Gene Ther Mol Biol Vol 4, 159-170. December 1999.

# Models of cationic liposome mediated transfection

**Research Article**

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**Key words**: cationic lipid, liposome, gene delivery, DOTAP, transfection

**Received: 3 December 1999; accepted: 21 December 1999**

## Summary

**Synthetic gene delivery vectors are being developed for *in vivo* gene transfer applications, and these systems may circumvent the risks inherent in the use of recombinant virus vectors. The majority of synthetic delivery systems are based on the use of cationic amphiphiles to coat and condense polynucleotides, and to facilitate the uptake and release of the polynucleotide payload into somatic cells. Cationic amphiphile-based vector technology has benefited from over a decade of mechanistic and structure-function analyses, but many aspects of the pharmacology, toxicology, and cell biology of these systems remain unresolved. Important outstanding issues include the structure and characteristics of active and inactive particles, *in vivo* distribution and clearance of particles, and the cellular events involved in binding, cytoplasmic delivery, and nuclear uptake. These processes are interdependent, and therefore difficult to isolate and examine experimentally. The wide range of compounds, methodologies, and polynucleotides used to study these phenomena further complicate development of general principles. This article focuses on the molecular and cellular processes involved in cationic amphiphile-mediated transfection. Both primary data and current literature will be used to illuminate the complexity that impacts on the development and application of this class of synthetic gene delivery vectors.**

## I. Introduction

Cationic lipid/DNA particles are not as active for transfection as their viral counterparts are for transduction. In a typical experiment, about 1 in 100 recombinant Adenovirus particles are successfully expressed, while only about 1 in 10,000 cationic lipid:DNA particles are effective. Transfection efficiency must be improved before cationic lipid:DNA complexes will become useful for creating effective genetic medicines. Many steps in the transfection process strongly influence overall efficiency and efficacy. Therefore analyzing, understanding and optimizing process components may identify new opportunities for developing or improving synthetic molecular medicines. From this reductionistic perspective, successful *in vivo* transfection may be considered as requiring: (**1**) formulation of the active particle; (**2**) distribution to the target cell and avoidance of clearance; **3**) Binding to the cell surface and cellular entry; (**4**) polynucleotide release from the synthetic complex; (**5**) entry into the nucleus; and (**6**) transgene expression. Modification of any of these steps often influences other aspects of the system, and this interrelationship complicates analysis of each process component. However, just as viral agents have evolved solutions to each of these steps, man-made preparations must also optimize each of these process components to enable gene-based therapies. The essential difference is that viral biology is optimized to maximize the reproductive fitness of the virus, whereas synthetic gene delivery systems must be engineered to maximize therapeutic benefit while minimizing toxicity.

In general, the preparation of synthetic vector formulations begins with the deceptively simple step of mixing a cationic compound with a polyanionic genetic molecule. The resulting interactions neutralize the anionic charge associated with the polynucleotide phosphate backbone and thereby facilitate condensation of the extended molecular structure typical of most polynucleotides. Although the genetic molecule is most often a double stranded covalently closed DNA circle (plasmid), non-viral systems are not restricted to delivering a single type of polynucleotide. mRNA, anti-sense

oligonucleotides, and other DNA structures can all be delivered by similar methods. This flexibility allows for a wide range of therapeutic strategies, and bypasses the constraints and risks associated with adapting highly evolved viral agents for therapeutic gene transfer applications. While polynucleotides share many structural and chemical features, differences in size, sugar (ribose and deoxyribose), and base pairing (single strand/intramolecular and antiparallel double stranded) significantly influence the structural and functional characteristics of the particles formed from polynucleotide/cationic amphiphile mixtures.

Most non-viral gene therapy research has focused on the delivery of plasmids. This class of polynucleotide is attractive for gene transfer applications due to flexibility and manufacturing considerations. Plasmids may be engineered to incorporate very large DNA segments, though practical aspects of cellular uptake and recombination during amplification often limit transgene length to roughly 3 KB. This capacity allows transfection of large open reading frames or combinations of genes. Additionally, molecular cloning techniques involved in synthesizing recombinant plasmids are well understood and easy to reproduce. Finally, once the recombinant molecule has been prepared and isolated, proven bacterial amplification methodology may be employed to prepare milligram to gram quantities of transgene-coding plasmid using pharmaceutically compatible processes. Unfortunately, plasmids are also associated with undesirable bacterial-associated modifications and contaminants such as unmethylated CpG sequences and endotoxin. Thus, although bacterially produced plasmids are the most frequently used polynucleotide, other polynucleotides may be preferred for some applications.

The most developed pharmaceuticals for non-viral genetic transfer are cationic lipids. The chemical structure of these compounds varies greatly, but all include a positively charged hydrophilic head group linked to a lipophilic body. Although the exact nature of the cationic lipid/polynucleotide complex is controversial (see below), it is documented that these particles successfully transfect cells *in vitro* (Felgner et al., 1987) and *in vivo.* The molecular structures of some of the important prototype cationic transfection lipids are summarized in **Figure 1**. The transfection activity of these compounds have led to the synthesis and testing of hundreds of chemical analogs. In some cases, analogs have been prepared, tested, and published in an attempt to correlate chemical structure with function. In other instances, development and testing have been driven by commerce. In these cases, information from the developmental process leading to disclosed or marketed compounds is usually not available to other scientists, making it difficult to infer which chemical modifications correlate with changes in biological activity. Although this diversity has lead to progress in the field, it also complicates the process of drawing broad conclusions about mechanism or structure/function relationships. Despite the lack of definitive mechanistic studies, the proven transfection activity associated with various cationic lipid formulations is likely to continue to attract scientific and commercial interest for the foreseeable future.

