Synthetic DNA delivery systems

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The ability to safely and efficiently transfer foreign DNA into cells is a fundamental goal in biotechnology. Toward this end, rapid advances have recently been made in our understanding of mechanisms for DNA stability and transport within cells. Current synthetic DNA delivery systems are versatile and safe, but substantially less efficient than viruses. Indeed, most current systems address only one of the obstacles to DNA delivery by enhancing DNA uptake. In fact, the effectiveness of gene expression is also dependent on several additional factors, including the release of intracellular DNA, stability of DNA in the cytoplasm, unpackaging of the DNA–vector complex, and the targeting of DNA to the nucleus. Delivery systems of the future must fully accommodate all these processes to effectively shepherd DNA across the plasma membrane, through the hostile intracellular environment, and into the nucleus.

Keywords; nonviral gene delivery, transfection, gene therapy, DNA, intracellular transport

Over the past 30 years, DNA delivery, especially via the nonviral route (i.e., transfection), has become a powerful and popular research tool for elucidating gene structure, regulation, and function. Indeed, a recent search of the keyword "transfection" using the National Center for Biotechnology Information's (NCBI; Rockville, MD) MEDLINE database (www.ncbi.nlm.nih.gov/PubMed/) yielded more than 50,000 papers. DNA delivery has also been pivotal in developing new approaches (e.g., gene therapy and DNA vaccination) for treating and controlling diseases that are likely to impact clinical medicine and biotechnology over the next few years. Before such applications can be realized, however, the relative inefficiency and cytotoxicity of modern synthetic DNA delivery systems must be addressed.

Crucial to the success of DNA as a pharmaceutical or a basic research tool is transfection efficiency: in general practice, too few cells receive and express the exogenous DNA. Efficiency of transfection is dependent on both the efficiency of DNA delivery (i.e., fraction of DNA molecules getting into the nucleus) and the efficiency of DNA expression (i.e., fraction of nuclear DNA molecules that undergo transcription). Although a greater efficiency of expression can usually be achieved with strong promoters and enhancers[1], improvements in the efficiency of DNA delivery per se have been difficult to achieve; thus, the number of cells receiving DNA in their nucleus is usually small. In addition, transfection efficiency in vitro and in vivo do not always correlate[2,3], making translation of positive results in cell culture into animals even more difficult.

Therefore, the challenge of DNA delivery is to develop a system that is both highly efficient in delivery/expression and applicable to basic research as well as clinical settings. This paper reviews current methods of nonviral DNA delivery, highlighting systems that exploit our understanding of basic mechanisms of DNA transport and metabolism.

Defining the problem

Traditionally, DNA delivery systems have been classified as viral vector-mediated systems and nonviral vector-mediated systems (the majority of which are synthetic systems). Currently, because of their highly evolved and specialized components, viral systems are by far the most effective means of DNA delivery, achieving high efficiencies (usually >90%) for both delivery and expression. In fact, around 75% of recent clinical protocols involving gene therapy use recombinant virus-based vectors for DNA delivery (http://www.wiley.com/genetherapy/clinical/vectors.html). As yet, however (except for a few anecdotal reports of success in individual patients), no definitive evidence has been presented for the clinical effectiveness of any gene therapy protocol (for a review, see ref. 4). The impotence of current methodology is attributable to the limitations of viral-mediated delivery, including toxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, recombination, and high cost[5,6]. Furthermore, the toxicity and immunogenicity of viral systems also hamper their routine use in basic research laboratories. For these reasons, nonviral systems, especially synthetic DNA delivery systems, have become increasingly desirable in both basic research laboratories and clinical settings.

