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DNA Nanoparticles and Development of DNA Delivery Vehicles for Gene Therapy†

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ABSTRACT: DNA transport through the cell membrane is an essential requirement for gene therapy, which utilizes oligonucleotides and plasmid DNA. However, membrane transport of DNA is an inefficient process, and the mechanism(s) by which this process occurs is not clear. Although viral vectors are effective in gene therapy, the immune response elicited by viral proteins poses a major problem. Therefore, several laboratories are involved in the development of nonviral DNA delivery vehicles. These vehicles include polyamines, polycationic lipids, and neutral polymers, capable of condensing DNA to nanoparticles with radii of 20−100 nm. Although the structural and energetic forces involved in DNA condensation have been studied by physical biochemists for the past 25 years, this area has experienced a resurgence of interest in recent years because of the influx of biotechnologists involved in developing gene therapy protocols to combat a variety of human diseases. Despite an intense effort to study the mechanism(s) of DNA condensation using a variety of microscopic, light scattering, fluorescence, and calorimetric techniques, the precise details of the energetics of DNA nanoparticle formation and their packing assembly are not known at present. Future studies aimed at defining the mechanism(s) of DNA compaction and structural features of DNA nanoparticles might aid in the development of novel gene delivery vehicles.

A major requirement for gene therapy is the efficient transport of DNA through the cell membrane by processes that are not well defined (*1*, *2*). The mechanistic pathway for gene transfection includes the collapse of extended DNA chains into compact, orderly particles containing only one or a few molecules (*3*, *4*). This process, known as DNA condensation, has received considerable attention in recent years due to its biological importance in DNA packaging in virus heads as well as in the development of gene delivery vehicles (*3*−*7*). Multivalent cations such as polyamines, positively charged polymers, and peptides are known to provoke the condensation of DNA to nanoparticles that appear as rods, toroids, or spheroids under the electron microscope. Recent studies indicate a columnar hexagonal liquid crystalline packaging of DNA in the presence of polyamines and other multivalent cations (*8*, *9*).

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DNA condensation is an example of polymer−globule transition, which is readily reversible and is favored by the association of multivalent cations around DNA phosphate groups (*3*, *10*−*14*). The cations cause localized bending or distortion of DNA at a critical extent of charge neutralization, which facilitates the formation of rods and toroid-like structures (*15*, *16*). In addition to decreasing the net charge on DNA, the cations aid in decreasing the unfavorable DNA segment−segment interactions. Thermodynamic analysis of polymer condensation was carried out by Post and Zimm based on the Flory−Huggins lattice theory of polymer solutions by including a third virial coefficient to take into account the high local concentration of polymer segments in the condensed state (*17*). Recently, two models, the spool model and the constant loop model, have been proposed to explain how the DNA is wound within the toroid (*18*, *19*). The ligands are believed to affect the rotational dynamics and, in turn, the hydrodynamic radius by altering the hydration layer and thereby the structure of DNA (*20*).

Nanoparticles formed by the condensation of DNA in the presence of cationic polymers and polyamine analogues are essential for the transport of oligonucleotides and plasmid DNA through the cell membrane (*1*, *2*, *4*−*7*, *21*−*24*). While small cationic molecules reversibly dissociate on dilution and also on polyanionic interactions, chemical modification of the condensing agent introduces stability to the complexes and enhances transfection efficiency (*25*, *26*). Pollard et al. (*27*) suggested that compaction of plasmid DNA into spherical particles rather than the ionic charge of the DNA complexes is important in nuclear trafficking mechanisms. However, there has not been any consensus regarding the relationships between the size and shape of the DNA particles, their ability to be taken up by cells, and the chemical structure of agents that cause the condensation of DNA. Confocal microscopic investigation tracking the intracellular path of polyethylenimine (PEI)−DNA complexes using fluorescently tagged PEI and fluorescent DNA provided evidence for the nuclear entry of both the delivery vehicle (PEI) and DNA (*28*). Nuclear accumulation was also found in polyamine-facilitated oligonucleotide transport in MCF-7 breast cancer cells (*29*). In this review, we summarize the DNA condensation process in an attempt to evaluate agents and processes that promote the cellular transport of oligonucleotides and plasmids that are used in gene therapy applications.