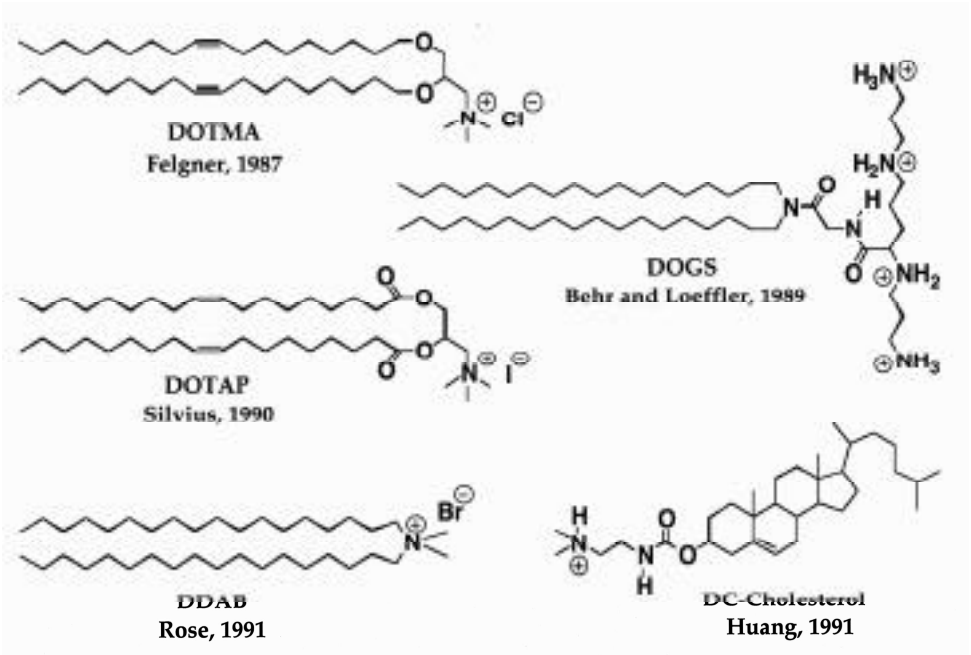


**Figure 1:** The molecular structures of some of the prototypical cationic lipid compounds.

This review will employ current literature and primary data from our lab to illustrate models that describe how cationic lipid:DNA complexes facilitate polynucleotide transfection, and will explore some of the molecular and cellular barriers to the overall utility of these particles. Our intention is to integrate the work of different research laboratories that address questions of transfection mechanism from different frames of reference. In particular, we wish to emphasize the difficulties associated with interpreting and comparing structural and mechanistic studies that are based on polymorphic formulations of different polynucleotides and cationic compounds.

## II. Complex formation, the first step in transfection

Cationic lipid mediated plasmid transfection begins with the formulation of a lipid:DNA complex. The mechanism of interaction between DNA and the cationic liposomes will determine the nature of the resulting transfection particle population, and in turn the characteristics of this population will strongly influence the transfection activity even before the solution is applied to the cells. Understanding these dynamic interactions is crucial to understanding the entire transfection process.

Standard laboratory practice involves simple mixing of a cationic lipid emulsion (80-400 nm multilamellar vesicles (MLV)) together with a bacterial plasmid that encodes the transgene of interest. Typically, these MLV are prepared by mixing the lipid components in an organic solvent, evaporating the solvent to yield a lipid film, and hydrating the film in a buffer. In many cases, the resulting emulsion is either sonicated or extruded to reduce the size and polydispersity of the MLV, although subsequent particle aggregation and fusion may reduce the long-term effects of such treatments. Such preparations are often purchased directly from commercial vendors, and the bench researcher may be unaware of the formulation process employed and structure of the particles that are mixed with the plasmid.

Incubation of cationic lipid MLVs and DNA will lead to a heterogeneous population of particles (Zabner et al., 1995). It is likely that only subsets of these particles are capable of transfection. The characteristics of transfection active particles have not been conclusively identified, and different subpopulations may interact to influence the usual outcome parameter, transgene expression. These ambiguities complicate the design and interpretation of experiments that focus on transfection mechanism and structure/function relationships. One research strategy employed to address such questions involves modifying formulation parameters, characterizing physical alterations in the resulting particle population, and then correlating changes in these physical characteristics to increases in transfection activity. In other cases, reagents may be employed which alter cellular processes posited to be involved in the transfection process. In such experiments, the active particles may be a very small percentage of the lipid particle population. Modifications that change the overall distribution of the entire population may alter important characteristics of the active particles, or may only alter an extraneous population. Therefore, the polydispersity of the population of lipid/polynucleotide complexes complicates all studies involving cationic lipid-mediated transfection. In light of these issues, it also becomes clear that even comparative gene expression studies involving transfection of different polynucleotides require rigorously standardized formulation protocols.

Although virtually all populations of cationic lipid/polynucleotide complexes are capable of transfection under some conditions, differences in the experimental conditions used for formulation lead to variation in both the population distribution and the resulting transfection activity. Evidence shows that the nature of the complex formed will vary based on: (**1**) the cationic amphiphile; (**2**) the helper lipids involved; (**3**) the ratio of lipid to DNA; (**4**) the time allowed for formation; (**5**) the temperature at which it is performed; (**6**) the concentration of lipid and plasmid during formulation; (**7**) the nature of the solvent; and (**8**) whether or not energy is imparted to the system through sonication or extrusion (Bennett et al., 1996; Ahearn, 1999). Analysis of any transfection study must consider the interactions between these various factors, as well as the complexity of the particle population mixture. An added level of complexity derives from the wide range of compounds, formulation conditions, and cellular models employed by different researchers. This fact makes it difficult to integrate the data derived from different laboratories into a cohesive model.

We propose that a simple thermodynamic model may greatly facilitate analysis and understanding of the process of particle formation, and of how this process affects the resulting population's transfection activity. Such a model may also account for how subtle changes in laboratory formulation conditions may result in huge variations in the population's final transfection activity. In this model, separate cationic liposomes and anionic plasmids join to form a metastable intermediate, then mature to become fully neutralized actively transfecting particles. Two antagonizing forces acting on the cationic lipid molecules, electrostatic interactions and hydrophobic bonding, are proposed to drive this process.

The first step in forming the transfection complex begins with initial association between MLV and polynucleotide. In the absence of polynucleotide, the cationic charges of lipids within MLV are neutralized by counterions. The nature of these counterions significantly influences the transfection activity associated with any one compound (Aberle et al., 1996). In this microenvironment, associative hydrophobic forces between lipidic side chains predominate, although the particle/fluid water interface remains highly positive (**Figure 2**, upper left panel). Upon mixing with polynucleotide, electrostatic interactions draw the positive surface of the MLV together with the negative

phosphate backbone of the plasmid. Cationic headgroups are then attracted to distribute along the backbone, but hydrophobic inter-lipid interactions slows this process of redistribution and prevents immediate coating and neutralization of the entire plasmid. Thus, the liposome binds to one region of the plasmid supercoil, forming a meta-stable complex with a positive pole –the liposome- and a negative pole – the uncoated plasmid. We will refer to this association as the macro-dipole intermediate. The "map pin" structures described by Sternberg (Sternberg et al., 1998) (see below) may be an example of such an intermediate.