Most DNA delivery systems operate at one of three general levels: DNA condensation and complexation, endocytosis, and nuclear targeting/entry. Negatively charged DNA molecules are usually condensed and/or complexed with cationic transfection reagents before delivery. These complexes are taken up by cells, usually through endocytosis, the route of uptake determining subsequent DNA release, trafficking, and lifetime in the cell. Endocytosis is a multistep process involving binding, internalization, formation of endosomes, fusion with lysosomes, and lysis. The extremely low pH and enzymes within endosomes and lysosomes usually bring about degradation of entrapped DNA and associated complexes. Finally, DNA that has survived both endocytotic processing and cytoplasmic nucleases must then dissociate from the condensed complexes either before or after entering the nucleus. Entry is thought to occur through nuclear pores (which are ~10 nm in diameter) or during cell division. Once inside the nucleus, the transfection efficiency of delivered DNA is mostly dependent on the composition of the gene expression system, which has been addressed in other recent reviews[1].

The low efficiency of DNA delivery from outside the cell to inside the nucleus is a natural consequence of this multistep process. As a result, the number of DNA molecules decreases at each step of the journey to the nucleus. Therefore, identifying and overcoming each hurdle along the DNA entry pathways can improve DNA delivery, and hence overall transfection efficiency, dramatically. There are three major barriers to DNA delivery: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting (see Fig. 1). The effect of delivery systems on these three major barriers is the focus of this review. In the following sections, we describe the use of different methods for enhancing DNA penetration of the plasma membrane (see Table 1),

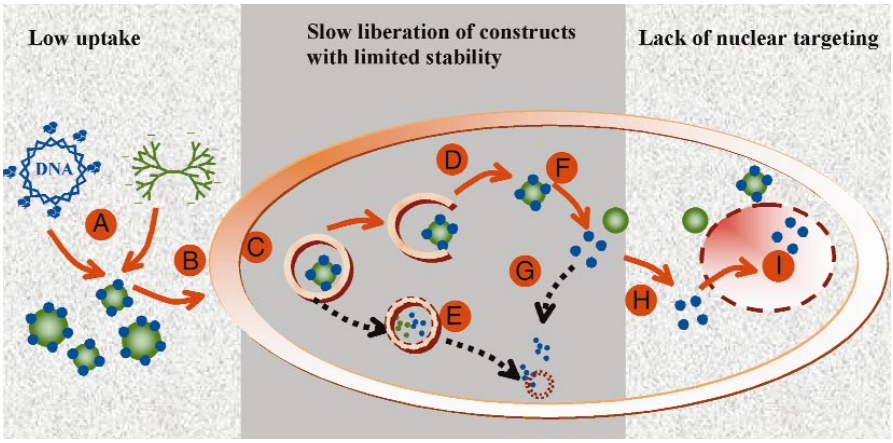

Figure 1. Schematic drawing of DNA delivery pathways with three major barriers: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting. (A) DNA–complex formation. (B) Uptake. (C) Endocytosis (endosome). (D) Escape from endosome. (E) Degradation (edosome. (F) Intracellular release. (G) Degradation (cytosol. (H) Nuclear targeting. (I) Nuclear entry and expression.

approaches for optimizing protection and intracellular release of DNA, and ways of enhancing targeting of DNA to the nucleus.

Mechanical and electrical methods

The direct injection of naked DNA (i.e., uncomplexed DNA) into a cell nucleus is perhaps the most conceptually simple, and therefore appealing, approach to gene delivery. One drawback of the approach, however, is that microinjection can be achieved only one cell at a time, which limits its use to applications in which individual cell manipulation is desired and possible, such as producing transgenic organisms. Though relatively efficient, the method is also rather slow and laborious and therefore neither appropriate for research with large numbers of cells nor practical for DNA delivery in vivo.

Recently, Mann and colleagues[7] have developed a pressure-mediated naked oligonucleotide transfection method using controlled, nondistending pressure. When they tested the approach on cardiovascular tissues, >50% efficiency of delivery was achieved. Whether or not this promising approach can be adapted to noncardiovascular tissues with plasmid DNA remains to be seen. Similarly, hydrodynamic force (rapid injection via tail vein) has been applied to deliver naked DNA to hepatocytes, with delivery efficiencies of ~40%[8,9]. Another method, ultrasonic nebulization, has also been used to deliver DNA–lipid complexes to cells[10].

