Themed Issue: Non-viral Gene Delivery: Opportunities and Challenges
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Nonviral Gene Delivery: What We Know and What Is Next

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Abstract

Gene delivery using nonviral approaches has been extensively studied as a basic tool for intracellular gene transfer and gene therapy. In the past, the primary focus has been on application of physical, chemical, and biological principles to development of a safe and efficient method that delivers a transgene into target cells for appropriate expression. This review summarizes the current status of the most commonly used nonviral methods, with an emphasis on their mechanism of action for gene delivery, and their advantages and limitations for gene therapy applications. The technical aspects of each delivery system are also reviewed, with a focus on how to achieve optimal delivery efficiency. A brief discussion of future development and further improvement of the current systems is intended to stimulate new ideas and encourage rapid advancement in this new and promising field.

Keywords: Gene delivery, gene therapy, nonviral vectors, transfection

Introduction

The primary challenge for gene therapy is to develop a method that delivers a therapeutic gene (transgene) to selected cells where proper gene expression can be achieved. An ideal gene delivery method needs to meet 3 major criteria: (1) it should protect the transgene against degradation by nucleases in intercellular matrices, (2) it should bring the transgene across the plasma membrane and into the nucleus of target cells, and (3) it should have no detrimental effects.

Viral vectors are able to mediate gene transfer with high efficiency and the possibility of long-term gene expression, and satisfy 2 out of 3 criteria. The acute immune response, immunogenicity, and insertion mutagenesis uncovered in gene therapy clinical trials have raised serious safety concerns about some commonly used viral vectors. The limitation in the size of the transgene that recombinant viruses can carry and issues related to the production of viral vectors present additional practical challenges. Methods of nonviral gene delivery have also been explored using physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery). Physical approaches, including needle injection,[1] electroporation,[2,3] gene gun,[4,5] ultrasound,[6] and hydrodynamic delivery,[7,8] employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer. The chemical approaches[9-12] use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells. Although significant progress has been made in the basic science and applications of various nonviral gene delivery systems, the majority of nonviral approaches are still much less efficient than viral vectors, especially for in vivo gene delivery. In this review, we will briefly discuss the advantages and limitations of the nonviral gene delivery systems that are shown to be active for in vivo gene delivery. We will also highlight approaches toward development of improved nonviral systems for human gene therapy. We hope that our discussion here will stimulate new thoughts and efforts toward advancement of this diverse and promising new field.

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Gene Transfer by Needle Injection of Naked DNA

Simple injection of plasmid DNA directly into a tissue without additional help from either a chemical agent or a physical force is able to transfect cells. Local injection of plasmid DNA into the muscle,[1] liver,[13-15] or skin,[16] or airway instillation into the lungs,[17] leads to low-level gene expression. Specific or nonspecific receptors on the cell surface that bind and internalize DNA have been implicated as a mechanism, though the details are sketchy at this point. Nevertheless, gene transfer with naked DNA is attractive to many researchers because of its simplicity and lack of toxicity. Practically, airway gene delivery and intramuscular injection of naked DNA for the treatment of acute diseases and DNA-based immunization, respectively, are 2 areas that are likely to benefit from naked DNA-mediated gene transfer, provided that further improvements are made in delivery efficiency and duration of transgene expression. A broad application of naked DNA–mediated gene transfer to gene therapy may not be conceivable because DNA, being large in size and highly hydrophilic, is efficiently kept out of the cells in a whole animal by several physical barriers. These

include the blood endothelium, the interstitial matrices, the mucus lining and specialized ciliate/tight junction of epithelial cells, and the plasma membrane of all cells. In addition, DNA degradation by intra- and extracellular nuclease activities further reduces the chance that DNA entering nuclei will be intact and functional. The current strategy for improving naked DNA–based gene transfer is to include in DNA solution substances capable of enhancing the efficiency of DNA internalization by target cells. For example, transferrin has been shown to enhance transfection in vitro.[18] The addition of water-immiscible solvents,[19,20] non-ionic polymers,[21] or surfactants,[22] or the use of hypotonic solution,[23] has also been shown to elevate gene transfer across cell membranes. Also, several nuclease inhibitors have been shown to enhance naked DNA–mediated gene transfer in cultured cells,[24] muscle,[25] and lungs.[26]

Gene Transfer by Physical Methods

Physical approaches have been explored for gene transfer into cells in vitro and in vivo. Physical approaches induce transient injuries or defects on cell membranes, so that DNA can enter the cells by diffusion. Gene delivery employing mechanical (particle bombardment or gene gun), electric (electroporation), ultrasonic, hydrodynamic (hydrodynamic gene transfer), or laser-based energy has been explored in recent years.

Transfer by Gene Gun

Particle bombardment through a gene gun is an ideal method for gene transfer to skin, mucosa, or surgically exposed tissues within a confined area.[4] DNA is deposited on the surface of gold particles, which are then accelerated by pressurized gas and expelled onto cells or a tissue. The momentum allows the gold particles to penetrate a few millimeters deep into a tissue and release DNA into cells on the path. Such a simple and effective method of gene delivery is expected to have important applications as an effective tool for DNA-based immunization. Further improvements could include chemical modification of the surface of the gold particles to allow higher capacity and better consistency for DNA coating, and fine-tuning of the expelling force for precise control of DNA deposition into cells in various tissues.[27]

Gene Transfer by Electroporation

Electroporation is a versatile method that has been extensively tested in many types of tissues in vivo,[2,3] among which skin and muscles are the most extensively investigated, although the system should work in any tissues into which a pair of electrodes can be inserted. For example, Hasson et al demonstrated that electroporation substantially increased transgene expression in isolated lung in an ex vivo organ culture setting,[28] and Dean et al showed that such a strategy also worked in live animals when a pair of electrodes was placed on the chest.[29] The level of reporter gene expression obtained was 2 to 3 orders of magnitude higher than that with plasmid DNA alone. DNA as large as 100 kb has been effectively delivered into muscle cells.[30] Long-term expression over 1 year after a single electroporation treatment was seen.[31] Gene transfer by electroporation showed less variation in efficiency across species than did direct DNA injection. The amount of DNA and how well the injected plasmid DNA distributes within the treated tissue prior to electroporation appear to have an important impact on transfection efficiency. It was also reported that age of the recipient animals affects the transfection efficiency in mice.[32] Treatment of muscle with hyaluronidase prior to injection of plasmid DNA to loosen up the surrounding extracellular matrix significantly enhanced transfection, possibly because of improved distribution of plasmid DNA in the tissue.[32,33] Alternatively, plasmid DNA administration through the portal vein followed by localized electroporation on rat liver resulted in widespread transfection in hepatocytes in the treated lobe but not in the surrounding lobes.[34] This result raises the possibility that one can supply cells with plasmid DNA via blood circulation and then apply electroporation to a selected area to achieve localized gene transfer. A short time interval between DNA administration and electroporation is critical to minimize DNA degradation by extracellular nucleases.

Several major drawbacks exist for in vivo application of electroporation. First, it has a limited effective range of ~1 cm between the electrodes, which makes it difficult to transfect cells in a large area of tissues. Second, a surgical procedure is required to place the electrodes deep into the internal organs. Third, high voltage applied to tissues can result in irreversible tissue damage as a result of thermal heating.[35] Ca^{2+} influx due to disruption of cell membranes may induce tissue damage because of Ca^{2+}-mediated protease activation.[36] The possibility that the high voltage applied to cells could affect the stability of genomic DNA is an additional safety concern. However, some of these concerns may be resolvable by optimizing the design of electrodes, their spatial arrangement, the field strength, and the duration and frequency of electric pulses.