It is hypothesized that macro-dipole intermediates may be resolved by one of three pathways; **1**) Binding additional plasmids to the cationic lipid portion will yield a larger and more complex anionic particle, **2**) Binding additional cationic MLV to the partially neutralized plasmid will facilitate liposome aggregation, and **3**) Redistribution of lipids along the polynucleotide axis will form a cationic lipid-coated particle. Once the surface of a lipid/polynucleotide particle becomes more uniformly cationic, it will repel other cationic particles (such as unbound MLV). We propose that the first two processes will lead to aggregation of particles and the formation of large charged complexes (**Figure** ), while the third process may yield smaller, less strongly cationic particles. Since the products of the third process are predicted to be smaller and less charged, they may be more likely to facilitate transfection. By this line of reasoning, either small, fully coated plasmid complexes, and/or small transition state intermediates (map-pins) comprise the majority of the transfection active particles. Under most conditions, such particles are typically a minor subpopulation of the overall mixture. Thus, the model proposes that the final transfection activity of the population will depend on the balance between aggregation (yielding large, inactive complexes) and the formation of smaller (transfectionally active) particles.

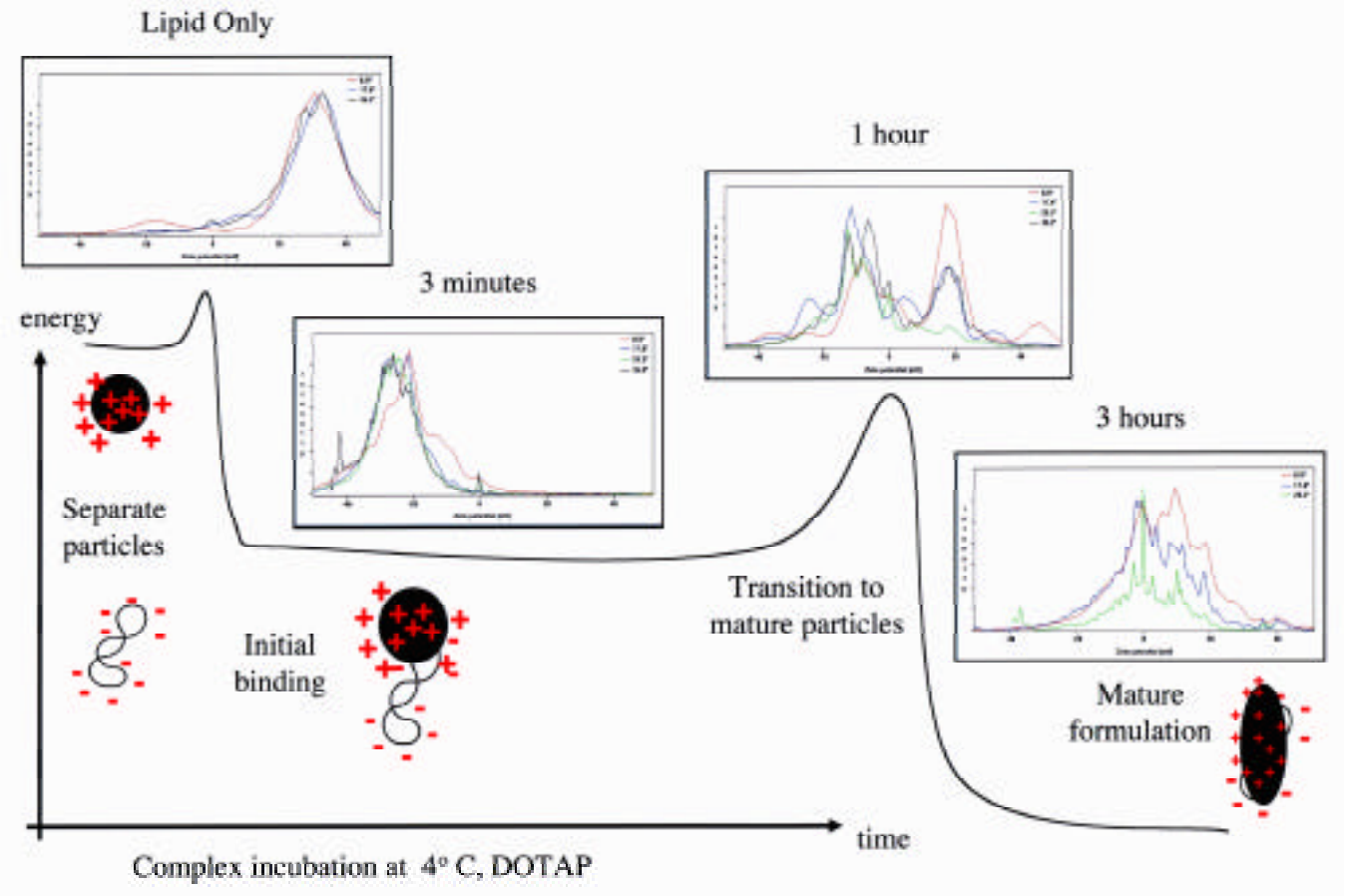


**Figure 2:** A hypothetical depiction of the thermodynamic energy barriers that must be overcome in order for mature particles to form. The inlays are actual measurements of the populations' zeta potentials measured at different angles (represented by different color tracings: Red: 8.9°; blue: 17.6°; green: 26.3°; black: 35.2°) using the Coulter Delsa 440. The formulations were stored at 4°C for 2 minutes, 1hour, and 3 hours after the plasmid was added. The inlays are placed on the curve to approximate the thermodynamic energy state of the majority of the particles in these populations.

In order to promote the process of lipid redistribution and charge neutralization, energetic barriers must be overcome to reach an equilibrium state of lipid-coated polynucleotide. Examples of such barriers include the free energy change associated with disruption of hydrophobic lipid-lipid interactions within MLV, and the disruption of lipid headgroup-counterion interactions. The sum of the free energy changes associated with disruption of the MLV structure may be the dominant component of the transition state activation energy. Forces favoring MLV disruption and lipid redistribution include the energy of association between the cationic headgroup and the polynucleotide phosphates. In an ideal system where aggregation does not occur, the balance of these different forces will determine the final equilibrium between macro-dipole intermediates and lipid-coated polynucleotides.