DNA Condensation by Cationic and Polymeric Agents

DNA compaction to toroids, rods, or spheroids that are ∼50−300 nm in diameter can be achieved in the presence multivalent cations, including polymers such as PEI, alcohols, and neutral or anionic polymers with monovalent salts (*3*, *4*, *7*, *9*−*14*, *18*, *22*−*28*, *30*−*34*). The most extensive work on DNA condensation has been carried out using the natural polyamines, spermidine [$H_2N(CH_2)_3NH(CH_2)_4NH_2$] and spermine [$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$], and cobalt hexamine [$Co(NH_3)_6^{3+}$], an inorganic cation that is nonreactive to DNA bases (*3*, *9*−*11*, *18*, *35*−*39*). Charge density and features of polyamine structure have remarkable effects on the DNA condensation and size of the nanoparticles that are formed. Compaction of T4 DNA using a series of diaminoalkanes indicated that compounds with three and five methylene groups showed a significant ability to compact DNA, while diamines with two, four, and six methylene groups were less effective (*40*). Although spermidine and cobalt hexamine bear the same number of three positive charges, cobalt hexamine condensed DNA 5-fold more efficiently than spermidine, with the size of the condensates generally smaller than that formed with spermidine (*36*). DNA condensation in the presence of a series of tri- and tetravalent polyamine homologues showed that the chemical structure of isovalent polyamines exerts a profound influence on the ability of these molecules to provoke DNA nanoparticle formation and the size of the nanoparticles (*36*, *39*). Table 1 summarizes some representative sizes of the DNA nanoparticles formed in the presence of cobalt hexamine, polyamines, and polymeric agents.

Table 1: DNA Condensing Agents and Size of DNA Nanoparticles

condensing agent	source of DNA	size of nanoparticles (nm)	method[a]	ref
spermidine	T4, T7, viral f29, PM2, λ, or calf thymus	45−130	DLS, EM, or AFM	*31*, *35*−*38*
spermine	λ or pEGlacZ	50−130	DLS or EM	*18*, *39*
$Co(NH_3)_6^{3+}$	pUC12, λ, calf thymus, or polynucleotides	39−45	DLS or EM	*36*−*38*
lipospermine	pSfiSVneo, pSfiSV19, or pCISfi-γIFN	50−70	SFM	*33*
poly(ethylenimine)	same as above	20−40	SFM	*33*
cationic thiol detergent	pCMV-Luc	35	TEM	*25*
PLL-AsOR	pCMV-Luc	50	AFM	*16*
galactosylated PLL	PCMV-Luc	30	EM	*23*
poly-L-lysine	plasmid DNA	10−100	DLS	*25*, *34*
peptide	CMV-β-gal	20−100	PCS	*32*
poly(4-hydroxyproline)	pSV-CAT	100	DLS	*24*

[a] DLS, dynamic light scattering; EM, electron microscopy; AFM, atomic force microscopy; SFM, scanning force microscopy; TEM, transmission electron microscopy; PCS, photon correlation spectroscopy.

DNA condensation by cationic silanes, which combine both the condensing properties of polyamines and cross-linking properties of silane, results in well-defined classical toroidal and rod-shaped structures (*10*, *32*). Consequently, solid supports, treated with monovalent or multivalent silanes followed by removal of the soluble molecules, present a platform for DNA condensation. Interestingly, silica particles coated with aminosilanes are reported to be effective carriers for gene delivery (*41*).

In contrast to small cations, such as polyamines and cobalt hexamine, that have been studied for understanding the mechanism(s) of DNA condensation, cationic polymers, including PEI and poly-L-lysine, are of interest because of their efficacy in transporting DNA through the cell membrane (*23*, *42*, *43*). The ability of PEI and poly-L-lysine to condense DNA and the hydrodynamic properties of DNA nanoparticles produced by these agents have been characterized (*23*, *27*, *34*). Although PEI with branched structure condenses DNA to a greater extent than linear ones, the size of the condensed particles formed by linear and branched PEI is comparable. However, significant differences were observed in the toxicity of branched versus linear PEI, and the ability of these molecules to transfect cells (*44*). Atomic force microscopic (AFM) studies indicate these complexes are 20−40 nm in

diameter, and electron microscopic (EM) studies show the presence of toroidal condensates 55 ± 12 nm in diameter (*27*, *33*). However, dynamic light scattering studies revealed complexes with apparent diameters of 90−130 nm (Table 1). The apparent discrepancies in the size of the particles determined by various methods might be related to sampling techniques. Use of dry samples in AFM and EM can lead to dehydration of the particles, with a decrease in the apparent size of the condensed particles. Light scattering methods provide *z* average values, where large particles contribute more than the small particles. In contrast, visualization techniques (AFM and EM) give number average values where both small and large particles contribute equally (*45*).