Ultrasound-Facilitated Gene Transfer

The discovery that ultrasound can facilitate gene transfer at cellular[37] and tissue levels[38] expands the methodology of gene transfer by physical methods. A 10- to 20-fold enhancement of reporter gene expression over that of naked DNA has been achieved. The transfection efficiency of this system is determined by several factors, including the frequency,

the output strength of the ultrasound applied, the duration of ultrasound treatment,[39] and the amount of plasmid DNA used. The efficiency can be enhanced by the use of contrast agents or conditions that make membranes more fluidic.[40,41] The contrast agents are air-filled microbubbles that rapidly expand and shrink under ultrasound irritation, generating local shock waves that transiently permeate the nearby cell membranes. Unlike electroporation, which moves DNA along the electric field, ultrasound creates membrane pores and facilitates intracellular gene transfer through passive diffusion of DNA across the membrane pores.[37,42] Consequently, the size and local concentration of plasmid DNA play an important role in determining the transfection efficiency. Efforts to reduce DNA size for gene transfer by methods of standard molecular biology or through proper formulation could result in further improvement. Interestingly, significant enhancement has been reported in cell culture and in vivo when complexes of DNA and cationic lipids have been used.[42,43] Since ultrasound can penetrate soft tissue and be applied to a specific area, it could become an ideal method for noninvasive gene transfer into cells of the internal organs. Evidence supporting this possibility has been presented: in one study, plasmid DNA was coadministered with a contrast agent to blood circulation, and this was followed by ultrasound treatment of a selected tissue.[44] So far, the major problem for ultrasound-facilitated gene delivery is low gene delivery efficiency.

Hydrodynamic Gene Delivery

Hydrodynamic gene delivery is a simple method that introduces naked plasmid DNA into cells in highly perfused internal organs (eg, the liver) with an impressive efficiency.[7,8] The gene delivery efficiency is determined by the anatomic structure of the organ, the injection volume, and the speed of injection. In a mouse model, the optimal condition involves 1.6 to 1.8 mL of DNA solution in saline for a 20 g mouse (8%-9% of the body weight) and an injection time of ~5 seconds via the tail vein. Mechanistically, the rapid tail vein injection of a large volume of DNA solution causes a transient overflow of injected solution at the inferior vena cava that exceeds the cardiac output. As a result, the injection induces a flow of DNA solution in retrograde into the liver, a rapid rise of intrahepatic pressure, liver expansion, and reversible disruption of the liver fenestrae.[45] Electron microscopy shows the existence of transient membrane defects in hepatocytes shortly after the hydrodynamic treatment, which could be the mechanism for plasmid DNA to enter the hepatocytes.[45] The gene transfer efficiency of this simple procedure is the highest so far achieved in vivo using nonviral approaches. Approximately 30% to 40% of the hepatocytes are transfected by a single hydrodynamic injection of less than 50 μg of plasmid DNA.[7] Various substances of different molecular weight and chemical structure—including small dye molecules, proteins, oligonucleotides, small interfering RNA, and linear or circular DNA fragments as large as 175 kb—have been delivered by this method.[30,46] The nonspecific nature of hydrodynamic delivery suggests that this method can be applied to intracellular delivery of any water-soluble compounds, small colloidal particles (molecular assembly), or viral particles. Hydrodynamic delivery allows direct transfer of substances into cytoplasm without endocytosis.

Such a simple, reproducible, and highly efficient method for gene delivery has been used to express proteins of therapeutic value such as hemophilia factors,[47,48] alpha-1 antitrypsin,[49-51] cytokines,[52] hepatic growth factors,[53] and erythropoietin[54] in mouse and rat models. Depending on the plasmid construct and the regulatory elements driving expression of the transgene, the level of gene expression in some cases has reached or exceeded the physiological level.[49-51] The fact that a bacterial artificial chromosome that contains an entire chromosomal transcription unit and replication origin (>150 kb) can be delivered successfully to the liver using this method[30] opens up many possibilities for gene therapy applications in liver-associated genetic diseases.

The real challenge for gene transfer by the hydrodynamic method is how to translate this simple and effective procedure to one that is applicable to humans. Rat liver can be transfected similarly through tail vein injection using an injection volume equivalent to 8% to 9% of body weight (T. Suda and D. Liu, unpublished data, 2006). If the same ratio is extrapolated to humans, one would have to inject up to 7.5 L of saline at a high rate, which is obviously many times over the maximal volume that a person can tolerate. However, successful liver transfection has been achieved using balloon catheter–based and occlusion-assisted infusion to specific lobes in rabbit[55] and swine models,[56,57] indicating that with modification, hydrodynamic gene delivery can become a clinically relevant procedure.

Gene Delivery by Chemical Methods

By far the most frequently studied strategy for nonviral gene delivery is the formulation of DNA into condensed particles by using cationic lipids or cationic polymers. The DNA-containing particles are subsequently taken up by cells via endocytosis, macropinocytosis, or phagocytosis in the form of intracellular vesicles, from which a small fraction of the DNA is released into the cytoplasm and migrates into the nucleus, where transgene expression takes place.

Cationic Lipid-Mediated Gene Delivery

Since 1987, when Felgner et al first reported that a double-chain monovalent quaternary ammonium lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride,

effectively binds and delivers DNA to cultured cells,[58] hundreds of new cationic lipids have been developed (for review, see Liu et al[10]). These lipids differ by the number of charges in their hydrophilic head group and by the detailed structure of their hydrophobic moiety. Although some cationic lipids alone exhibit good transfection activity, they are often formulated with a noncharged phospholipid or cholesterol as a helper lipid to form liposomes. Upon mixing with cationic liposomes, plasmid DNA is condensed into small quasi-stable particles called lipoplexes. DNA in lipoplexes is well protected from nuclease degradation. Lipoplexes are able to trigger cellular uptake and facilitate the release of DNA from the intracellular vesicles before reaching destructive lysosomal compartments.

Most of our understanding of lipid-mediated gene delivery derives from characterization work on lipoplexes prepared in low-salt solution and transfection tests on cells in the absence of interfering substances such as serum. Under these conditions, the transfection efficiency of lipoplexes is affected by (1) the chemical structure of the cationic lipid, (2) the charge ratio between the cationic lipid and the DNA, (3) the structure and proportion of the helper lipid in the complexes, (4) the size and structure of the liposomes, (5) the total amount of the lipoplexes applied, and (6) the cell type. The first 4 factors determine the structure, charge property, and transfection activity of the lipoplexes. The remaining 2 define the overall toxicity to the treated cells, and the susceptibility of the cells to a particular lipid-based transfection reagent. The chemical structure of the cationic lipid has a major impact on the transfection efficiency. In general, multivalent lipids with long and unsaturated hydrocarbon chains tend to be more efficient than monovalent cationic lipids with the same hydrophobic chains. Transfection typically requires that the cationic lipid be in slight excess over DNA such that the lipoplexes have net positive charges on the surface. Spontaneous mixing between cationic lipids and cellular lipids in the membrane of the endocytic vesicles is crucial to the endosome-releasing process.[59] Spontaneous lipid mixing in endosomes becomes more profound when a non-bilayer-forming lipid such as dioleoylphosphatidylethanolamine (DOPE) is used as the helper lipid, rather than a bilayer-forming lipid, dioleoylphosphatidylcholine.[60,61] Inclusion of DOPE is believed to increase membrane fluidity and facilitate lipid exchange and membrane fusion between lipoplexes and the endosomal membrane. A high local concentration of DOPE, which has a strong tendency to form an inverse hexagonal phase, may lead to a nonbilayer lipid structure and cause membrane perturbation and endosome destruction.[62] However, some multivalent lipids have intrinsic transfection activity, and a helper lipid does not have a major impact on overall transfection activity, indicating that multivalent cationic lipids work on a different mechanism.[63,64] Often, these cationic lipopolyamines have protonable amine groups that apparently intercept the endosome maturation by absorbing protons to slow down the acidification process inside the endosomes, preventing the endosome-lysosome transition. It has also been suggested that a local destabilization effect of some micelle-forming cationic lipids on the endosomal membrane's integrity is part of the underlying mechanism of lipid-based gene delivery.[63]