If there is sufficient thermal energy available to each nascent complex (macro-dipole) to overcome the activation energy, the lipid molecules will flow from the liposome structure to coat the plasmid. We suggest that the completely coated polynucleotide particle is the thermodynamically favored product, and that the coated particle formation rate is limited by the amount of energy available in the system. **Figure 2** shows a hypothetical plot of the free energy of the system and a simplified representation of proposed intermediates.

The energy boundary between the intermediate state and the final product is crucial, because it will determine how long it will take for the active particle to form at any one temperature. This energy boundary will be partly determined by the strength of the hydrophobic interactions of the chosen lipids. Manipulation of cohesive forces within the MLV membranes is therefore predicted to influence the frequency of active particle formation. For example, addition of the helper lipid dioleoylphosphatidylethanolamine (DOPE) has been shown to destabilize the cationic liposomes and to increase transfection activity (Farhood et al., 1995). Likewise, selection of a cationic lipid that has better surface hydration leads to increased transfection (Bennett, 1996). It is logical that these changes would lower the energy barrier required for the lipids to dissociate from one another and move to a new position on the plasmid, thus increasing the efficiency of the formation of the final product.

Although the above description model purports to account for interactions between a single MLV and plasmid in excess solvent, it does not account for the interactions that occur between these complexes. As discussed above, two aggregation pathways complicate the outcome of the formulation process. We propose that the charged ends of the macro-dipole intermediates tend to facilitate aggregation by binding to other immature particles, unbound MLVs and unbound plasmids (**Figure 3**). In this way, immature particles may bind to each other forming larger and larger complexes that eventually fall out of solution. This aggregation process is influenced by the concentration and kinetic energy associated with MLV, plasmid, macro-dipole intermediates, and mature particles within the solvent. It has been clearly documented that precipitated DNA-lipid complexes are not active for transfection *in vitro* or *in vivo* (Sternberg, 1998; Ahearn, 1999). Atomic force microscopy has also shown that vesicles larger than 1.4 μm are not good for transfection (Kawaura et al., 1998). Thus, in order to optimize the transfection activity of cationic lipid/DNA formulations, one must consider aggregation as well as polynucleotide coating and condensation.

This model, based on ionically driven association, subsequent coating, and non-productive aggregation, provides an explanation of why parameters such as time, temperature, and the concentration of components effect transfection activity of the resulting particle population. These parameters determine the amount of energy available to overcome the activation energy required for mature particle formation, and also determine the frequency of aggregation. For example, as temperature increases, more nascent particles are predicted to be converted to coated particles over any given time interval. These considerations may also explain the enhanced activity associated with different functional groups such as the lipopolyamines. Cooperative binding between polyamine headgroups and multiple backbone phosphates may enhance the free energy changes associated with a shift from MLV hydrophobic interactions to coated polynucleotide. Increased temperature will also impart increased kinetic energy, which will facilitate initial interaction between free plasmid and MLV. However, predicting the exact results of changes in these parameters is difficult because increased time, temperature, and concentration will increase aggregation, shifting the resulting particle distribution toward large, inactive complexes. Concentration cuts both ways, because lower concentrations lead to less aggregation, but *in vivo* protocols usually require high concentrations of complexes. Only empiric multi-parameter analysis will enable each of these variables to be simultaneously optimized.

Based on these considerations, we predict that formulation parameters that facilitate complete neutralization of polynucleotide charge and smaller particle size will provide the highest level of active particle uptake and thus the highest transfection activity. This is distinguished from total particle uptake, which includes non-productive binding and/or endocytosis of large, inactive aggregates. In order to test this hypothesis, we attempted to correlate measurable parameters of the particle population, namely size and surface charge, with the transfection activity of the population. An expression plasmid encoding the *P. pyralis* Luciferase reporter gene was mixed with DOTAP liposomes at a 3:1 positive to negative charge ratio. These formulations were then stored at 4°C for various periods of time resulting in different particle populations that could be physically analyzed and used for transfection.

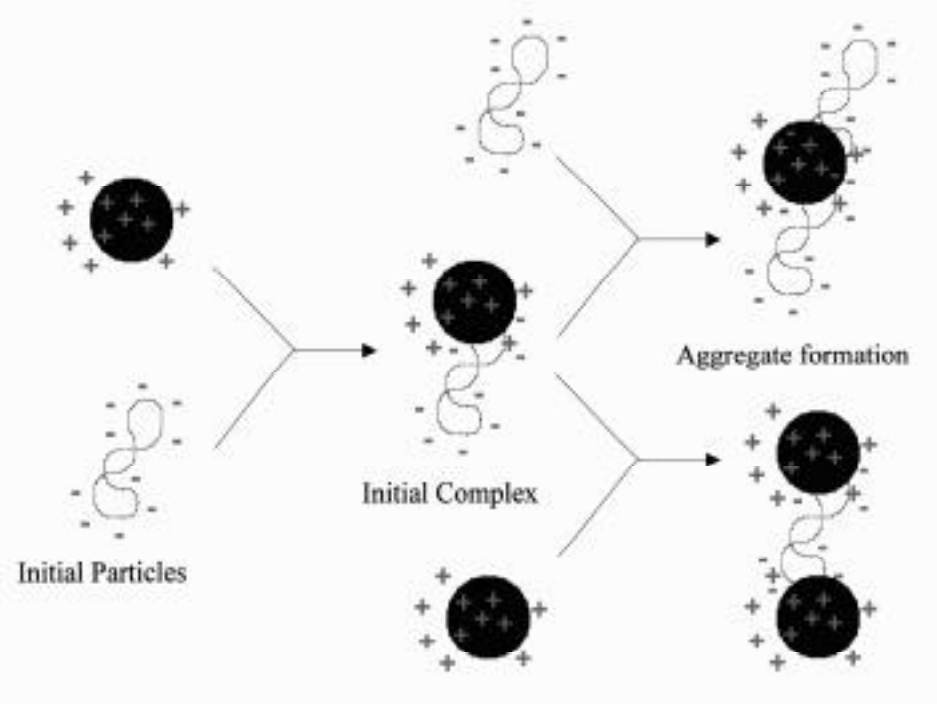


**Figure 3:** This Figure is a schematic representation of the process of aggregation. Particles that have not undergone maturation easily stimulate aggregation because of their dipole nature. Aggregated particles can become quite large (> 1500 nm) and are probably inactive for transfection.