Poly-L-lysine interacts with DNA cooperatively at high NaCl concentrations and in excess of DNA, and produces DNA particles with various structures, depending upon the concentration of monovalent ions in the medium (*23*). A significant advantage of poly-L-lysine is that it can be conjugated with ligands to achieve receptor-mediated uptake. Efficient receptor-mediated transfection occurred only when cooperative interactions were present and the condensed DNA complex (with poly-L-lysine) had a spherical shape and a diameter of 15−30 nm. On the other hand, McGregor et al. reported that condensates of ∼500 nm formed in the presence of a novel group of cationic gemini surfactants could promote transfection of a plasmid DNA (*46*). It is not known whether different uptake pathways are involved in facilitating the small (10−20 nm) versus large (500 nm) DNA particles. The condensed particles could also be formed from oligonucleotides using cationic polymers (*47*−*50*). Polybutylcyanoacrylate and polyisohexylcyanoacrylate are often used with antisense oligonucleotides. Because of the negative surface potential of the polymer particles, a cationic copolymer or a cationic hydrophobic detergent is combined with polyalkylcyanoacrylate (PACA) polymers (*47*). Oligonucleotide particles prepared in this manner have a size range of 50−500 nm (*48*, *50*).

Mechanistic Considerations

The ability of cationic molecules to condense DNA indicates that a major factor governing the compaction of DNA is charge neutralization by electrostatic interaction between the negatively charged phosphate groups of DNA and the positively charged groups of the cations (*3*, *51*−*54*). Due to its highly organized chemical structure and negative charge, double-stranded DNA is inherently rigid. Volume occupied by a random coil DNA is dependent upon its molecular weight, size, and persistence length. The flexibility of DNA is characterized by the persistence length and the distance between its ends, manifested by excluded volume (*55*, *56*). The persistence length of DNA has two components, the electrostatic contribution due to the repulsion between the two strands and the intrinsic stiffness of the uncharged helix. Due to the large electrostatic repulsion, DNA remains in the elongated coiled state. Under high-salt conditions, the level of electrostatic interactions is reduced by shielding, resulting in a decrease in the persistence length (*57*). Investigations into the effect of spermidine on the persistence length of DNA showed that cross-linking by the polyamine was required for DNA collapse (*53*). Under these conditions, excluded volume is minimized and DNA forms a highly ordered liquid crystalline cholesteric phase or a more dense regular hexagonally packed phase by attractive interactions between the parallel rods (*8*, *9*, *11*). Therefore, the ionic strength of the medium and the number of positive charges of the counterions play important roles in DNA condensation. The cations can also provoke DNA condensation by making DNA−solvent interactions less favorable (*51*, *53*, *54*).

Manning (*58*) proposed a model for cation-induced DNA condensation in which DNA bends spontaneously at a critical extent of charge neutralization, leading to the formation of toroids, which are the natural physical state of stably bent DNA. According to the counterion condensation theory, 76, 88, 92, and 94% of DNA phosphate charge is neutralized by mono-, di-, tri-, and tetravalent cations, respectively. Wilson and Bloomfield (*35*) studied the compaction of T7 bacteriophage DNA with putrescine (2+), spermidine (3+), and spermine (4+) and found that a positive tri- or tetracation was necessary to collapse DNA in an aqueous solution. Using the counterion condensation theory, they calculated the total charge neutralization by a mixture of mono- and multivalent cations and found that DNA condensation occurred at >89% charge neutralization. The critical concentration of the multivalent cation required for DNA condensation increased with an increase in monovalent ion concentration in solution (*35*, *39*, *59*). However, DNA condensation did not occur at 1.5 M Na^+ even when the spermine concentration was increased to a level 6 orders of magnitude higher than that required for DNA condensation at 1 mM Na^+ (*59*).