Lipoplexes form spontaneously when cationic liposomes are mixed with DNA. The process involves an initial rapid association of polycationic liposomes and polyanionic DNA through electrostatic interaction, followed by a slower lipid rearrangement process.[65] The structure of lipoplexes is influenced by multiple factors, including the charge ratio, the concentration of individual lipids and DNA, the structure of the cationic lipid and the helper lipid, the physical aggregation state of the lipids (multilamellar or unilamellar liposomes, or micelles), the salt concentration, and the method of preparation. Lipoplexes come in various forms, including fully condensed lipid/DNA complexes, partially condensed lipid/DNA complexes, DNA sandwiched between cationic lipid bilayers, lipid-coated DNA arranged in a hexagonal lattice, or partially condensed DNA surrounded by a lipid bilayer.[66,67] With the same lipid composition and charge ratio, lipoplexes that are prepared from multilamellar liposomes with a size of ~500 nm and those that are intrinsically less stable exhibit better activity in transfection.[68,69]

The simplest way to prepare lipoplexes is to mix diluted solutions of plasmid DNA and preformed liposomes. The resulting lipoplexes are generally heterogeneous in size and morphology. The heterogeneity is primarily due to the relatively large sizes of DNA and liposomes, and the multivariant nature of the interaction between the DNA and liposomes. Alternative methods involving forms of lipid assembly other than liposomes have been designed to overcome these problems. For example, direct addition of DNA solution to a dried film of cationic lipid and DOPE promotes entrapment of DNA within multilamellar liposomes, rather than sandwiching of DNA between liposomes.[70] A method of lipoplex preparation by slow dialysis has also been developed. This procedure involves DNA condensation in mixed micelles consisting of cationic lipid and non-ionic detergent, and removal of the detergent by dialysis.[70] At a concentration below the critical micelle concentration of single-chain cationic lipids, DNA collapses into unimolecular lipid-DNA nanoparticles that are much smaller (20-30 nm). Small particles are preferred for in vivo gene delivery because of their slower clearance rate in the blood and, therefore, their high probability of reaching the target cells. Conjugation to these small-sized complexes with polyethylene glycol (PEG) and targeting ligands on their surface makes it possible to construct target-specific gene carriers.[71]

Many cationic lipids show excellent transfection activity in cell culture, but most do not perform well in the presence of serum, and only a few are active in vivo.[10] A dramatic change in size, surface charge, and lipid composition occurs when lipoplexes are exposed to the overwhelming amount of negatively charged and often amphipathic proteins and polysaccharides that are present in blood, mucus, epithelial lining fluid, or tissue matrix. Once administered in vivo, lipoplexes tend to interact with negatively charged blood components and form large aggregates that could be absorbed onto the surface of circulating red blood cells, trapped in a thick mucus layer, or embolized in microvasculatures, preventing them from reaching the intended target cells in the distal location. Some even undergo dissolution after they are introduced to the blood circulation.[72,73]

Despite these undesirable characteristics, lipoplexes have been used for in vivo gene delivery to the lungs by intravenous[74-76] and airway[77-79] administration. Transgene expression was clearly detectable but in most cases was insufficient for a meaningful therapeutic outcome. For airway gene delivery to the lungs, animal studies using lipoplexes prepared from 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl]cholesterol (DC-Chol) and DOPE have shown that this procedure was mild to the host and partially effective in correcting genetic defects in a cystic fibrosis transmembrane regulator mutant model.[80] By screening a large cationic lipid library in earlier studies, the Genzyme group revealed some structure-activity relationships important to the transfection activity of cationic lipids in a mouse model.[81] Several cholesterol derivatives with polyamine groups linked to cholesterol through a carbamoyl bond exhibited significantly higher activity in the lung compared with that of naked DNA or DC-Chol/DOPE lipoplexes.[81] Most transfected cells were found in the lower airways in the alveoli region, not the intended bronchial epithelial cells.

Several inhibitory factors for lipoplex-based gene delivery have been identified for airway gene transfer.[82,83] A critical factor is that upper-airway epithelial cells are covered by a negatively charged and viscous mucus layer that often traps and neutralizes the surface charges of the lipoplexes. In patients with cystic fibrosis, the epithelial cells are further covered with a thick layer of sputum that contains genomic DNA released from dead cells and bacteria. In lower airways, the surfactant layer enriched with several phospholipids and surfactant proteins is also believed to inhibit the transfection activity of lipoplexes. In addition, well-differentiated upper airway epithelial cells are known to be less active in taking up lipoplexes than are those in the lower airways. Proper shielding of surface charges of lipoplexes to reduce nonspecific protein/mucin association, inclusion of a target ligand to enhance specific binding, and substitution of a portion of the cationic lipids with less toxic and membrane-active peptide seem to be logical and incremental steps to solving some of these problems.

In vivo gene transfer by systemic administration of lipoplexes mainly transfects endothelial cells in the pulmonary vasculature.[74] A large excess of cationic lipids was needed to mediate optimal gene transfer.[74] Although using DOPE as the helper lipid makes the formulation more efficient for airway gene delivery, it has an adverse effect on intravenous transfection. Cholesterol was found to be a better helper lipid for systemic transfection.[74] In systemic gene delivery, cholesterol seems to stabilize the lipoplex structure in blood, while formulations containing DOPE tend to fall apart more easily in the presence of blood components.[72,74] Moreover, it has been shown that one can efficiently transfect pulmonary endothelial cells by injecting free cationic liposomes and following shortly thereafter with a second injection of naked DNA solution,[84] which suggests that various forms of lipoplex structures that seem to be important for transfection of cells in tissue culture are not critical for transfection by intravenous injection. The expression of a reporter gene in transfected endothelial cells in the lung follows a quick onset, which reaches its peak level ~8 to 16 hours posttransfection, then declines rapidly. The rapid decline is not solely due to DNA degradation, as a second transfection 1 week later did not result in significant expression,[74] suggesting that it is likely that initially transfected cells become resistant to the same type of transfection. The lack of cellular response by the transfected cells to subsequent transfection suggests a negative transcription regulatory mechanism.

Toxicity related to gene transfer by lipoplexes has been observed. Acute inflammation reactions have been reported in animals treated with airway instillation or intravenous injection of lipoplexes.[74] Detailed toxicological studies on one of the Genzyme Lipid formulations, GL-67/DOPE, revealed that the cationic lipid contributes significantly to the toxicity observed.[85,86] Similar toxic effects are also noticeable in systemic gene delivery via the tail vein with other types of cationic lipids. Symptoms include acute pulmonary hypotension, induction of inflammatory cytokines, tissue infiltration of neutrophils in lungs, decrease in white cell counts, and in some cases tissue injury in liver and spleen.[87] In humans, various degrees of adverse inflammatory reactions, including flulike symptoms with fever and airway inflammation, were noted among subjects who received aerosolized GL67 liposomes alone or lipoplexes.[85] These early clinical data suggest that these lipoplex formulations are inadequate for use in humans.