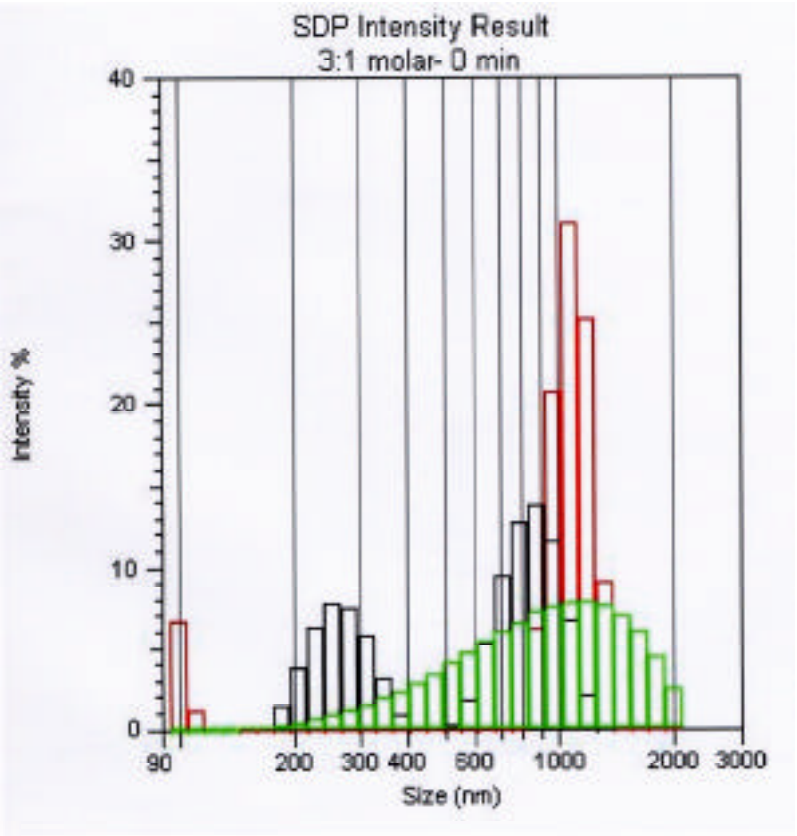


**Figure 4:** This Figure shows the SDP analysis of the population size for particles incubated for 2 min (Black), 1 hour (Red), and 4.5 hours at 4°C. Note that at 2 minutes two populations of particles exist: one at about 250 nm and one at roughly 1000 nm. It is believed that the smaller population represents free liposomes and that the larger population represents small complexes- either initial particles or mature ones. At 1 hour almost all the particles belong to the second category. However, after 4.5 hours the particles have begun to aggregate and therefore appear in larger compartments. Note that SDP analysis calculates all particles larger than 2000 nm as 2000 nm.

Particle size analysis was performed after incubating the mixture for 2 minutes, 1 hour, and 4 hours using a Coulter N4 plus submicron particle sizer, which employs multi angle laser refraction with photon correlation spectroscopy to estimate the distribution of particle sizes within a suspension. The results of this analysis are summarized in **Figure 4**. At 2 minutes, two separate populations are seen, one with a mean of about 250 nm and one with a mean of about 1000 nm. 250 nm is the approximate size of an average free liposome (MLV), representing particles that have not yet encountered plasmid, and the population around 1000 nm represents complexes of plasmid and liposomes. After an hour of incubation, the majority of the resulting particles cluster around the 1000 nm population mean. However, after 4.5 hours more large particles are seen and fewer were measured at 1000 nm, indicating that aggregation has progressed and shifted the population toward the very large particles that are predicted to be inactive for transfection.

The zeta potential is a measurement of charge on the structured water surrounding the particles, and may be measured using the Coulter DELSA 440SX Doppler electrophoretic light scattering analyzer**.** This machine measures zeta potential by propelling the particles in an oscillating electric field and using the Doppler shift of laser-generated photons to measure the speed of the particle movement. This speed is directly proportional to the surface charge of the particle and is used to calculate the population estimates seen in the inlays of **Figure 2**. As expected, the cationic DOTAP liposomes alone have a strong positive surface charge. Two minutes after the addition of the plasmid, the particles become quite negative. We interpret this data as indicating that very little of the phosphate backbone has been neutralized at this time. After one to three hours of incubation, the surface charge becomes progressively more positive, and this appears to indicate that more and more of the negative charges are neutralized as the lipid coats the DNA. The inlays of **Figure 2** are placed to correspond to relative energy states that we predict to exist using this model.

To correlate temporal alterations in size and surface charge with transfection activity, particle populations were incubated for different times and then used to transfect cultured fibroblasts. Particle populations formed by mixing DOTAP and DNA for 2 minutes, 2 hours, 8hours, 24 hours, and 30 hours were used to transfect NIH 3T3 cells plated into 24 well plates one day prior to all of the transfection treatments. The formulation conditions were identical to those used to generate the data summarized in **Figures 2** and **4**. As shown in **Figure 5**, activity dramatically improved when incubation was extended from 2 minutes to 2 hours, but declined at incubations of 8 hours and continued to decline at incubation periods up to 30 hours. Transfections of DNA without added lipid were used as a negative control, and resulted in less than 1000 total luciferase counts (data not shown). As predicted, the peak transfection activity of the particles correlate with populations that have a measured mean size of around 1000 nm, but not larger, and have a positive surface potential. This data is consistent with that obtained in other laboratories. For example, it has been demonstrated that a positive zeta potential directly correlates with the transfection activity of the particles (Takeuchi et al., 1996).

The thermodynamic model described above is consistent with the findings of other formulation studies. For example, Yang and Huang have reported studies focused on avoiding the decrease in transfection activity that occurs when complexes enter human serum (Yang and Huang, 1998). This study demonstrated that allowing lipid:DNA complex to incubate without serum for sufficient time nullifies this drop in activity, even if the particles are subsequently placed in serum. To relate this finding to our model, we suggest that the charged components of the serum may bind to the metastable particles, and either facilitate aggregation or interfere with polynucleotide coating. If sufficient time is allowed for mature particles to form before the serum is added, there will be no decrease in the distribution of active particles and thus no decrease in transfection activity. Higher temperatures, higher concentrations, and higher ratios of lipid to DNA all increased the speed at which serum resistance was obtained (Yang and Huang, 1998). The finding that this serum- resistance was obtained faster under conditions that promote thermodynamic conversion further supports this hypothesis.