Particle bombardment, which is also called biolistic particle delivery, can introduce DNA into many cells (including cell-walled plant cells) simultaneously. In this procedure, DNA-coated microparticles (composed of metals such as gold or tungsten) are accelerated to high velocity to penetrate cell membranes or cell walls[11–13]. Bombardment is widely employed in DNA vaccination, where limited local expression of delivered DNA (in cells of the epidermis or muscle) is adequate to achieve immune responses[14,15]. Because of the difficulty in controlling the DNA entry pathway, this procedure is applied mainly adherent cell cultures and has yet to be widely used systemically.

An alternative approach is to use high-voltage electrical pulses to transiently permeabilize cell membranes, thus permitting cellular uptake of macromolecules. This process, called electroporation, was first used to deliver DNA to mammalian cells in 1982 (refs 16,17). Since that time, electroporation has been used to deliver DNA to myriad cell types in vitro, including bacteria and yeast. It is one of the most efficient gene transfer methods, but it is limited because of the high mortality of cells after high-voltage exposure and difficulties in optimization.

Although electroporation is difficult to apply in vivo, some progress has been achieved in skin[18], corneal endothelium[19], and muscle[20]. An electrical permeabilization method using low-voltage, high-frequency electric pulses, reported recently by Rizzuto and colleagues[21], produced similar DNA uptake in muscle with only transient tissue damage, represents a significant improvement.

Chemical methods

The use of uptake-enhancing chemicals—which is arguably the easiest, most versatile, most effective, and most desirable of the DNA delivery methods—was demonstrated more than 30 years ago[22]. The general principle is based on complex formation between positively charged chemicals (usually polymers) and negatively charged DNA molecules. These techniques can be broadly classified by the chemical involved into those based on 2-(diethylamino)ether (DEAE)-dextran, calcium phosphate, artificial lipids, protein, dendrimers, or others.

The earliest chemical methods for DNA delivery were introduced in the late 1950s. These techniques used high salt concentration and polycationic proteins to enhance nucleic acid entry into the cell (for a review of early work, see ref. 23). Over a 10-year period starting in 1965, a variety of other chemicals were introduced, including DEAE-dextran[22] and calcium phosphate[24], which interact with DNA to form DEAE-dextran–DNA and calcium phosphate–DNA complexes, respectively. After the complexes are deposited onto cells, they are internalized by endocytosis.

DEAE-dextran and calcium phosphate methods are simple, effective, and still widely used in the laboratory for in vitro transfection. Even so, both methods are hampered by cytotoxicity and the difficulty of applying them to in vivo studies. In addition, DEAE-dextran can be used neither with serum in culture medium nor for stable transfection. The calcium phosphate method also suffers from variations in calcium phosphate–DNA sizes, which causes variation among experiments.

Since the early 1970s, many other polymers have been demonstrated to increase DNA uptake by cells. The most noticeable improvement is the development of artificial lipid-based DNA delivery systems. Felgner and colleagues developed the cationic lipid Lipofectin in 1987. Lipofectin–DNA complexes can be handled easily and, therefore, became one of the first chemical systems that could be used in animals.

In addition, DNA has been successfully complexed with cationic, anionic, and neutral liposomes, as well as various mixtures thereof. New lipid formulations and new recipes of blending have yielded

Table 1. Summary of DNA transfection methods.

Approach	Method
Mechanical	Microinjection Pressure Particle bombardment
Electrical	Electroporation (high voltage) Electroporation (low voltage)
Chemical	DEAE-dextran Calcium phosphate Artificial lipids Proteins Dendrimers Other polymers (including controlled-release polymers)

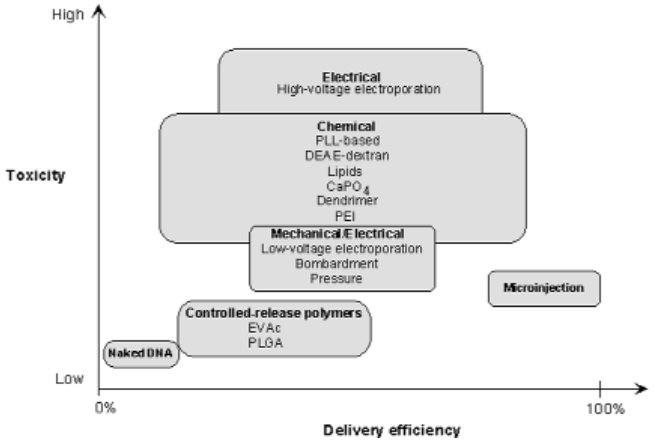

Figure 2. Comparison of delivery efficiency versus toxicity for various DNA transfection methods.