Part of the inflammation response seen in treated lungs is related to the unmethylated CpG (umCpG) sequences found in the plasmid of bacterial origin. A potent immune stimulant, umCpG triggers release of proinflammatory cytokines.[88,89] Cationic lipids in lipoplexes are capable of enhancing the umCpG effect.[90] Another factor related to the severity of

transfection-related side effects is complement activation[91] and adsorption of serum proteins onto their surface, which in turn act as opsonins to trigger the uptake of opsonized particles by macrophages and other immune cells. Various strategies have been considered to deal with the toxic responses. For example, covering the lipoplex surface with inert polymers could, in principle, reduce protein adsorption and their affinity to immune cells and thereby minimize the toxic responses. Toward this end, PEG-lipid conjugates have been incorporated into the lipoplexes to minimize the nonspecific interaction of lipoplexes with blood components.[92-94] It is believed that PEG, being hydrophilic and unable to interact with either DNA or cationic lipids, provides longer circulation times of liposomes in blood circulation by minimizing the binding of blood components and lipoplexes. Unfortunately, inclusion of such bulky PEG lipids into lipoplexes causes dose-dependent inhibition in transfection activity. For this reason a different length of hydrocarbons in PEG-lipid derivatives was used to adjust the time of PEG-lipid association with lipoplexes. The objective of this strategy is to use the PEG as a cover for lipoplexes before they reach the target cells. Once at the target cells, PEG-lipids fall off, revealing highly active lipoplexes.[94] More recent work from Szoka's group showed that the PEG-lipid shield for lipoplexes can be made detachable using an acid-sensitive linkage through which the PEG moiety can be removed when lipoplexes are inside the endocytic compartment.[95] In addition, reduction of the toxicity associated with cationic lipids can be achieved by improved chemical design of lipids with increased biodegradability.[96-98] Cationic lipids that are charged only at mildly acidic but not at neutral pH,[99] or those whose charge groups can be eliminated after lipoplex formation,[97] may also be a potential solution to the toxicity issues of current lipoplexes.

Cationic Polymer-Mediated Gene Transfer

Synthetic and naturally occurring cationic polymers constitute another category of DNA carriers that have been used widely for gene delivery. In fact, poly-L-lysine is one of the first group of cationic polymers used in gene transfer in vivo.[100] Over the years, a significant number of cationic polymers in linear or branched configuration have been explored as carriers for in vitro and in vivo gene delivery. These include polyethylenimine (PEI),[101-103] polyamidoamine[104-106] and polypropylamine dendrimers,[107] polyallylamine, cationic dextran,[108] chitosan,[109-112] cationic proteins (polylysine, protamine, and histones),[113] and cationic peptides.[114,115] Although most cationic polymers share the function of condensing DNA into small particles and facilitating cellular uptake via endocytosis through charge-charge interaction with anionic sites on cell surfaces, their transfection activity and toxicity differ dramatically.

PEI is perhaps the most active and most studied polymer for gene delivery. PEI is made either from an acid-catalyzed aziridine ring opening reaction that leads to branched polymers, or by hydrolysis of poly(2-ethyl-2-oxazolium) that results in linear polymers. Jean Paul Behr's group first introduced PEI as an efficient and economic synthetic polymeric gene transfer agent.[101] Chemically, PEI is one of the most densely charged polymers: one third of the atoms are nitrogen, and one sixth of the nitrogen atoms carry a positive charge at physiological pH. Branched PEI has a ratio of primary:secondary:tertiary amine groups close to 1:1:1, according to a recently revised estimate.[116] For PEI-mediated transfection, DNA-to-PEI ratios, the molecular weight and configuration of PEI, the concentration of DNA and polymer, and the ionic strength of the solvent for preparation are all important factors that determine the physical properties of the DNA/PEI complexes (polyplexes) and their transfection activity. Most of the amines in linear PEI are secondary amines except the terminal groups. Both linear PEI (LPEI) and branched PEI (BPEI) have excellent transfection activities in vitro and exhibit moderate transfection activity in vivo. LPEI is reportedly less toxic to cells than its branched counterparts. When added to cells, LPEI/DNA complexes lead to higher and faster gene expression than BPEI/DNA complexes. This coincides with the fact that LPEI/DNA complexes are less condensed and are able to dissociate more efficiently than BPEI/DNA complexes when inside a cell. Studies have also revealed that LPEI/DNA complexes enter the nucleus more readily than branched PEI/DNA complexes.[117]

One drawback in the use of PEI as a transfection reagent relates to its nonbiodegradable nature.[118] It is known that the toxicity and transfection activity of PEI is molecular weight–dependent. The most active PEI from a commercial source is 25 k for BPEI and 22 k for LPEI. PEI with a molecular weight larger than 25 k is also active but exhibits greater toxicity. BPEI of 5 to 10 k appears to be more active in transfection and less toxic when compared with a standard "benchmark" of 25 k BPEI.[119] PEI of 2 k or smaller is relatively nontoxic but not active in transfection.

Treatment of low–molecular weight PEI with several bifunctional cross-linking reagents generates PEI oligomers that are transfectionally active. Cross-linking of small PEI with a biodegradable bond such as a disulfide or ester bond resulted in oligomers that were as active as PEI 25 k but significantly less toxic to cells.[120,121] Strategies to coat the surface of noncharged nanoparticles such as gold nanoparticles,[122] polymethylacrylate nanogels,[123] and silica gels[124] with PEI have generated active transfection reagents. PEI derivatives with lipid conjugation form aggregated micelles that are biologically active.[125,126] Conjugation of lytic peptides (melittin) to PEI 2 k was shown to result in a significant increase in transfection activity.[127,128]

To study the effects of charge density of PEI on transfection activity, various chemical modifications of amine groups have been performed. Most amine groups of PEI are weak bases, and only a fraction of these are protonated at physiological pH. Permethylation of these amines to form stronger basic quaternary ammonia groups reduces the transfection activity.[125] Careful modifications to reduce a portion of the positive charges by conjugation of relatively bulky groups (proteins, peptides, PEG) or smaller groups (sugar, small polymers, acyl group) have yielded PEI derivatives that are more efficient in transfection and less toxic to cells.[129] However, too much modification or elimination of too many amine groups of PEI leads to reduction or loss of transfection activities.

LPEI outperforms BPEI for in vivo transfection when intravenous injection or intratracheal instillation into the lungs is performed. Intravenous injection of polyplexes prepared from LPEI was as efficient as intravenous injection of lipoplexes in transfecting pulmonary endothelial cells in mice.[130] One study reported that polyplexes prepared at lower PEI-to-DNA charge ratios (N/P = 1:3) than that for optimal transfection in vitro (1:5) worked better by intratumor injection.[131] The enhanced in vivo activity of polyplexes made at lower PEI-to-DNA ratios may be due to less extensive nonspecific interaction between serum proteins and the PEI/DNA complexes. Surface modification with PEG can drastically reduce the surface charge of PEI and the tendency to form large aggregates in the presence of serum. Conjugation to PEI with a ligand, such as transferrin, antibody, avidin, or sugar moieties, can also provide shielding to polyplexes and the possibility of target-specific gene delivery.[129] Such a strategy has been proven effective for targeted gene delivery to tumor cells in mice bearing inoculated tumor cells, although only cells close to the blood vessels were transfected.[132]

BPEI works well for airway gene delivery. The small particle size and good stability of polyplexes prepared from PEI and DNA are well suited for airway gene delivery by aerosols. Low-dose and well-dispersed polyplexes administered by aerosolization appear to be relatively nontoxic, while large doses administered by direct instillation are clearly toxic and injurious to the respiratory tract.[133]

More biodegradable cationic polymers have been designed to reduce the toxicity associated with cationic polymer-based gene delivery systems such as PEI and poly-L-lysine. One of the successful classes of newly designed cationic polymers is aminoesters.[134,135] The intrinsic ester bonds make these polymers less stable over time; therefore, the overall toxicity is reduced. Early studies suggested that branched polyaminoesters are chemically more stable than those with linear configurations when the polymer contains primary amine groups.[135,136] Cationic polymers with disulfide bond linkages assembled from low-molecular-weight polyamines also showed good transfection efficiency and relatively low toxicity.

A proton sponge hypothesis has been put forward to explain the mechanism of cationic polymer-based gene delivery, especially gene transfer by PEI and its derivatives. The majority of PEI's amine groups are not fully protonated under physiological pH. However, they could be protonated when the pH drops below 6.0 in the endosome compartment, buffering the endosome pH. Proton entrance into the endosomes also brings chloride counterions into the endosomes, raising the osmotic pressure and causing these vesicles to swell and rupture.[101,137] Interestingly, neither lipids nor naturally occurring cationic polymers (polylysine, histone, chitosan) are very efficient in triggering endosome rupture, yet they are still able to transfect cells in vitro and in vivo. As is clear from these observations, more needs to be learned about nonviral vector-mediated gene delivery.

