicity. Cholesterol-containing cationic liposomes were developed for *in vivo* application. The presence of cholesterol stabilizes the cationic lipidic membrane structures against the destructive activity of serum components and can provide better activity for *in vivo* transfection when serum components are present (70).

The choice of a colipid has a quite dramatic effect on the overall performance of cationic liposomes. In general, a lipid composition that provides high degree of membrane fluidity allows better transfection. It has been found that DOPE as a colipid promotes hexagonal phase lipid polymorphism that is in favor of membrane fusion, lipid mixing, and boost of

Table I. Lipids Commonly Used for Gene Transfer

Lipid	Abbreviation	Feature
1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine	DOPC	Helper
1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine	DOPE	Helper
Cholesterol		Helper
N-[1-(2,3-Dioleyloxy)propyl]*N*,*N*,*N*-trimethylammonium chloride	DOTMA	Cationic
1,2-Dioleoyloxy-3-trimethylammonium-propane	DOTAP	Cationic
Dioctadecylamidoglycylspermine	DOGS	Cationic
N-(3-Aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide	GAP-DLRIE	Cationic
Cetyltrimethylammonium bromide	CTAB	Cationic
6-Lauroxyhexyl ornithinate	LHON	Cationic
1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium	2Oc	Cationic
2,3-Dioleyloxy-*N*-[2(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate	DOSPA	Cationic
1,2-Dioleyl-3-trimethylammonium-propane	DOPA	Cationic
N-(2-Hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide	MDRIE	Cationic
Dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide	DMRI	Cationic
3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)-carbamoyl]cholesterol	DC-Chol	Cationic
Bis-guanidium-tren-cholesterol	BGTC	Cationic
1,3-Dioleoxy-2-(6-carboxy-spermyl)-propylamide	DOSPER	Cationic
Dimethyloctadecylammonium bromide	DDAB	Cationic
Dioctadecylamidoglicylspermidin	DSL	Cationic
rac-[(2,3-Dioctadecyloxypropyl)(2-hydroxyethyl)]-dimethylammonium chloride	CLIP-1	Cationic
rac-[2(2,3-Dihexadecyloxypropyl-oxymethyloxy)ethyl]trimethylammonium bromide	CLIP-6	Cationic
Ethyldimyristoylphosphatidylcholine	EDMPC	Cationic
1,2-Distearyloxy-*N*,*N*-dimethyl-3-aminopropane	DSDMA	Cationic
1,2-Dimyristoyl-trimethylammoniumpropane	DMTAP	Cationic
O,*O*′-Dimyristyl-*N*-lysyl aspartate	DMKE	Cationic
1,2-Distearoyl-sn-glycero-3-ethylphosphocholine	DSEPC	Cationic
N-Palmitoyl D-erythro-sphingosyl carbamoyl-spermine	CCS	Cationic
N-*t*-Butyl-*N*0-tetradecyl-3-tetradecylaminopropionamidine	diC14-amidine	Cationic
Octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride	DOTIM	Cationic
*N*1-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine	CDAN	Cationic
2-(3-[Bis-(3-amino-propyl)-amino]propylamino)-*N*-ditetradecylcarbamoylme-ethyl-acetamide	RPR209120	Cationic

transfection efficiency *in vitro* (71). The fusogenic properties of DOPE facilitate the endosomal escape of lipoplexes through membrane destabilization. In addition, DOPE induces the displacement of the anionic lipids from the cytoplasm-facing monolayer of the endosomal membrane to the opposite direction via a flip-flop mechanism (5,72). Displaced anionic lipids then interact with the endosomally engaged cationic lipids causing the formation of neutral lipids that subsequently help in decomplexation of the DNA from the cationic lipid leading to the release of the transgene in the cytoplasm as a free or as a lipoplex. Although DOPE-containing formulations are among the best for *in vitro* transfection in the absence of serum, the serum components are known to inactivate and destabilize the lipoplex structures that contain DOPE (73).