Although the interaction between multivalent and polymeric cations with DNA is electrostatic in origin, geometric and chemical considerations also play a significant role in DNA condensation. Schellman and Parthasarathy (*60*) showed that the structural arrangement of DNA precipitated by spermidine homologues had a strong dependence on the chemical structure of the polyamines. The Bragg spacing and the calculated interhelical spacing for a hexagonal packing model of DNA varied systematically with the length of the methylene bridge. Investigations of λ-DNA condensation by spermine homologues showed that the midpoint concentration of polyamine homologues for condensing DNA and the hydrodynamic radii of the condensates were dependent on the polyamine structure (*39*). This result indicates that the number of positive charges and the charge spacing of polyamine molecules affect the size of DNA nanoparticles formed by multivalent cations. Polyamine homologues also exhibited significant differences in their ability to provoke liquid crystalline textures of the aggregates of genomic DNA and oligonucleotides (*9*, *61*).

Early physical and biochemical studies supported a mechanism of toroid formation by circumferential winding of DNA, often denoted as the spool model (*18*, *62*, *63*). Hud et al. (*19*) proposed an alternate model that is compatible with the experimental data obtained from EM studies. Accordingly, DNA in toroids is organized within a series of equally sized contiguous loops around the toroid axis. Theoretical considerations based on this model predict that the kinetic process of spontaneous loop formation is the dominant factor in the determination of toroid dimensions. It was also suggested that formation of toroids is a coil−globule transition (*3*, *11*, *61*, *64*). Dunlap et al. (*33*) observed individual DNA strands, separated by ∼3.2 nm, in the condensed state by atomic force microscopy. It was shown that toroids were formed by the opening of condensed DNA

rods, at least under certain conditions (*16*, *33*). Alternately, the model proposed by Ha and Liu (*65*) postulates an infinite number of rods that bundle into a condensate due to the counterion correlation across the bundle, and suggest that nonelectrostatic forces might be responsible for bundle formation. X-ray diffraction and polarizing microscopic results are consistent with local hexagonal ordering of DNA strands with interaxial spacing of 25−30 Å (*8*, *11*, *66*).

Bloomfield and co-workers (*51*, *67*) found that multivalent cations significantly reduce the magnitude of the long-range repulsive forces and allow the attractive forces to dominate. Free energy changes associated with electrostatic interactions, bending, hydration, and mixing drive DNA condensation; however, electrostatic interaction is the dominant force in DNA condensation. In this process, Coulombic interactions should be stronger than entropic interactions so the entropy loss can be overcome. Hence, multivalent ions with a valence of ≥ 3 are required for DNA condensation. When Coulombic interactions are strong enough to dominate entropic interactions, DNA forms a charged ordered structure, such as a toroid or rod (*3*, *51*).

The effects of sequence and secondary structure on the morphology of DNA condensates were studied by Reich et al. (*68*), who found that toroids of unusually small inner radius can be obtained by using DNA fragments with intrinsically bent A-tracts. Shen et al. (*69*) also investigated the effect of static loops on toroid formation by condensing DNA with intrinsically bent A-tracts. Their results support the hypothesis that toroid size is influenced by the size of the first loop upon which DNA is condensed. In terms of flexible rod models and crossover points, Park et al. (*70*) described the size invariance of the toroidal condensates formed from DNA in the range of 400−5000 base pairs. This model explains that the size of the torus is determined by the torus supporting only a certain number of topological defects the minimum number of which is determined by the number of loops or number of strands in the cross section of the torus. Even a short, single-stranded oligonucleotide of 18 bp was shown to form toroids upon complexation with asialoorosomucoid-conjugated polylysine (*71*). A tendency to decrease the surface area and the need to minimize the bend seem to make for a plausible explanation for the formation of toroids. However, the basis of predominant toroid formation in preference to other structures remains unresolved.

Toroids and rods were found to be the end products of DNA condensation induced by polyamines, cobalt hexamine, and polymeric peptides of interest in gene transfection. Using electron microscopy, Böttcher et al. (*18*) showed the presence of a series of complex multimolecular condensates with parallel bundles of DNA in the presence of spermidine. Atomic force microscopic images revealed early precollapse intermediates with interesting morphologies such as flower- and disklike structures formed by the crossing over of multiple loops on the DNA strand at the same point (*10*, *16*, *32*, *33*). With an increasing concentration of cations, multimolecular flowers were formed by the association of two or more strands, which later appear to be disk-shaped condensates, indicating that several intermediates with intra- and intermolecular contacts are involved in the early stages of DNA condensation. When cationic concentrations that are higher than that required for producing complete rods and toroids are used, condensation occurs by an abrupt two-state process. Evidence for a multistage condensation process has been obtained recently from isothermal calorimetry experiments on the interaction of polyamines, cobalt hexamine, and cationic lipids with DNA (*72*−*74*).