Cationic peptides have been explored in recent years as a carrier for gene delivery. For example, an arginine-rich peptide derived from a small basic protein, protamine, was able to transfect cells in vitro at least as efficiently as PEI, while the whole protein showed minimal activity.[115] Synthetic peptides that contain a dimer of arginine-rich peptide derived from HIV TAT protein were reported to be active in transfection.[138] Lysine-rich peptides derived from histone H1, H2A,[114] anti-DNA antibody[139] can also transfer genes into cells. Recently, short cationic peptides have also been studied as membrane-penetrating molecules bringing into cells a variety of substances ranging from small molecules and proteins to nanoparticles, though the exact mechanism that governs transport across the cell membrane is still not fully understood. It is hoped that the eventual understanding of this mechanism can be employed to enhance the efficiency of polyplex-mediated gene delivery. Future improvement of current polycation-based gene delivery systems could also take a more rational approach by incorporating into the complexes an active motif to enhance membrane permeability, a nuclear localization signal to enhance nuclear import, a targeting ligand to membrane receptors to increase binding affinity, and/or compounds to facilitate DNA dissociation from the complexes when inside the cytoplasm.

Lipid-Polymer Hybrid System

The reported lipid-polymer hybrid systems include DNA precondensed with polycations, then coated with either cationic liposomes,[140,141] anionic liposomes,[142] or amphiphilic polymers with or without helper lipids.[143] Linear poly-L-lysine, protamine, histone, and several synthetic polypeptides have been used as the DNA condensation component; the polyplexes formed are then coated with a lipid layer. DNA is better protected in these lipid-wrapping polyplexes. The 3-part system appears to be more efficient in transfection than lipid-DNA complexes in vitro[140,141] and is equally active in vivo.[144]

When anionic, DOPE-rich liposomes are added to DNA-polycation complexes, an extensive reorganization of the lipid membranes takes place following the initial contact, resulting in lipid-polymer-DNA complexes with anionic lipid coatings.[142] This strategy overcomes the surface charge issue associated with cationic lipid-polymer-DNA complexes. The cytotoxicity of the complexes is reduced, making the receptor-mediated targeting possible without interference of nonspecific charge-charge interaction. A parallel approach using amphipathic peptide derivatives as DNA packing agents, and a bulk of neutral helper lipid to prepare DNA complexes by the detergent dialysis method, also resulted in a functional complex that can be used for targeted gene delivery.[145] Several aspects related to lipid composition, the presence of shielding PEG-lipid conjugates, and the nature of chemical bonding that contributes to the biodegradability of the PEG-lipid conjugates in cells have been thoroughly studied.[146,147] These concepts are substantially different from the original lipid-DNA or polymer-DNA complexes and certainly deserve further exploration, particularly in the area of in vivo targeted gene delivery.

Conclusion

Numerous nonviral gene delivery systems have been developed in the last 20 to 25 years. This article has discussed those that are at more advanced stages of development. The advantages and limitations of each method for gene delivery are summarized in Table 1. It is important to point out that therapeutic applications of these nonviral gene delivery systems are rather limited despite the progress in vector design and the understanding of transfection biology. Continuous effort to improve currently available systems and to develop new methods of gene delivery is needed and could lead to safer and more efficient nonviral gene delivery. For a better nonviral system, it appears essential for us to identify the critical parameters limiting gene delivery in the current systems. The challenge for in vivo gene delivery is to pinpoint the limiting factors and implement strategies to enhance gene delivery efficiency with minimal tissue damage. For physical methods, differences in extracellular and intracellular structures in various tissues and organs appear to play a dominant role in determining whether a particular method of gene delivery will be safe and effective. A thorough understanding of the differences among various organs, tissues, and types of cells in response to physical impacts will provide clues on how to develop an effective device or procedure applicable to humans.

Practically, cationic lipids, cationic polymers, and other naturally occurring compounds have proven to be extremely effective for in vitro gene delivery. However, all of the

Table 1. Advantages and Limitations of Current Nonviral Gene Delivery Systems

Method	Route of Gene Delivery	Advantages	Limitations
Needle injection	Intratissue	Simplicity and safety	Low efficiency
Gene gun	Topical	Good efficiency	Tissue damage in some applications
Electroporation	Topical Intratissue	High efficiency	Limited working range; need for surgical procedure for nontopical applications
Hydrodynamic delivery	Systemic Intravascular	High efficiency, simplicity, effectiveness for liver gene delivery	Extremely effective in small animals; surgical procedure may be needed for localized gene delivery
Ultrasound	Topical Systemic	Good potential for site-specific gene delivery	Low efficiency in vivo
Cationic lipids	Topical Intratissue Systemic Airway	High efficiency in vitro; low to medium high for local and systemic gene delivery	Acute immune responses; limited activity in vivo
Cationic polymers	Topical Intratissue Systemic Airway	Highly effective in vitro; low to medium high for local and systemic gene delivery	Toxicity to cells; acute immune responses
Lipid/polymer hybrids	Intratissue Systemic Airway	Low to medium-high efficiency in vitro and in vivo; low toxicity	Low activity in vivo

cationic molecule-based systems have failed or been unimpressive in clinical trials because of low delivery efficiency and toxicity, such as complement activation and acute inflammation. The low in vivo transfection efficiency appears to be related to the cationic nature of the gene carrier. While effective in protecting DNA from DNA degradation, the polycations in either lipoplexes or polyplexes have the intrinsic property of causing significant aggregation in biological matrices full of negatively charged molecules, and of preventing effective release of DNA once inside the cells. Inclusion of PEG-lipids, target ligands, endosomolytic peptides, and nuclear peptides into DNA complexes to convert a simple DNA complex into a more sophisticated multicomponent gene carrier appears to be a reasonable approach to equip the complexes with more function. The drawback of this approach is that the intended function of the added component tends to be compromised because of its nonspecific interaction with other components in the complexes. In addition to the effort to solve the problems associated with polycationic carriers (lipids and polymers), progress has been noted in developing different types of DNA-containing particles, including calcium phosphate-DNA coprecipitates,[148] hydrogel-based nanoparticles,[149] and DNA complexes with membrane-active peptides.[150] One advantage of these gene carriers is that the DNA complexes tend to be more homogeneous in size and structure. Successful gene transfer with these particles has been seen in vitro. Further studies are needed to explore their possible use in vivo.

From a mechanistic point of view, a better understanding of intracellular trafficking of various types of DNA complexes in different cell types may help in rational design of more diverse nonviral carriers. The biological and cellular responses to the process of physical stimulation involving gene transfer also deserve more attention. Innovation in applying the principles of physics, chemistry, and biology to the development of a safe and effective method for gene delivery is the key to making the urgently needed breakthroughs in nonviral gene therapy.

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References

1. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle *in vivo. Science*. 1990;247:1465-1468.

2. Heller LC, Ugen K, Heller R. Electroporation for targeted gene transfer. *Expert Opin Drug Deliv*. 2005;2:255-268.

3. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J*. 1982;1:841-845.

4. Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci USA*. 1990;87:9568-9572.

5. Yang NS, Sun WH. Gene gun and other non-viral approaches for cancer gene therapy. *Nat Med*. 1995;1:481-483.

6. Lawrie A, Brisken AF, Francis SE, Cumberland DC, Crossman DC, Newman CM. Microbubble-enhanced ultrasound for vascular gene delivery. *Gene Ther*. 2000;7:2023-2027.

7. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther*. 1999;6:1258-1266.

8. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther*. 1999;10:1735-1737.

9. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J Gene Med*. 2005;7:992-1009.