Due to the excessive surface charge, the circulation half-life of cationic lipids in blood is very short. Systemic elimination of cationic lipids takes place upon formation of larger aggregates via their interactions with the negatively charged serum molecules or cellular components (primarily erythrocytes and platelets). Lipoplexes tend to initially accumulate in the pulmonary vasculature because of the so-called first passage effect (74). However, the lipoplexes redistribute to the liver in about 60 min after the injection owing to active uptake by Kupffer cells. As a result, pulmonary vascular endothelial cells and some airway epithelial cells are predominant cells that are transfected (75). The drawback of a fast clearance of cationic lipids from the circulation limits their utility in gene delivery to cells located beyond vascular endothelial cells. Surface shielding through the use of hydrophilic and charge neutral polymers such as polyethylene glycol (PEG) to reduce excessive charge–charge interaction appears very effective in prolonging the circulation half-life of lipoplexes (76).

It is important to note that the presence of the bulky PEG moiety on the surface prevents an intimate interaction between lipoplexes and cell membrane, thus reduces the overall transfection efficiency. Several strategies have been used to make the PEG shielding conditional and nonpermanent. PEG–lipid conjugates with single lipid anchor of a shorter fatty chain length have been used to make these molecules diffusible over time to achieve a conditional deshielding by kinetic means. Various forms of pH-sensitive or reduction-sensitive chemical bonds have been developed to allow the deshielding within acidified endosome compartments.

Prolonged circulation time due to surface modification makes targeted gene delivery to interstitially located cell populations possible. Successfully targeted delivery of DNA and siRNA to tumor cells using lipoplexes coated with antitransferrin receptor monoclonal antibodies to bind to overexpressed transferrin receptors has been reported (77). Gene transfer of anticancer therapeutic genes, either with tumor-suppresser or enzyme-directed prodrug or by siRNA-based gene knockdown approaches, has been explored as a possible therapeutic intervention for cancer gene therapy. In addition, monoclonal antibody-mediated transcytosis for lip-

oplex-based brain gene delivery has also been studied for gene therapy of neuronal diseases and brain tumor (78).

In addition to systemic application, significant efforts have been made for topical and regional gene and siRNA delivery with lipoplexes to respiratory mucosa and airway epithelial cells for the treatment of cystic fibrosis, acute lung injuries (79), as well as to cornea tissues for degenerative ocular diseases (80).

Major hurdles for practical use of lipoplex-mediated transfection are acute toxicity and short duration of gene expression. Cationic lipids, when combined with unmethylated CpG-containing plasmid DNA or other nucleic acid compositions, can stimulate potent inflammatory response in the hosts. Rapid production of cytokines followed by clearance of transfected cells after the administration of uncoated DNA lipoplexes contributes to both treatment-related toxicity and shortened duration of gene expression. In some extreme cases, a subsequent transfection of the same vectors is less effective because of the cytokine effect from previous transfection. Systematic deletions and mutations of CpG sequences from plasmid sequences have generated some promising data in suppressing the levels of cytokine production after the administration of lipoplexes (81). Surface shielding with PEG–lipid can also minimize the inflammatory response (76,82).

In general, cationic lipids have the advantages of being inexpensive to produce and can be engineered to have targeted specificity. However, their transfection efficiency needs to be further improved, and the significant toxicities such as formation of aggregates in blood and the tendency to induce inflammatory response have to be solved for *in vivo* application. As of March 2009, lipoplexes have been used in clinical trials and represent 7.1% (n=109) of total human gene therapy trials mainly for cancer and cystic fibrosis studies (http://www.wiley.co.uk/genetherapy/clinical/).

Cationic Polymers

Cationic polymers have also been used extensively for gene transfer. Upon mixing with DNA, these polymers form nanosized complexes, often called polyplexes. Typically, polyplexes are more stable than lipoplexes. Table II summarizes some polymers that are commonly used in gene delivery.