continuous improvements in transfection efficiency. Currently, lipid-based systems, which have been reviewed extensively elsewhere[23–31], are probably the most commonly used methods of DNA delivery and have been used in human clinical trials. Still, lipid-based systems have important drawbacks, including the lack of targeting, the poorly understood structure of DNA–lipid complexes, and variations arising during fabrication.

The major limitation of the above approaches is toxicity upon systemic administration[32]. In some situations, such as intramuscular injection[33] and inhalation[34], directly administered DNA (i.e., naked DNA) is expressed in tissue cells in the absence of vector; thus, the mechanism of lipid enhancement and the role of charged lipids in human gene therapy are still unclear.

Another approach that is gaining increasing prominence is the use of protein-based methods for DNA delivery, in some part as a result of the versatility offered by the addition of other chemicals. The cationic peptide poly-L-lysine (PLL) can condense DNA for more efficient uptake[35]. Kim and colleagues[36] have devised a "terplex" system where PLL, low-density lipoprotein (LDL), and DNA produced a fivefold increase in reporter gene expression in vitro. A significant advantage of PLL conjugates as delivery systems is the possibility of chemical modification for cell-specific transfection. For example, PLL has been conjugated with ligands, such as asialoorosomucoid (ASOR), which binds to a liver-specific asialoglycoprotein receptor to achieve receptor-mediated uptake[37]. Conjugates of PLL, DNA, and epidermal growth factor (EGF) have provided an elegant system for examining the importance of ligand density and conjugate properties on DNA delivery to cells expressing the EGF receptor[38]. However, the polydispersity of PLL preparations resulting from the procedures used in its synthesis leads to variable DNA delivery and difficulty in forming DNA complexes.

Other cationic peptides are also capable of condensing DNA and enhancing uptake. For example, Huang and colleagues[39] have combined the polycationic peptide protamine sulfate with cationic liposomes to enhance DNA delivery in vitro. Similarly, Schwartz and colleagues[40] have synthesized short peptides derived from human histone or protamine and formed peptide–DNA–lipid complexes that enhanced DNA delivery both in vitro and in vivo.

In another approach, a novel bifunctional fusion protein, Gal4-invasin, has been used to form complexes with PLL and DNA; Gal4 is an *Escherichia coli* DNA-binding protein, and invasin is a cell-binding protein from *Yersinia pseudotuberculosis.* Together, Gal4-invasin, PLL, and DNA complexes have been demonstrated in vitro to deliver DNA in an invasin receptor-specific fashion[41]. Of course, one drawback of this approach is that bacteria fusion proteins may present immunological problems when used in vivo; synthetic, short peptide-based approaches, on the other hand, are much less immunogenic and may provide another viable design alternative (for a review, see ref. 42).

Polyamidoamine (PAMAM) dendrimers are a class of highly branched cationic polymers[43] that, unlike PLL, have a well-defined architecture. Similar to PLL, these dendrimers are capable of condensing DNA[44] and delivering it to a variety of cell lines with minimum cytotoxity[45]. Szoka and colleagues[46] have improved the expression level 50-fold by partially degrading dendrimers. Since their introduction, PAMAM and other dendritic polymers have gained popularity because of their versatility and simplicity in transfection.

Synthetic polymers often can be improved by chemical modification. For example, Choi and colleagues[47] have conjugated linear polyethylene glycol (PEG) with a dense globular PLL dendrimer forming PEG–block–PLL–dendrimer, a water-soluble polyionic spherical complex. Other chemicals (mostly polymers) have also been explored for enhancing DNA delivery, including the following: polyethylenimine (PEI)[48] and its conjugates, such as mannose–PEI[49], transferrin–PEI[50], and linear PEI (IPEI)[51]; gelatin[52] and chitosan[53]; methacrylate/methacrylamide polymers[54]; and pyridinium surfactants[55].