Sternberg et al have employed electron microscopy to identify structures that may represent different stages of maturation. When image analysis is performed less than 20 minutes after the initial mixing of lipid and DNA, structures described as “map pins” become apparent. These “map pins” consist of a large lipid head and DNA tail. Larger aggregations of lipid and DNA are also seen in these preparations.

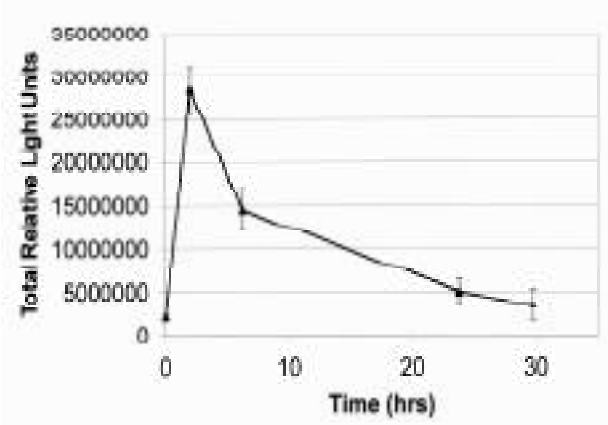


**Figure 5:** This shows the relative light activity of several populations of particles with the same DNA to lipid ratio, but incubated for different periods of time at 4°C. Transfection activity is highest at 2 hours and declines at 8 hours and beyond. This activity corresponds with the populations that have positive zeta potentials but have not yet formed large aggregates.

Formulations with a greater proportion of "map pins" and fewer large aggregates show higher transfection activity (Sternberg, 1998). It is possible that these structures represent metastable particles that have not yet aggregated and still retain the potential to mature to form active particles. Alternatively, the map pin intermediate may be the active particle. The thermodynamic model and the data summarized above predicts that further incubation of one or two hours may shift the proportion of particles to yield an increased fraction of small, fully coated particles. If a progression were found from map pin structures toward evenly coated particles, then fully coated plasmids would be more likely to be the functional particle. On the other hand, an increase in map-pin structures during incubation would support the importance of what may be the macro-dipole intermediate.

Based on the assertion that the conditions of formulation are as important as the lipid being utilized, the current search for new lipids to improve activity may be less efficient than refining the thermodynamic conditions used with existing lipids. Focusing on this aspect would allow the choice of lipid to be based on issues such as toxicity. We suggest that further development of these models may provide a more complete understanding of the structure and formation of the active particle components of the overall mixture. Consideration of such interactions during formulation may also aid rational design of experiments aimed at improving the transfection activity of synthetic cationic lipid-based vectors.

## III. Distribution to target cells and avoiding clearance

Synthetic vectors must diffuse or be transported to target cells before transfection can occur. *In vitro,* this process is relatively simple, as particles simply diffuse through the medium to cellular membranes. The only potential obstacles are serum-associated proteins and other molecules that bind lipidic particles, and either do not allow them to reach the cellular membrane or reduce their activity. For this reason, transfections are typically performed in serum free media. On the other hand, the process of distribution to the target cell is much more complicated *in vivo.* For example, complex modification by serum factors and sequestration either by the reticuloendothelial system or by lodging in the microvasculature can reduce transfection of the intended target cells. The importance of particle maturation in preventing this serum inactivation has already been discussed (see above). Any design of the lipid:DNA complexes for systemic administration must overcome these effects in order to be effective.

Systemically administered particles are often sequestered by reticuloendothelial cells including macrophages. After injection, high levels of DNA are observed in the phagocytic cells of the liver and spleen (Liu et al., 1995). This phenomenon can lead to unexpected results that again illustrate the complexity of these systems. Smith et al used ultracentrifugation and a sucrose gradient to separate the free liposomes from the lipid:DNA complexes that they had prepared (Smith et al., 1998). They predicted that purified lipid:DNA complexes would have better activity than the heterogeneous mixture of both liposomes and complexes. When purified particles were administered by intravenous injection, the expression observed in lung was actually lower than the mixture of both free liposomes and lipid:DNA complexes. However, when directly administered to the lung by tracheal instillation, the purified complexes yielded higher expression levels. This difference in expression levels may be mediated by the reticuloendothelial system (RES). The free liposomes may saturate RES clearance and prevent sequestration of some of the lipid:DNA complexes. This observation predicts that RES blockade wiith free liposomes, followed by administration of lipid:DNA particles, will provide higher levels of transfection at the intended site than is observed after administering the heterogeneous mixture.

In general, more research that focuses on maximizing delivery to target tissue must be performed. Avoiding both macrophage clearance and transfection of non-target tissue will increase the effectiveness of these therapies and reduce the side effects associated with treatment.

## IV. Cellular binding and uptake

Cationic lipid-plasmid complexes must cross cell membranes before transgene expression may occur, but the mechanism involved is not well understood. Cationic lipid:DNA particles clearly associate with the cell surface, and over time are readily detected within endosomal compartments. Based on this observation, as well as studies involving agents that alter endosomal acidification or transport, it has been inferred that endosomal uptake is required for transfection. However, the large endosomal aggregates typically observed in such studies may not be transfectionally active. In general, the highly polymorphic nature of the transfection particle population makes it very difficult to design definitive experiments to address these issues, and as a result most studies establish correlation rather than prove causation.

Since formulations of negatively charged plasmids and anionic liposomes are relatively inactive for transfection, it has been proposed that the positive charge associated with the cationic lipids is essential for cellular binding. One explanation for this difference is that positive surface charge may encourage interaction with anionic cell surfaces. If tissue culture cells are pre-treated with Pronase, an enzyme that removes negatively charged glycoproteins form the cell surface, then the level of transfection activity decreases by fifty percent (Hui et al., 1996). However, removing cytoplasmic membrane glycoproteins disrupts cellular signaling and decreases the rate of cell division, factors that play a key role in transfection (Zabner, 1995). Therefore, these studies do not definitively resolve this issue.