10. Liu D, Ren T, Gao X. Cationic transfection lipids. *Curr Med Chem*. 2003;10:1307-1315.

11. Huang L, Hung MC, Wagner E. *Nonviral Vectors for Gene Therapy.* San Diego, CA: Academic Press; 1999.

12. Mahato RI, Kim SW. *Pharmaceutical Perspectives of Nucleic Acid-Based Therapeutics.* London, UK: Taylor & Francis; 2002.

13. Hickman MA, Malone RW, Lehmann-Bruinsma K, et al. Gene expression following direct injection of DNA into liver. *Hum Gene Ther*. 1994;5:1477-1483.

14. Zhang G, Vargo D, Budker V, Armstrong N, Knechtle S, Wolff JA. Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. *Hum Gene Ther*. 1997;8:1763-1772.

15. Budker V, Zhang G, Knechtle S, Wolff JA. Naked DNA delivered intraportally expresses efficiently in hepatocytes. *Gene Ther*. 1996;3:593-598.

16. Choate KA, Khavari PA. Direct cutaneous gene delivery in a human genetic skin disease. *Hum Gene Ther*. 1997;8:1659-1665.

17. Meyer KB, Jr, Thompson MM, Jr, Levy MY, Jr, Barron LG, Jr, Szoka FC, Jr. Intratracheal gene delivery to the mouse airway: characterization of plasmid DNA expression and pharmacokinetics. *Gene Ther*. 1995;2:450-460.

18. Sato Y, Yamauchi N, Takahashi M, et al. In vivo gene delivery to tumor cells by transferrin-streptavidin-DNA conjugate. *FASEB J*. 2000;14:2108-2118.

19. Schughart K, Rasmussen UB. Solvoplex synthetic vector for intrapulmonary gene delivery. Preparation and use. *Methods Mol Med*. 2002;69:83-94.

20. Schughart K, Bischoff R, Rasmussen UB, et al. Solvoplex: a new type of synthetic vector for intrapulmonary gene delivery. *Hum Gene Ther*. 1999;10:2891-2905.

21. Desigaux L, Gourden C, Bello-Roufai M, et al. Nonionic amphiphilic block copolymers promote gene transfer to the lung. *Hum Gene Ther*. 2005;16:821-829.

22. Freeman DJ, Niven RW. The influence of sodium glycocholate and other additives on the in vivo transfection of plasmid DNA in the lungs. *Pharm Res*. 1996;13:202-209.

23. Lemoine JL, Farley R, Huang L. Mechanism of efficient transfection of the nasal airway epithelium by hypotonic shock. *Gene Ther*. 2005;12:1275-1282.

24. Ross GF, Bruno MD, Uyeda M, et al. Enhanced reporter gene expression in cells transfected in the presence of DMI-2, an acid nuclease inhibitor. *Gene Ther*. 1998;5:1244-1250.

25. Walther W, Stein U, Siegel R, Fichtner I, Schlag PM. Use of the nuclease inhibitor aurintricarboxylic acid (ATA) for improved non-viral

intratumoral in vivo gene transfer by jet-injection. *J Gene Med*. 2005;7:477-485.

26. Glasspool-Malone J, Malone RW. Marked enhancement of direct respiratory tissue transfection by aurintricarboxylic acid. *Hum Gene Ther*. 1999;10:1703-1713.

27. O'Brien J, Lummis SC. An improved method of preparing microcarriers for biolistic transfection. *Brain Res Brain Res Protoc*. 2002;10:12-15.

28. Hasson E, Slovatizky Y, Shimoni Y, Falk H, Panet A, Mitrani E. Solid tissues can be manipulated ex vivo and used as vehicles for gene therapy. *J Gene Med*. 2005;7:926-935.

29. Dean DA, Machado-Aranda D, Blair-Parks K, Yeldandi AV, Young JL. Electroporation as a method for high-level nonviral gene transfer to the lung. *Gene Ther*. 2003;10:1608-1615.

30. Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Cooke H, Huxley C, Wagner E. In vitro and in vivo delivery of intact BAC DNA—comparison of different methods. *J Gene Med*. 2004;6:195-209.

31. Molnar MJ, Gilbert R, Lu Y, et al. Factors influencing the efficacy, longevity, and safety of electroporation-assisted plasmid-based gene transfer into mouse muscles. *Mol Ther*. 2004;10:447-455.

32. McMahon JM, Wells DJ. Electroporation for gene transfer to skeletal muscles: current status. *BioDrugs*. 2004;18:155-165.

33. McMahon JM, Signori E, Wells KE, Fazio VM, Wells DJ. Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase—increased expression with reduced muscle damage. *Gene Ther*. 2001;8:1264-1270.

34. Sakai M, Nishikawa M, Thanaketpaisarn O, Yamashita F, Hashida M. Hepatocyte-targeted gene transfer by combination of vascularly delivered plasmid DNA and *in vivo* electroporation. *Gene Ther*. 2005;12:607-616.

35. Durieux AC, Bonnefoy R, Busso T, Freyssenet D. *In vivo* gene electrotransfer into skeletal muscle: effects of plasmid DNA on the occurrence and extent of muscle damage. *J Gene Med*. 2004;6:809-816.

36. Gissel H, Clausen T. Excitation-induced Ca^{2+} influx and skeletal muscle cell damage. *Acta Physiol Scand*. 2001;171:327-334.

37. Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. *Hum Gene Ther*. 1996;7:1339-1346.

38. Liang HD, Lu QL, Xue SA, et al. Optimisation of ultrasound-mediated gene transfer (sonoporation) in skeletal muscle cells. *Ultrasound Med Biol*. 2004;30:1523-1529.

39. Huber PE, Jenne J, Debus J, Wannenmacher MF, Pfisterer P. A comparison of shock wave and sinusoidal-focused ultrasound-induced localized transfection of HeLa cells. *Ultrasound Med Biol*. 1999;25:1451-1457.

40. Nozaki T, Jr, Ogawa R, Jr, Feril LB, Jr, Kagiya G, Fuse H, Kondo T. Enhancement of ultrasound-mediated gene transfection by membrane modification. *J Gene Med*. 2003;5:1046-1055.

41. Ogawa R, Jr, Kagiya G, Jr, Feril LB, Jr, et al. Ultrasound mediated intravesical transfection enhanced by treatment with lidocaine or heat. *J Urol*. 2004;172:1469-1473.

42. Koch S, Pohl P, Cobet U, Rainov NG. Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects. *Ultrasound Med Biol*. 2000;26:897-903.

43. Anwer K, Kao G, Proctor B, et al. Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration. *Gene Ther*. 2000;7:1833-1839.

44. Unger EC, Hersh E, Vannan M, Matsunaga TO, McCreery T. Local drug and gene delivery through microbubbles. *Prog Cardiovasc Dis*. 2001;44:45-54.

45. Zhang G, Gao X, Song YK, et al. Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther*. 2004;11:675-682.

46. Al-Dosari MS, Knapp JE, Liu D. Hydrodynamic delivery. *Adv Genet*. 2005;54:65-82.

47. Miao CH, Thompson AR, Loeb K, Ye X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer *in vivo*. *Mol Ther*. 2001;3:947-957.

48. Miao CH, Ye X, Thompson AR. High-level factor VIII gene expression *in vivo* achieved by nonviral liver-specific gene therapy vectors. *Hum Gene Ther*. 2003;14:1297-1305.

49. Zhang G, Song YK, Liu D. Long-term expression of human alpha1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. *Gene Ther*. 2000;7:1344-1349.

50. Alino SF, Crespo A, Dasi F. Long-term therapeutic levels of human alpha-1 antitrypsin in plasma after hydrodynamic injection of nonviral DNA. *Gene Ther*. 2003;10:1672-1679.

51. Stoll SM, Sclimenti CR, Baba EJ, Meuse L, Kay MA, Calos MP. Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. *Mol Ther*. 2001;4:122-129.

52. Jiang J, Yamato E, Miyazaki J. Intravenous delivery of naked plasmid DNA for *in vivo* cytokine expression. *Biochem Biophys Res Commun*. 2001;289:1088-1092.