The methods described above all expose cells to a pulse of DNA; thus, DNA and transfection agents are available for uptake into cells only for a short period of time. Recently, biocompatible controlled-release polymers have also been introduced into the synthetic DNA delivery field. Several groups have successfully encapsulated naked DNA into biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) microparticles[56] and PLGA microspheres[57–59] for long-term and controlled DNA release. DNA has also been encapsulated into highly biocompatible poly(ethylene-*co*-vinyl acetate) (EVAc) matrices, resulting in the controlled and predictable release of bioactive DNA for several months[59]. These low-cost and adjustable controlled-release DNA delivery systems, which are made of materials approved by the US Food and Drug Administration (FDA; Rockville, MD), have important advantages over other DNA delivery methods, including DNA protection before release, site-specific delivery using implantable polymers, and long-term release without repeat administration. In fact, Mooney and colleagues[60] have recently demonstrated a significant tissue response using PLGA to deliver functional DNA locally. To date, only naked DNA has been tested in controlled-release systems; combinations of controlled release with other chemical enhancers may offer additional benefits.

The above classification is provided for convenience in reviewing the field; it is not meant to suggest that these methods must be independent. Novel combinations of mechanical, electrical, and chemical methods may provide us with better DNA delivery systems in the near future. A summary of the efficiencies of various methods is shown in Figure 2.

Protection and intracellular release of DNA

While improvements in the efficiency of DNA uptake by cells are being made, it is also essential to protect DNA from both extracellular and intracellular degradation during the long journey to the cell nucleus. Extracellularly, DNA can be protected by complex formation with various polymers and lipids, as mentioned above. If delivered systemically, however, DNA is susceptible to blood clearance. A process known as "opsonization" removes 80–90% of hydrophobic particles in blood, and is thus a major limiting factor for DNA delivery using artificial lipids. A recent review describes the biodistribution of various nonviral gene delivery systems, including comparison of potential delivery routes, stability in blood circulation, and extravasation into tissues[61].

Intracellularly, DNA must escape from normal endosomal pathways, which lead to degradation. Indeed, by comparing three different cationic lipid compounds, Ouahabi et al.[62] have observed that the efficiency of DNA delivery is correlated not only with uptake,

but also with destabilization and escape from endosomes. Methods to enhance DNA early release from endosomal pathways are thus actively being explored. Chloroquine, which raises endosomal pH, has been used to decrease DNA degradation by inhibiting lysis[63]. Branched cationic polymers, such as PEI or starburst dendrimers, also promote early release of DNA[45,48,64]. Synthetic peptides that include a carrier domain and an amphipathic domain for pH-dependent endosome lysis have also been investigated[65]. Some attempts have been made to completely bypass endosomal pathways; subunits of toxins, such as *Diphtheria* toxin and *Pseudomonas* exotoxin, have been incorporated into DNA complexes to enhance transfection[66], but this approach can be limited by the inherent toxicity and immunogenicity of the subunits. Viruses have evolved to totally escape and/or bypass endocytosis with extremely high efficiency. For example, the viral capsid of the adenovirus is capable of association with the endosomal membrane, which results in pore formation and lysis of the endosome[67]. Similarly, hemagglutinin HA2 peptide from influenza virus can insert itself into the endosomal membrane, which also leads to lysis[68]. Incorporation of these viral components can enhance transfection efficiency; Plank and colleagues[69] have demonstrated that fragments of the virus hemagglutinin enhance the efficiency of DNA delivery when incorporated into PLL–DNA complexes.