Electron microscopic studies indicate that formation of DNA toroids and rods depends on the counterion and solvent used in DNA condensation. The toroid size was also dependent on the topology of DNA and the nature of the cations (*75*). Toroids of arbitrary sizes, with the outer diameter varying between 90 and 200 nm, were formed by the progressive release of T5 phage DNA into proteoliposomes reconstituted with the receptor FhuA and spermine (*76*). A comparable situation is involved in DNA compaction in the presence of cationic lipids that are used in gene transfection (*77*−*79*). An early model of lipid−DNA complex formation suggested that DNA is encapsulated in the interior of large unilamellar liposomes formed by liposome fusion in the presence of DNA (*80*). Other studies suggest that DNA is entrapped in the water channels between lipids in the hexagonal lipid phase (*81*). Liquid crystalline phases have also been found in both polyamine−DNA and lipid−DNA complexes that are relevant to gene therapy (*9*, *11*, *13*, *82*, *83*).

Cellular Transport of DNA

The most important goal of gene therapy is to develop site-specific gene delivery systems for the controlled expression of transgenes in targeted cells or tissues (*84*−*86*). In most instances, it is accomplished by the proper selection of an appropriate vector with the desired expression system. Gene delivery vectors generally fall into two categories: viral and nonviral. DNA can also be directly injected into the target tissue and expressed to induce an immune response (*87*−*89*). While viral vectors are mainly replication defective viruses with some or all of the coding sequences being replaced by therapeutic genes of interest, the nonviral system relies on DNA nanoparticle formation in the presence of DNA compacting agents, including polyamines and their derivatives as well as liposomes. Viral vectors currently employed in gene therapy include retroviruses, adenoviruses, and adeno-associated viruses, and each one of them has its own unique advantages (*90*−*94*). While retroviruses can infect only dividing cells, adenoviruses can be used for both dividing and nondividing cells. On the other hand, adeno-associated viruses can produce progenies only in the presence of a helper virus such as adenovirus. Viral vectors are highly effective in achieving high efficiency for both gene delivery and expression, and exhibit stable long-term expression of a foreign gene when the recombinant DNA is integrated into the chromosomal DNA. Major limitations of virally mediated gene delivery include limited DNA carrying capacity, toxicity, potential replication, and high cost. Nonviral gene delivery vehicles are being developed to overcome the deficiencies of viral vectors.

Nonviral vectors rely on the basics of supramolecular chemistry in which anionic DNA molecules are condensed into compact, ordered nanoparticles that are ~50−200 nm in diameter by complexing DNA with an appropriately designed cationic molecule. The polycations reduce the size of the complex, and confer excess cationic charge to the

complex, thereby enhancing their cellular uptake by an endocytosis pathway. While viral vectors impose a restriction on the size of the DNA that can be used, DNA molecules as large as 2.3 Mb can be condensed into compact particles that are suitable for gene delivery (*95*). Since very large DNA molecules can be condensed into compact particles, nonviral vectors permit the incorporation of the gene regulatory regions that may afford better control of gene expression. Once DNA condensation has been established with synthetic vectors, it is possible to incorporate functional groups into the carrier molecules so that cell-specific targeting and nuclear localization can be facilitated (*96*). Ideal gene delivery systems should be stable, nontoxic, and biodegradable and protect DNA from degradation (*1*, *2*, *97*−*101*). They should also mediate cellular uptake by cell-specific plasma membrane receptors and facilitate endosomal release (*34*, *97*).