53. Yang J, Chen S, Huang L, Michalopoulos GK, Liu Y. Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth. *Hepatology*. 2001;33:848-859.

54. Maruyama H, Higuchi N, Kameda S, Miyazaki J, Gejyo F. Rat liver-targeted naked plasmid DNA transfer by tail vein injection. *Mol Biotechnol*. 2004;26:165-172.

55. Eastman SJ, Baskin KM, Hodges BL, et al. Development of catheter-based procedures for transducing the isolated rabbit liver with plasmid DNA. *Hum Gene Ther*. 2002;13:2065-2077.

56. Alino SF, Herrero MJ, Noguera I, Dasi F, Sanchez M. Pig liver gene therapy by noninvasive interventionist catheterism. *Gene Ther*. 2007;14:334-343.

57. Yoshino H, Hashizume K, Kobayashi E. Naked plasmid DNA transfer to the porcine liver using rapid injection with large volume. *Gene Ther*. 2006;13:1696-1702.

58. Felgner PL, Gadek TR, Holm M, et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*. 1987;84:7413-7417.

59. Xu Y, Jr, Szoka FC, Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*. 1996;35:5616-5623.

60. Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta*. 1995;1235:289-295.

61. Wrobel I, Collins D. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta*. 1995;1235:296-304.

62. Litzinger DC, Huang L. Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. *Biochim Biophys Acta*. 1992;1113:201-227.

63. El Ouahabi A, Thiry M, Pector V, Fuks R, Ruysschaert JM, Vandenbranden M. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett*. 1997;414:187-192.

64. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci USA*. 1989;86:6982-6986.

65. Pedroso de Lima MC, Simoes S, Pires P, Faneca H, Duzgunes N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. *Adv Drug Deliv Rev*. 2001;47:277-294.

66. Sternberg B, Sorgi FL, Huang L. New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett*. 1994;356:361-366.

67. Lin AJ, Slack NL, Ahmad A, George CX, Samuel CE, Safinya CR. Three-dimensional imaging of lipid gene-carriers: membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes. *Biophys J*. 2003;84:3307-3316.

68. Thierry AR, Rabinovich P, Peng B, Mahan LC, Bryant JL, Gallo RC. Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA. *Gene Ther*. 1997;4:226-237.

69. Koltover I, Salditt T, Radler JO, Safinya CR. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science*. 1998;281:78-81.

70. Hofland HE, Shephard L, Sullivan SM. Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc Natl Acad Sci USA*. 1996;93:7305-7309.

71. Dauty E, Remy JS, Zuber G, Behr JP. Intracellular delivery of nanometric DNA particles via the folate receptor. *Bioconjug Chem*. 2002;13:831-839.

72. Li S, Tseng WC, Stolz DB, Wu SP, Watkins SC, Huang L. Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther*. 1999;6:585-594.

73. Simberg D, Weisman S, Talmon Y, Faerman A, Shoshani T, Barenholz Y. The role of organ vascularization and lipoplex-serum initial contact in intravenous murine lipofection. *J Biol Chem*. 2003;278:39858-39865.

74. Song YK, Liu F, Chu S, Liu D. Characterization of cationic liposome-mediated gene transfer in vivo by intravenous administration. *Hum Gene Ther*. 1997;8:1585-1594.

75. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol*. 1997;15:647-652.

76. Thierry AR, Lunardi-Iskandar Y, Bryant JL, Rabinovich P, Gallo RC, Mahan LC. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci USA*. 1995;92:9742-9746.

77. Hyde SC, Southern KW, Gileadi U, et al. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther*. 2000;7:1156-1165.

78. Noone PG, Hohneker KW, Zhou Z, et al. Safety and biological efficacy of a lipid-CFTR complex for gene transfer in the nasal epithelium of adult patients with cystic fibrosis. *Mol Ther*. 2000;1:105-114.

79. Bragonzi A, Dina G, Villa A, et al. Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. *Gene Ther*. 2000;7:1753-1760.

80. Middleton PG, Caplen NJ, Gao X, et al. Nasal application of the cationic liposome DC-Chol:DOPE does not alter ion transport, lung function or bacterial growth. *Eur Respir J*. 1994;7:442-445.

81. Lee ER, Marshall J, Siegel CS, et al. Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum Gene Ther*. 1996;7:1701-1717.

82. Duncan JE, Whitsett JA, Horowitz AD. Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells *in vitro*. *Hum Gene Ther*. 1997;8:431-438.

83. Rosenecker J, Naundorf S, Gersting SW, et al. Interaction of bronchoalveolar lavage fluid with polyplexes and lipoplexes: analysing the role of proteins and glycoproteins. *J Gene Med*. 2003;5:49-60.

84. Song YK, Liu F, Liu D. Enhanced gene expression in mouse lung by prolonging the retention time of intravenously injected plasmid DNA. *Gene Ther*. 1998;5:1531-1537.

85. Ruiz FE, Clancy JP, Perricone MA, et al. A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. *Hum Gene Ther*. 2001;12:751-761.

86. Scheule RK, St George JA, Bagley RG, et al. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum Gene Ther*. 1997;8:689-707.

87. Yew NS, Scheule RK. Toxicity of cationic lipid-DNA complexes. *Adv Genet*. 2005;53:189-214.

88. Krieg AM. Direct immunologic activities of CpG DNA and implications for gene therapy. *J Gene Med*. 1999;1:56-63.

89. McLachlan G, Stevenson BJ, Davidson DJ, Porteous DJ. Bacterial DNA is implicated in the inflammatory response to delivery of DNA/DOTAP to mouse lungs. *Gene Ther*. 2000;7:384-392.

90. Yew NS, Wang KX, Przybylska M, et al. Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid: pDNA complexes. *Hum Gene Ther*. 1999;10:223-234.

91. Plank C, Jr, Mechtler K, Jr, Szoka FC, Jr, Wagner E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum Gene Ther*. 1996;7:1437-1446.

92. Fenske DB, MacLachlan I, Cullis PR. Long-circulating vectors for the systemic delivery of genes. *Curr Opin Mol Ther*. 2001;3:153-158.

93. Song LY, Ahkong QF, Rong Q, et al. Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes. *Biochim Biophys Acta*. 2002;1558:1-13.

94. Ambegia E, Ansell S, Cullis P, Heyes J, Palmer L, MacLachlan I. Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. *Biochim Biophys Acta*. 2005;1669:155-163.

95. Guo X, Jr, Szoka FC, Jr. Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG-diortho ester-lipid conjugate. *Bioconjug Chem*. 2001;12:291-300.

96. Wetzer B, Byk G, Frederic M, et al. Reducible cationic lipids for gene transfer. *Biochem J*. 2001;356:747-756.

97. Huang Z, Jr, Li W, Jr, MacKay JA, Jr, Szoka F, Jr. Thiocholesterol-based lipids for ordered assembly of bioresponsive gene carriers. *Mol Ther*. 2005;11:409-417.

98. Tang F, Hughes JA. Use of dithiodiglycolic acid as a tether for cationic lipids decreases the cytotoxicity and increases transgene expression of plasmid DNA *in vitro*. *Bioconjug Chem*. 1999;10:791-796.

99. Singh RS, Goncalves C, Sandrin P, Pichon C, Midoux P, Chaudhuri A. On the gene delivery efficacies of pH-sensitive cationic lipids via

endosomal protonation: a chemical biology investigation. *Chem Biol*. 2004;11:713-723.

100. Wu GY, Wu CH. Receptor-mediated gene delivery and expression in vivo. *J Biol Chem*. 1988;263:14621-14624.

101. Boussif O, Lezoualch F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA*. 1995;92:7297-7301.

102. Goula D, Remy JS, Erbacher P, et al. Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. *Gene Ther*. 1998;5:712-717.