The cytosol is an unfriendly environment for DNA. Movement of DNA through the cytosol toward the nucleus probably occurs by diffusion, a relatively slow process during which DNA must be protected. Coating with PEG can stabilize plasmid DNA encapsulated in lipid particles and protect them from degradation by nucleases[26]. Katayose and Kataoka[70] have investigated a block copolymer, PEG–PLL, and have shown that copolymer–DNA complexes are highly resistant to DNase I attack. Similar stabilization and protection of DNA has been achieved by Wiehle and collaborators[71] using PLL, epidermal growth factor (EGF), and streptavidin complexes in in vitro transfection experiments. DMI-2, an acid nuclease inhibitor, has also been noted to enhance reporter gene expression[72]. These studies represent progress in an area that is critical for enhancing DNA delivery efficiency.

Since most DNA delivery systems involve complex formation with other molecules, liberation of DNA molecules from a macromolecular assembly must occur before transcription can proceed. The stability of complex formation, and therefore the rate of DNA "unpackaging," must influence efficiency of gene expression. Although DNA release from vector complexes is often neglected, recent work by Schaffer, Lauffenburger, and colleagues[73] demonstrates the importance of this step. Maximal gene expression occurs at intermediate stability, because stable complexes restrict DNA transcription and unstable complexes permit rapid DNA degradation.

Nuclear targeting

Finding the nucleus is the final obstacle for DNA delivery, and in this respect, knowledge concerning nuclear targeting is still relatively rudimentary. Certain synthetic polymers, such as PEI, but not cationic lipids, protect DNA in the cytoplasm and are known to promote entry into the nucleus[74], and a recent paper has studied the pathway for PEI transport[75]. Overall, however, synthetic systems are notably more inefficient than viral vectors at targeting the nucleus.

Dohrman and collaborators[76] have investigated the subcellular distribution and integrity of a small DNA fragment delivered by cationic lipids. As expected, only a small portion (0.3%) of input DNA was detected inside the nucleus after 24 h.

Viral nuclear localization signals (NLSs) are a logical addition to synthetic DNA delivery systems. Indeed, a fusion protein between a NLS and a peptide nucleic acid (PNA) has been shown to facilitate nuclear transport of transfected DNA[77]. Similarly, other fusion proteins, such as Gal4-NLS, have been employed to enhance transfection efficiency. Plant NLS proteins have also been complexed with DNA and demonstrated to be efficiently imported into mammalian nuclei[78].

Most recently, nuclear-targeting peptide scaffolds have been conjugated and synthesized for lipid-based transfection of nondividing mammalian cells; greater than 80% delivery efficiency and a 63-fold increase in reporter gene expression was achieved[79]. Since the final destiny of transfection is the nucleus, it is clear that progress in understanding and exploiting nuclear targeting should greatly increase the efficiency of DNA delivery.

In addition to the three major barriers to the efficiency of cellular DNA delivery, several other hurdles also need to be addressed, including optimization of DNA condensation, size of DNA complexes, route of administration, biodistribution, bioavailability, cell and tissue targeting, and cytotoxicity. These issues have all been discussed in recent reviews[1,28,61,80].

Perspective

Based on previous studies (Fig. 2), the ideal synthetic DNA delivery system should possess the following properties: ease of assembly (e.g., it should use modular components that can be "packaged" in vitro); efficient delivery leading to total transfection (i.e., DNA should be physically delivered to the majority of intended cells); stabilization of DNA before and after uptake (e.g., using nontoxic biocompatible materials); capability of bypassing or escaping from endocytotic pathways (e.g., by incorporating viral components); efficient decomplexation or "unpackaging" (e.g., intracellular controlled release); efficient nuclear targeting; and high, persistent, and adjustable expression of therapeutic levels of proteins.

While no current synthetic systems have all these properties, many viruses do and have evolved them to enable efficient infection. Lessons from viruses have already improved synthetic delivery systems; for example, expression efficiency has been improved significantly by inclusion of viral DNA components (such as promoters and enhancers). There is little doubt that viral protein components will be incorporated into future synthetic DNA delivery systems, but it is not necessary that delivery systems of the future must be "virus-like" particles. Instead, by understanding and incorporating the extremely efficient mechanisms of infection by viruses, future DNA delivery systems will be viruslike in function, not necessarily in shape—just as our aviation systems mimic the function of birds, but not always their morphology.

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