Nonviral systems should be sufficiently stable to serum inactivation and provide protection of DNA from degradation (*1*, *2*, *97*−*103*). Parameters such as lipid and DNA concentrations, particle size distribution, turbidity, and ζ potential affect the expression of the transfected gene. The charge and stability of the particles can be predicted to a certain extent by ζ potential measurements. While DNA molecules condensed with low-molecular weight cations are susceptible to aggregation under physiological conditions, DNA condensed by polymers can resist aggregation and is stable (*103*). The aggregation may be explained by DLVO (Derjaguin Landau Verwey Overbeek) theory, which predicts that colloids are stable if the net electrostatic repulsions are stronger than the attractive forces (*45*). The surface charge of the complex depends on the nature of the condensing agent and also on the ratio of the condensing agent to DNA. Addition of salt overcomes the repulsive interactions and leads to the aggregation of the complex. DNA condensates formed in the presence of some cationic polymers exhibit excess positive surface charge that prevents it from aggregating under physiological salt conditions.

For cationic lipid- and polyamine-mediated transfection, the first step is believed to be the electrostatic interaction between the carrier−DNA complex and the anionic plasma membrane. A positively charged structure is desirable since it can preferentially adhere to the negatively charged cell surface receptors, leading to endocytosis (*104*, *105*). A recent study indicates that the amount of negative charges on the cell surface and the size of the liposome−DNA complex play important roles in determining successful gene delivery (*106*). Receptor-mediated endocytosis, pinocytosis, and phagocytosis can occur depending on the size of the liposome−DNA complex (*102*). Inside the cytoplasm, endosomes are destabilized and release the DNA, although this is an inefficient process in many cases (*107*). A schematic diagram of DNA uptake in cells is shown in Figure 1.

Several approaches have been used in an attempt to facilitate the cytoplasmic release of DNA. Fusogenic peptides facilitate the cytoplasmic delivery of DNA when such peptides are complexed with liposome−DNA complexes. Chloroquine is known to enhance transfection by facilitating endocytosis (*108*, *109*). Chloroquine accumulates in the acidic vesicles, leading to osmotic swelling and subsequent destabilization of the endosome. Once DNA is released into the cytoplasm, it may enter the nucleus by the support of

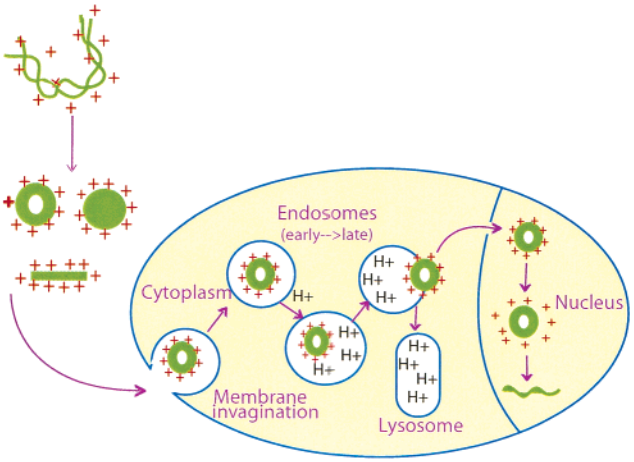

FIGURE 1: Schematic representation of DNA uptake by mammalian cells. DNA is compacted in the presence of polycations into ordered structures such as toroids, rods, and spheroids. These particles interact with the anionic proteoglycans at the cell surface and are transported by endocytosis. The cationic agents accumulate in the acidic vesicles, increase the pH of the endosomes, and inhibit the degradation of DNA by lysosomal enzymes. They also sustain a proton influx, which destabilizes the endosome, and release DNA. The DNA then is translocated to the nucleus either through the nuclear pore or with the aid of nuclear localization signals, and decondenses after separation from the cationic delivery vehicle.

nuclear localization signals (*96*). Conjugation of a nuclear localization signal peptide to plasmid DNA at a molar ratio greater than 40 was shown to enhance the nuclear transport of DNA (*96*, *110*). The transport of DNA across the nuclear envelope occurs through the nuclear pore (*111*). While smaller particles of 25 nm can freely diffuse through the nuclear pore that is ~55 nm in diameter, larger molecules enter the nucleus through a nuclear pore complex (*111*, *112*). Inside the nucleus, DNA should dissociate from the cation to be transcriptionally active. Release of DNA takes place by the interaction of cellular cations, histones, or liposomes with compositions that mimic that of the cytoplasmic monolayer of the plasma membrane. Studies on intracellular trafficking of the polylysine−liposome−DNA complex showed that modulation of endocytosis alters the activity of the transfected gene, indicating that cytoplasmic delivery of the gene involves fusion-related events in the endosome (*113*). Alternatively, the DNA−carrier complex may directly enter cells through the cell membrane. The transition to a two-dimensional columnar phase in DNA cationic liposome complexes was found to favor efficient transfection due to the ability of this liquid crystalline phase to rapidly fuse and release DNA after its attachment to anionic vesicles (*114*). Thus, interactions at the level of the endosomes are critical to the activity of the transfected gene.