103. Chemin I, Moradpour D, Wieland S, et al. Liver-directed gene transfer: a linear polyethylenimine derivative mediates highly efficient DNA delivery to primary hepatocytes in vitro and in vivo. *J Viral Hepat*. 1998;5:369-375.

104. Haensler J, Jr, Szoka FC, Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug Chem*. 1993;4:372-379.

105. Tang MX, Jr, Redemann CT, Jr, Szoka FC, Jr. *In vitro* gene delivery by degraded polyamidoamine dendrimers. *Bioconjug Chem*. 1996;7:703-714.

106. Rudolph C, Lausier J, Naundorf S, Muller RH, Rosenecker J. *In vivo* gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J Gene Med*. 2000;2:269-278.

107. Schatzlein AG, Zinselmeyer BH, Elouzi A, et al. Preferential liver gene expression with polypropylenimine dendrimers. *J Control Release*. 2005;101:247-258.

108. Hosseinkhani H, Azzam T, Tabata Y, Domb AJ. Dextran-spermine polycation: an efficient nonviral vector for *in vitro* and *in vivo* gene transfection. *Gene Ther*. 2004;11:194-203.

109. Leong KW, Mao HQ, Truong-Le VL, Roy K, Walsh SM, August JT. DNA-polycation nanospheres as non-viral gene delivery vehicles. *J Control Release*. 1998;53:183-193.

110. Erbacher P, Zou S, Bettinger T, Steffan AM, Remy JS. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. *Pharm Res*. 1998;15:1332-1339.

111. Venkatesh S, Smith TJ. Chitosan-mediated transfection of HeLa cells. *Pharm Dev Technol*. 1997;2:417-418.

112. Lee KY, Kwon IC, Kim YH, Jo WH, Jeong SY. Preparation of chitosan self-aggregates as a gene delivery system. *J Control Release*. 1998;51:213-220.

113. Balicki D, Beutler E. Histone H2A significantly enhances in vitro DNA transfection. *Mol Med*. 1997;3:782-787.

114. Balicki D, Putnam CD, Scaria PV, Beutler E. Structure and function correlation in histone H2A peptide-mediated gene transfer. *Proc Natl Acad Sci USA*. 2002;99:7467-7471.

115. Park YJ, Liang JF, Ko KS, Kim SW, Yang VC. Low molecular weight protamine as an efficient and nontoxic gene carrier: *in vitro* study. *J Gene Med*. 2003;5:700-711.

116. von Harpe A, Petersen H, Li Y, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. *J Control Release*. 2000;69:309-322.

117. Wightman L, Kircheis R, Rossler V, et al. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med*. 2001;3:362-372.

118. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials*. 2003;24:1121-1131.

119. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res*. 1999;16:1273-1279.

120. Gosselin MA, Guo W, Lee RJ. Efficient gene transfer using reversibly cross-linked low molecular weight polyethylenimine. *Bioconjug Chem*. 2001;12:989-994.

121. Forrest ML, Koerber JT, Pack DW. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug Chem*. 2003;14:934-940.

122. Thomas M, Klibanov AM. Conjugation to gold nanoparticles enhances polyethylenimine's transfer of plasmid DNA into mammalian cells. *Proc Natl Acad Sci USA*. 2003;100:9138-9143.

123. Zhu J, Tang A, Law LP, et al. Amphiphilic core-shell nanoparticles with poly(ethylenimine) shells as potential gene delivery carriers. *Bioconjug Chem*. 2005;16:139-146.

124. Manuel WS, Zheng JI, Hornsby PJ. Transfection by polyethyleneimine-coated microspheres. *J Drug Target*. 2001;9:15-22.

125. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc Natl Acad Sci USA*. 2002;99:14640-14645.

126. Han S, Mahato RI, Kim SW. Water-soluble lipopolymer for gene delivery. *Bioconjug Chem*. 2001;12:337-345.

127. Ogris M, Carlisle RC, Bettinger T, Seymour LW. Melittin enables efficient vesicular escape and enhanced nuclear access of nonviral gene delivery vectors. *J Biol Chem*. 2001;276:47550-47555.

128. Boeckle S, Fahrmeir J, Roedl W, Ogris M, Wagner E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J Control Release*. 2006;112:240-248.

129. Kichler A. Gene transfer with modified polyethylenimines. *J Gene Med*. 2004;6:S3-10.

130. Goula D, Benoist C, Mantero S, Merlo G, Levi G, Demeneix BA. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther*. 1998;5:1291-1295.

131. Sweeney P, Karashima T, Ishikura H, et al. Efficient therapeutic gene delivery after systemic administration of a novel polyethylenimine/DNA vector in an orthotopic bladder cancer model. *Cancer Res*. 2003;63:4017-4020.

132. Wagner E. Strategies to improve DNA polyplexes for *in vivo* gene transfer: will "artificial viruses" be the answer? *Pharm Res*. 2004;21:8-14.

133. Gautam A, Densmore CL, Xu B, Waldrep JC. Enhanced gene expression in mouse lung after PEI-DNA aerosol delivery. *Mol Ther*. 2000;2:63-70.

134. Akinc A, Anderson DG, Lynn DM, Langer R. Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery. *Bioconjug Chem*. 2003;14:979-988.

135. Lim YB, Kim SM, Suh H, Park JS. Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier. *Bioconjug Chem*. 2002;13:952-957.

136. Lim YB, Han SO, Kong HU, et al. Biodegradable polyester, poly[alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm Res*. 2000;17:811-816.

137. Sonawane ND, Jr, Szoka FC, Jr, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J Biol Chem*. 2003;278:44826-44831.

138. Rudolph C, Plank C, Lausier J, Schillinger U, Muller RH, Rosenecker J. Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. *J Biol Chem*. 2003;278:11411-11418.

139. Avrameas A, Ternynck T, Nato F, Buttin G, Avrameas S. Polyreactive anti-DNA monoclonal antibodies and a derived peptide as vectors for the intracytoplasmic and intranuclear translocation of macromolecules. *Proc Natl Acad Sci USA*. 1998;95:5601-5606.

140. Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. *Biochemistry*. 1996;35:1027-1036.

141. Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. *Gene Ther*. 1997;4:961-968.

142. Lee RJ, Huang L. Folate-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J Biol Chem*. 1996;271:8481-8487.

143. Lee LK, Williams CL, Devore D, Roth CM. Poly(propylacrylic acid) enhances cationic lipid-mediated delivery of antisense oligonucleotides. *Biomacromolecules*. 2006;7:1502-1508.

144. Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Ther*. 1997;4:891-900.

145. Murphy EA, Waring AJ, Haynes SM, Longmuir KJ. Compaction of DNA in an anionic micelle environment followed by assembly into phosphatidylcholine liposomes. *Nucleic Acids Res*. 2000;28:2986-2992.

146. Murphy EA, Waring AJ, Murphy JC, Willson RC, Longmuir KJ. Development of an effective gene delivery system: a study of complexes composed of a peptide-based amphiphilic DNA compaction agent and phospholipid. *Nucleic Acids Res*. 2001;29:3694-3704.

147. Longmuir KJ, Haynes SM, Dickinson ME, Murphy JC, Willson RC, Waring AJ. Optimization of a peptide/non-cationic lipid gene delivery system for effective microinjection into chicken embryo in vivo. *Mol Ther*. 2001;4:66-74.

148. Maitra A. Calcium phosphate nanoparticles: second-generation nonviral vectors in gene therapy. *Expert Rev Mol Diagn*. 2005;5:893-905.

149. Megeed Z, Jr, Haider M, Jr, Li D, Jr, O'Malley BW, Jr, Cappello J, Ghandehari H. *In vitro* and *in vivo* evaluation of recombinant silk-elastinlike hydrogels for cancer gene therapy. *J Control Release*. 2004;94:433-445.

150. Wagner E. Application of membrane-active peptides for nonviral gene delivery. *Adv Drug Deliv Rev*. 1999;38:279-289.