Fasbender et al studied a wide range of lipid and helper lipid combinations, and demonstrated that different lipid combinations strongly influence the fraction of DNA which becomes associated with the cells (Fasbender et al., 1997). However, the transfection activity of these formulations did not correlate with the amount of DNA bound. Zabner also showed that only 15% of the cellular associated DNA is taken up by cells (Zabner, 1995) in one experimental system. These findings suggest that cellular binding is probably not the main factor determining the rate of transfection.

If cellular binding is not the limiting factor in transfection, it becomes increasingly important to understand how the cationic liposome coat facilitates polynucleotide transport across the cytoplasmic membrane. Two theories have been developed to describe this process. The first theory, originally proposed by Felgner (Felgner, 1987), is based on the idea that cationic lipids directly fuse with the cytoplasmic membrane, permitting direct DNA transfer from the extracellular space to the cytoplasmic compartment. In the original paper describing cationic liposome-mediated transfection, it was asserted that this is the predominant mechanism for DNA entry into the cell. The second major theory proposes that transfection is mediated by endocytosis, and that DNA enters the cytoplasm only after undergoing endocytosis (Gao and Huang, 1995). It is important to note that these theories are not mutually exclusive. In general, the second theory (endosome uptake) has become the most widely accepted, and many experiments have focused on increasing the number of particles that are endocytosed.

After interaction with the cellular membrane, lipid:DNA particles are clearly taken up into endosomal vesicles. This process has been demonstrated by EM and florescence studies (Huebner et al., 1999). As many viruses enter the cell via endosomes and then escape into the cytoplasm, it is reasonable to hypothesize that cationic liposome/DNA complexes also follow this pathway. Although the overwhelming majority of plasmid DNA inside the cells is in vesicular compartments, a small amount can be seen in the cytoplasmic fraction (Friend et al., 1996). This cytoplasmic fraction may have escaped from the endosomes, or entered directly through the cellular membrane. Though the majority of DNA enters endosomes, this step is not proven to be the pathway of transfection.

Wrobel and Collins have attempted to show that DNA entry involves fusion of cationic liposomes with the endocytic compartments (Wrobel and Collins, 1995). The florescence pattern of N-NBD-PE, a helper lipid, changes upon mixing with another lipid present in either free liposomes or cellular membranes. The amount of lipid mixing between cationic lipid complexes and cellular membranes dictates the magnitude of the change in florescence. The study showed high amounts of lipid mixing under conditions favoring endocytosis. When the cells were subjected to low temperature or the poison Monensin, endocytosis stops and lipid mixing is reduced.

Presuming lipid mixing is crucial for cellular entry, Xu and Szoka focused on the differences between the intracellular and extracellular faces of the cellular membrane(Xu and Szoka, 1996). Employing a purely liposomal model, it was demonstrated that the interaction of cationic lipid:DNA complexes and anionic liposomes that mimic the lipid composition of the cytoplasmic face of the cellular membrane will uncoat plasmid DNA. DNA is not released when liposomes mimicking the extracellular face are used. This finding was interpreted to indicate that cationic lipid complexes disrupt the normal structure of endosomal membranes after endocytosis. It was concluded that disruption allows lipid from the cytoplasmic face to flip over to the extracellular face and interact with the endocytosed cationic lipid. In this model, the two lipids neutralize each other, stimulating release of the plasmid leading to an opening in the cellular membrane that allows for plasmid release into the cytoplasm.

Other observations are not consistent with this theory. The lipid mixing observed by Collins et al and Xu and Szoka requires the helper lipid DOPE, but many successfully transfecting cationic lipid formulations function without helper lipid. Also, the lipid-mixing studies performed by Stegmann showed absolutely no correlation between lipid mixing and transfection efficiency (Stegmann and Legendre, 1997). These studies imply either that mixing is not the mechanism for cellular entry or that another process determines the final transfection efficiency.

To summarize, there is no evidence that conclusively demonstrates that the small amount of DNA in the cytoplasm originates from endosomes. Endosomal and cytoplasmic membranes may be equally permeable, rendering endocytosis unnecessary. As the hostile endosomal environment may damage the plasmid DNA, development of non-endocytosed lipids would be beneficial. Also, if cationic particles open endosomal compartments, then the cytoplasm of the transfected cells is exposed to this harsh environment. Avoiding of endocytosis may reduce the toxicity seen with cationic lipids and increase effective transfection.

## V. Dissociation of lipid and DNA

Somewhere between particle uptake and transgene expression, the cationic lipid must dissociate from the plasmid DNA. In theory, this disassociation may occur after particle transport into the nucleus, perhaps even during transcription. To examine this possibility, Zabner et al injected oocyte nuclei with lipid:DNA complexes, but found very little expression of the transgene (Zabner, 1995). This study suggests that dissociation generally occurs before the plasmid passes into the nucleus.

As discussed above, Xu and Szoka have shown that DNA becomes exposed when cationic lipid:DNA particles

interact with anionic liposomes resembling the cytoplasmic face of the cellular membranes. These liposomes released the DNA at a charge ratio of 1:1. On the other hand, a 100-fold excess of charged molecules such as ATP, DNA, RNA, spermidine, and histone do not stimulate release (Capecchi, 1980). It is possible that cellular membranes mediate plasmid release. However, it remains unclear if this event is simultaneous with cellular entry. It is also possible that this process mediates release of the DNA without being the mechanism for cellular entry. After all, particles that have traversed the cellular membrane are exposed to the anionic surfaces of both the cytoplasmic membrane and the membranes of cellular organelles. Finally, if the map-pin structures observed by Sternberg are transfectionally active, it may be that the majority of the polynucleotide is not coated prior to uptake, and the process of migration across cellular membranes requires minimal disassociation of lipid from plasmid.

## VI. Nuclear entry

An essential step for gene expression is the entry of plasmid DNA into the nucleus (Capecchi, 1980). Surprisingly, nuclear entry may be the most inefficient process involved in lipid-mediated transfection. To examine the role of nuclear transport in the transfection process, Zabner et al designed an expression plasmid with a T7 promoter rather than the usual eurkaryotic transcriptional elements. This vector provides cytoplasmic transgene transcription when cells are co-infected with vaccinia virus expressing the T7 RNA polymerase (Zabner, 1995). Using this system, it was demonstrated that the majority of a sample of cultured cells could be productively transfected using 0.01 microgram of transfected plasmid. In contrast, 100 fold more of the CMV-promoter driven plasmid yielded transgene expression in only 10% of the cells under similar conditions. This data suggests that movement from the cytoplasm to the nucleus is probably the biggest limiting factor in transfection.