Among cationic polymers, PEI is considered one of the most effective polymer-based transfection agents. PEI was first used in gene transfer in 1995 (83). It exists in either branched or linear structures. PEI has a high density of amine groups of which majority are nonprotonated at the physiological pH. The nonprotonated amines in the PEI exert the so-called proton sponge effect, which effectively stops the acidification of the endosomal pH by neutralizing the protons that are pumped by an active membrane transporter, ATPase (6,84). Ultimately, it leads to an influx of chloride counter ions within the compartment and a buildup of osmotic pressure that causes the swelling and rupture of the endosomal membrane.

Transfection efficiency and toxicity of PEI depends on its molecular weight (MW), configuration, and the charge ratio of polymer to DNA used. Several studies showed that high

Table II. Polymers Commonly Used for Gene Transfer

Polymer	Abbreviation	Unique feature
Poly(ethylene)glycol	PEG	Inert
Polyethylenimine	PEI	Cationic
Dithiobis(succinimidylpropionate)	DSP	Biodegradable PEI
Dimethyl-3,3′-dithiobispropionimidate	DTBP	Biodegradable PEI
Poly(ethylene imine) biscarbamate	PEIC	Biodegradable PEI
Poly(L-lysine)	PLL	Cationic
Histidine modified PLL		Biodegradable
Poly(*N*-vinylpyrrolidone)	PVP	Neutral
Poly(propylenimine)	PPI	Dendromer
Poly(amidoamine)	PAMAM	Dendromer
Poly(amido ethylenimine)	SS-PAEI	Biodegradable
Triethylenetetramine	TETA	Cationic
Poly(β-aminoester)		Biodegradable
Poly(4-hydroxy-L-proline ester)	PHP	Biodegradable
Poly(allylamine)		Cationic
Poly(α-[4-aminobutyl]-L-glycolic acid)	PAGA	Biodegradable
Poly(D,L-lactic-co-glycolic acid)	PLGA	Biodegradable
Poly(*N*-ethyl-4-vinylpyridinium bromide)		Cationic
Poly(phosphazene)s	PPZ	Biodegradable
Poly(phosphoester)s	PPE	Biodegradable
Poly(phosphoramidate)s	PPA	Biodegradable
Poly(*N*-2-hydroxypropylmethacrylamide)	pHPMA	Cationic
Poly (2-(dimethylamino)ethyl methacrylate)	pDMAEMA	Cationic
Poly(2-aminoethyl propylene phosphate)	PPE-EA	Biodegradable
Chitosan		Polysaccharide
Galactosylated chitosan		Synthetic chitosan
N-Dodecylated chitosan		Synthetic chitosan
Histone		Natural
Collagen		Natural
Dextran–spermine	D-SPM	Polysaccharide

MW PEI (greater than 25,000 Da) is toxic to cells while polymers with medium to low MW (5,000–25,000 Da) are more efficient and less toxic (85). Synthetic, high MW PEI derived from low MW PEIs by polymerization either through biodegradable disulfide linkages or cross-linked with inert polymers, particularly those biodegradable polymers, have been shown to be more efficacious and less toxic than the PEI of the same MW (86). Polyplexes between DNA and linear PEI are active *in vivo* when administrated intravenously (87). The degree of free amine content, MW, and the polymer-to-DNA ratios and the solution used to prepare the polyplexes are important factors for *in vivo* transfection. Branched PEI, on the other hand, has high toxicity and low transfection efficiency than the polyplexes prepared from linear PEI (88). However, they are useful for forming stable nanoparticles that are suitable for airway gene delivery through aerosol for treating airway epithelial cells or lung cancers (89).