The search for a suitable gene carrier which can overcome the barriers to gene therapy, such as membrane fusion, endosomal release, or nuclear targeting, has led to a wide spectrum of DNA compaction agents that produce a moderate increase in the level of expression of the transfected gene. These include proteins, cationic lipids, liposomes, novel synthetic polymers, or combinations thereof since many of these agents are capable of interacting with DNA to form compact particles. Cationic lipids possess some common structural similarities, such as a hydrophobic moiety, a positively charged headgroup, and a linker functional group such as an ester, amide, or carbamate to bind these moieties

together covalently. These positively charged liposomes provoke secondary conformational transitions of the DNA molecules from the native B form to the C motif (*115*). However, these complexes do not possess the symmetric morphology of the DNA condensates produced by multivalent cations. A novel packaging mechanism was described where DNA molecules are partially embedded in a hexagonal micellar scaffold and partially condensed into a highly packed structure (*116*). DNA complexes with lamellar or columnar inverted hexagonal structures are formed, depending on the lipid composition and cationic lipid to DNA ratio. Univalent cationic lipids can interact as a polyvalent entity forming self-assembled bilayers, leading to the formation of <500 nm compact particles (*113*).

Complexes formed between cationic liposomes and negatively charged DNA are widely used for gene transfection experiments. Lipoplex formation studied by atomic force microscopy revealed a three-step mechanism, which includes a strong tendency toward orientation ordering where the DNA molecules enwrap themselves with the amphiphile molecules in a multilamellar fashion (*117*). Since liposomes are capable of forming complexes with 100% DNA and mediate interactions with the cell membrane, they exhibit high transfection activity. Polylysine, in combination with lipofectamine and DNA, exhibits higher transfection activity than the lipofectamine−DNA complex alone (*118*). A series of cholesterol derivatives have also been shown to improve transfection activity with reduced toxicity (*119*). Conjugation of polyamines to cholesterol can condense DNA, and the products can effectively function as efficient DNA delivery vehicles.

In contrast to low-molecular weight multivalent cations, cationic polymers bind to DNA irreversibly and can associate with excess DNA, leading to a strong positive ζ potential (*24*). This large surface charge can stabilize the complex and prevent it from aggregating by electrostatic repulsions. Most of the polymeric condensing agents contain vinyl or amide bonds and tend to accumulate in the endosomal compartment of the cell. The search for biodegradable polymers led to the discovery of polymeric cations based on proline or glycolic acid that bind and compact DNA like poly-L-lysine (*120*, *121*). DNA condensation by polymers composed of hydroxyproline, a constituent of gelatin, formed stable complexes with DNA, with an average size of 200 nm (*111*). These complexes were capable of transfecting the β-galactosidase gene into mammalian cells, thereby using a biodegradable gene delivery vehicle.

Primary polyplexes, which are smaller than individual DNA molecules, are formed by rapid mixing of DNA and poly-L-lysine (*98*). The primary polyplexes aggregate over a period of time, and the ultimate size of these aggregates is determined by the relative concentrations of DNA and poly-L-lysine. Poly-L-lysine produced a 5-fold increase in the level of reporter gene expression in combination with a low-density lipoprotein (*113*). Poly-L-lysines are also amenable to chemical modification. The specificity of uptake can be increased by conjugating poly-L-lysine with cell binding ligands that can trigger internalization by receptor-mediated endocytosis (*96*, *122*−*131*). Reduction of the positive charges of poly-L-lysine decreases its level of electrostatic interaction with DNA, favoring dissociation of the complexes inside the cell. Partial substitution of the amino groups of poly-L-lysine with the carbohydrate moiety can also facilitate the dissociation of polyplexes (*132*). Poly-L-lysine conjugated with asialoorosomucoid or epidermal growth factor condenses DNA and acts as a receptor-mediated vehicle (*16*, *133*). However, efficient transfection occurred only when the DNA nanoparticles had a spherical diameter of 15−30 nm. The polydispersity of these particles can lead to variable efficacy in DNA delivery (*134*).