One approach to overcoming this barrier is using the natural cellular trafficking to carry DNA into the nucleus. Zanta et al. used a single nuclear localizing signal to increase the entry into the nucleus (Zanta et al., 1999). The reporter gene was cleaved out of a plasmid and capped. A targeting peptide sequence that appears in nuclear proteins was then ligated to the DNA, increasing transfection between ten and 1,000 fold (depending on the cell type). Insertion of a signal mutation into the peptide sequence, eliminates this effect. Thus, it seems that nuclear import machinery recognizes the peptide sequence and is responsible for the increase in transfection.

## VII. Expression

Once plasmid DNA successfully enters the nucleus, the final step in transfection is expression of the gene. This process requires transcription, mRNA export, translation, and proper protein folding. Fortunately, this process is handled by the normal cellular machinery and is not usually a barrier to transfection. The only key issue is which promoter should be used. Currently, almost all constructs rely on the CMV promoter, because it is known to give the very high levels of expression. When this therapy is actually applied to a therapeutic situation, this promoter may not be the best choice. Much research has been done to discover tissue specific promoters that allow high levels of expression in a target tissue (Peel et al., 1997). Selection of a naturally occurring promoter may improve long-term gene expression since it is less likely to be inactivated by the cell..

## VIII. Conclusion

Although our understanding of cationic lipid-based transfection continues to improve, there are many parts of the puzzle that must still be pieced together. Resolving these issues will allow us to improve the effectiveness of non-viral gene delivery. When first reported in 1987, cationic lipid-mediated transfection was considered a laboratory curiosity of marginal significance. Since that time, this technology has become the most widely used method for experimentally transfecting cultured cells. The widespread acceptance of the method derives from it's efficiency and apparent simplicity. However, although apparently simple, the process involves a complex set of interactions and particle populations which confound thorough analysis and elude complete understanding. If the efficiency of cationic-lipid:DNA complexes can be improved, then the resulting improvements in transgene expression may create new opportunities to develop genetic medicines. However, we suggest that further development of the system to yield effective, well defined and reproducible pharmaceuticals will require a much more complete understanding of the processes involved in formulating and applying this promising class of synthetic gene delivery vectors.

## Material and Methods

**A. Liposome Preparations.** Bulk DOTAP, suspended in chloroform, was purchased from Avanti Polar Lipids. In glass vials, one mg of DOTAP was mixed with 500 µl of chloroform. The vials were then desiccated overnight, resulting in a lipid thin film, and stored at –20°C until needed. Just before the formulations, the lipid was resuspended in one ml of purified water for injection. The vial was then sonicated in a bath sonicator for two minutes and placed on ice.

**B. Plasmid Preparations.** For analysis of gene transfer and reporter gene expression, a plasmid encoding an enhanced *Photinus pyralis* luciferase (pND2Lux) was employed. The pUC19 replicon-based plasmid was constructed in a fashion similar to that described previously (Chapman et al., 1991), To prepare milligram quantities of plasmid DNA for transfection, pND2Lux was transformed into *E. coli* DH-5α (18258-012; Life

Technologies, Gaithersburg, MD), isolated by growth on ampicillin plates, amplified, and purified using either the glass powder/sodium iodide method (MonsterPrep; Merlin Core Services, Bio 101, Vista, CA) or base lysis with double CsCl banding.

**C. Cationic Lipid Formulations.** In general, cationic lipid:DNA formulations were prepared by mixing 1 µg of plasmid DNA with enough DOTAP to bring the final charge ratio to 3:1. This charge ratio was previously determined to be optimal for transfection (unpublished data). These formulations were performed in a total volume of 1 ml in serum free DMEM media and incubated for varying lengths of time at 4°C.

**D. Transfections.** NIH 3T3 cells were plated at 5 X $10^4$ cells in 24 well plates, and allowed to grow in DMEM with calf serum for 24 hours. The medium was aspirated, and 200 µl of the transfection mixtures was added to four wells for each time point. The plates were incubated for 2 hours and then 800 µl of DMEM with calf serum was added to each well. They were then incubated for 24 hours.

**E. Luciferase assays.** Relative luciferase activity was determined with the enhanced luciferase assay kit (556-866; PharMingen, San Diego, CA) and Monolight luminometer (2010; Analytical Luminescence Laboratories, San Diego, CA) as per the manufactures recommendations. The cells were lysed with 200 µl of 1X lysis buffer and incubated on wet ice for 30 minutes. Luciferase light emissions from 20 µl of the lysate were integrated over a 10-sec period, and results were expressed as a function of the total lysis volume. The data shown is a mean result of the 4 wells measured and the error bars represent one standard deviation.

**F. Characterization of the Size Distributions.** Size distributions of transfection particles were measured using the Coulter N4 Plus Submicron Particle Sizer according to the manufactures recommendations. 200 µls of the formulation mixtures were mixed with 3 mls of water, which had been purified using a 0.2 micron filter, in standard plastic cuvettes. Small adjustments in concentration were made in order to fall within the particle counts recommended by the machine. Laser refraction was performed on these mixtures for 425 sec at angles of 30° and 90° at a temperature of 20°C. SDP analysis of these results was performed using 30 bins with a range of 100 nm to 2000 nm.

**G. Zeta Potential.** The distribution of the surface charges of the particles were determined using the Coulter Delsa 440. The same mixture of particles used for the size experiments was used for these measurements. Zeta potential was determined by Doppler shift measurements of the movement inducted by an oscillating electric field. Measurements at 4 different angles are recorded on each graph.

## Acknowledgements

The authors would like to thank Martin Woodle for initial training in the use of the Delsa and the N4. We also thank Caroline Toll, Stella Somiari, Jim Mixson, and Joe Drabick for patient assistance in proofreading this manuscript. This work was supported by funding from the Molecular and Cellular Biology Program and the MD-Ph.D. Program at the University of Maryland, Baltimore, and the National Institutes of Health (USPHS training grant HL07612-14 and NIH-RO1RR12307 for AA, NIH-KO2A101370 and NIH-RO1RR12307 for RM).

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