Upon systemic administration, these polyplexes of small particle size tend to aggregate to form larger complexes and accumulate in major tissues including lung and liver. The levels of cytokine induction by polyplexes appear less severe than that of lipoplexes (90). Additional improvement can be achieved through conjugation to an inert polymer such as PEG (5,000 Da), pluronic triblock polymers (P_{127}), or dextran to reduce the nonspecific interactions. Nanoparticles coformulated from biodegradable anionic polymers such as albumin, dextran sulfate, or other anionic polymers to modify the surface charge of the polyplexes are another interesting idea to overcome the toxicity and nonspecific binding issues. Along the same direction, encapsulating the polyplexes inside neutral or anionic liposomes, solid biodegradable nanoparticles, or polymer-based hydrogels has shown promising improvement in either enhancing the transfection activity or reducing cytotoxicity of PEI-based polyplexes.

Recently, more polymers with improved biocompatibility and biodegradability have been reported demonstrating equal or superior performance comparing to nondegradable PEIs. Among these are aminoesters or oligoamines polymerized through disulfide linkages or polyamino acid derivatives with proton absorption capacities (91). Besides PEI and more recent polyamines of varied structures, synthetic or natural polypeptides and their derivatives have been explored as gene delivery vehicles. These include poly(L-lysine) (PLL), polyornithine, polyarginine, histones, and protamines that have excellent ability to condense DNA.

PLL is among the first synthetic polymers being used for constructing target-specific gene carrier by Wu and Wu and several other groups for liver, lung, and tumor-specific gene delivery (92). Recently, PEG–PLL conjugates with defined chemical composition have been shown to be improved vector for DNA formulation and delivery to various organs (93). Such polyplexes are being investigated in phase I and phase II clinical trials for potential treatment of cystic fibrosis and ocular degenerative diseases (94). Improved transfection has been shown using PLL with dendritic configuration and imidazole modified linear PLL (95). Other polymers such as dendromers, chitosans, synthetic amino derivatives of dextran, and cationic acrylic polymers have been shown to possess significant levels of gene transfer activity (96).

Inorganic Nanoparticles

Inorganic nanoparticles are usually prepared from metals (e.g., iron, gold, silver), inorganic salts, or ceramics (e.g., phosphate or carbonate salts of calcium, magnesium, or silicon) (97). The metal ion-based salts produce complexes with typical size range of 10–100 nm in diameter. The surface of these nanoparticles can be coated to facilitate DNA binding or targeted gene delivery. The small particle size offers several advantages including that they usually bypass most of the physiological and cellular barriers and produce higher gene expression (98). They can also be transported through the cellular membranes via specific membrane receptor or nucleolin which delivers nanoparticles directly to the nucleus skipping the endosomal–lysosomal degradation (99). Nanoparticles have the ability to efficiently transfect postmitotic cells *in vivo* and *in vitro*. Additionally, they tend to show no or low toxicity and are inert to immune responses. Supraparamagnetic iron oxide-based nanoparticles can also provide magnetic responsiveness in a magnetic field and can provide magnetic field guided targeted delivery (100). Progress in the *in vivo* application of the inorganic nanoparticles has picked up considerable speed recently. However, extensive studies are still required to assess the effect of their types, sizes, and shapes on transfection efficiency. It is certain that further studies focusing on long-term safety and surface functionalization can accelerate their clinical applications.

FUTURE PERSPECTIVES

Nonviral approaches were developed to facilitate transfer of exogenous genes into target cells without the complication of immunogenicity or insertion mutation commonly seen in viral vectors. These methods differ widely in their transfection efficiency and toxicity. In the past few years, the work continued in developing new nonviral methods, particularly in the area of chemical vectors. However, the last few years showed broad successful applications of the physical methods for *in vivo* gene transfer. As a whole, the transfection efficiency reported so far for the nonviral approaches is still below that of the highly efficient viral vectors. Further improvements to increase the efficiency and reduce the toxicity of nonviral vectors are needed before their clinical implication can be met. These improvement will rely on our better understanding of the limiting steps that nonviral vector must overcome. Developing new vectors that are more target specific will also be necessary. The strategies that merge nonviral and viral vectors might be helpful to achieve more, efficient, long-lasting, and nontoxic gene delivery systems.

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