The versatility and simplicity of polyamidoamine (PAMAM) dendrimers make them a new class of cationic polymers that are effective for DNA condensation and transfection (*135*, *136*). PEI dendrimers are capable of condensing DNA into compact nanoparticles that can transfect mammalian cells. These biodegradable polymers have three-dimensional structure with a tertiary amine interior structure. They are relatively nontoxic and efficient gene carriers. Controlled release of DNA can be accomplished by encapsulating DNA into these biocompatible ethylene−vinyl acetate copolymers (*137*).

PEI−DNA complexes exhibit a remarkable transfection efficiency, comparable with those of the best currently available synthetic vectors (*44*, *138*, *139*). The high transfection efficiency of PEI is related to its buffering capacity, which provokes a massive influx of Cl^- ions, leading to endosome swelling and disruption with release of the DNA into the cytoplasm. The transfection efficiency of PEI is also determined by the ratio of the PEI nitrogen atoms to the DNA phosphate charges. Addition of smaller PEI molecules to preformed PEI−DNA complexes strengthens the packing of amines around the DNA, yielding complexes with higher buffering capacity and transfection efficiency (*140*). They can also be coupled to ligands, such as RGD peptide sequence or anti-CD3 antibody for cell targeting (*141*, *142*). However, PEI is not easily metabolized by the cellular enzymes and tends to accumulate in the nucleus because it is an organic polymer (*44*). Toxicity can be decreased by conjugating PEI with cross-linking agents such as dithiobis(succinimidyl propionate) and dimethyl 3,3′-dithiobispropionimidate that utilize reducible cross-links (*143*). The reducing environment of the cytoplasm cleaves the disulfide bond and promotes the reversion of high-molecular weight complexes back to their low-molecular weight counterparts, thus reducing toxicity. Low-molecular weight peptide DNA condensates can be stabilized by incorporating multiple cysteine residues, which oxidize to form interpeptide disulfide bonds, once these molecules are bound to DNA (*25*, *144*). The stability of the cross-linked peptide condensates increased with cysteine residues and led to a decrease in the particle size, relative to the control peptide DNA condensates. These caged DNA condensates increase the stability and enhance transfection efficiency. Disulfide-containing cationic polymers, prepared by substituting the amino groups of poly-L-lysine with 3-(2-aminoethyldithio)propionyl groups, form stable polyplexes that decondense, releasing plasmid DNA upon reduction with glutathione and glutathione reductase (*144*). Conjugates of PEI with mannose, gelatin, chitosan, and methacrylate−methacrylamide polymers have also been exploited as gene delivery vehicles.

Poly-L-ornithine, formed from the monomer ornithine, which differs from polylysine by an additional CH_2 group in the side chain of lysine, also condenses plasmid DNA that is suitable for cellular uptake (*145*). Compared to poly-

L-lysine, plasmid DNA condensed with poly-L-ornithine exhibits a significant increase in the level of in vitro transfection of a reporter gene. It is interesting to note here that ornithine is the precursor amino acid involved in the synthesis of polyamines, which have been extensively studied for their ability to condense DNA to nanoparticles (*3*, *35*−*39*, *51*).

Future Directions

Research in the area of DNA nanoparticle formation is gaining significant momentum because of its link to therapeutic gene transfer. The search for efficient gene delivery vehicles has led to the discovery of a wide spectrum of novel DNA compacting agents. Although significant progress has been made in the development of DNA condensation agents, the technology is complicated by a lack of understanding of the mechanism(s) of action of gene delivery vehicles and the multiple morphologies and structures of the DNA complexes with these vehicles. Invariance in the size of the toroidal condensates with the nature of the condensing agent and DNA source makes it appear that condensing agents contribute little to the condensation process. However, the transfection activity of the DNA−carrier complexes is highly dependent on the morphology of the condensates, indicating that the nature of the condensing agent strongly influences the properties of the condensates. A lack of a clear structure−activity relationship between transfection agents and transfection efficiency makes the rationale for the design of new delivery vehicles with high transfection activity difficult. Hence, studies on the mechanism of DNA nanoparticle formation by potential gene delivery vehicles can give a wealth of new information about designing efficient DNA packaging systems and novel gene delivery vehicles.

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