ADVANCED GENE DELIVERY
From Concepts to Pharmaceutical Products

Edited by

Alain Rolland

GeneMedicine Inc., The Woodlands, Texas, USA

harwood academic publishers
Australia • Canada • China • France • Germany • India • Japan
Luxembourg • Malaysia • The Netherlands • Russia • Singapore
Switzerland

5. CATIONIC LIPID-BASED GENE DELIVERY SYSTEMS

D.D. LASIC[1], D. RUFF[2], N.S. TEMPLETON[3], P. BELLONI[4], T. ALFREDSON[4] and R. PODGORNIK[5]

[1]*Liposome Consultations, 7512 Birkdale Dr. , Newark, CA 94560*
[2]*Perkin Elmer, Applied BioSystems Division, Foster City, California*
[3]*National Cancer Institute, Frederick, Maryland*
[4]*Roche BioSciences, Palo Alto, California*
[5]*Laboratory of Physical and Structural Biology, National Institute of Child Health and Human Development, National Institute of Health, Bethesda, Maryland*

INTRODUCTION

Modern biotechnology often requires effective and safe delivery of recombinant DNA molecules into different plant and animal cells *in vitro* and *in vivo*. However, in vivo gene transfer requires more sophisticated delivery systems. These include the administration of DNA integrated in viruses or associated with synthetic colloidal particles, such as liposomes, micelles, emulsion droplets, polymers and combinations of these delivery systems.

Cationic lipids and liposomes are especially popular because they are easy to prepare in large quantities and can be easily sterilized. They are stable and researchers in general claim that they are safe and non-immunogenic. Many of the lipids used are also biodegradable.

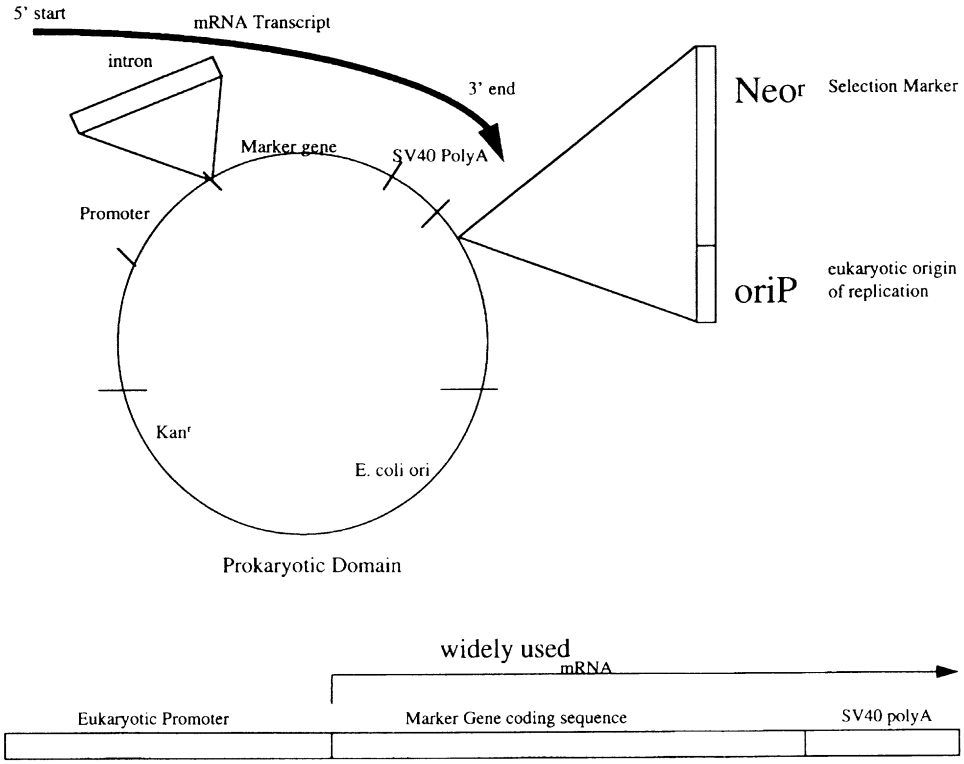
In addition to transfection and expression of genes for basic research and biotechnology, genes can be used therapeutically in medical treatment of various diseases. The aim of initial medical applications has been to express wild type genes, in cases where existing genes are mutated or when certain cellular changes are advantageous, or to suppress the expression of the unwanted genes. More recently gene therapy has been used to provide continuous delivery of vectors encoding recombinant proteins, such as growth factors or cytokines. Among several delivery vehicles for systemic and localized parenteral or topical administration of nucleic acids, cationic liposomes, possibly in conjunction with certain (block) co-polymers, seem to be a promising delivery vehicle. Ultimately, it is hoped, that most of the development in the field of liposomal drug delivery, such as long circulating and targeted liposomes, can be applied to DNA-lipid complexes (genosomes). The former ones can increase the concentation of plasmids in tissues with leaky vasculature, such as in tumors and sites of infection and inflammations, while the latter one have a potential to selectively target cells which express appropriate receptors. Besides DNA plasmids, ribozymes and antisense oligonucleotides can be administered to selectively limit gene expression. They can be delivered by using (cationic) liposomes.

DNA PLASMID DESIGN, TRANSFECTION AND GENE EXPRESSION

Cells store the genetic information necessary to encode proteins in chromosomal DNA molecules (Watson *et al.*, 1983). Non-coding chromosomal sequences direct the spatial and temporal cues required to regulate gene expression. Extrachromosomal DNA, such as plasmids and viruses, can effectively utilize the host cell genetic machinery to direct the production of large quantities of proteins. With the advent of recombinant DNA techniques, plasmids encoding virtually any engineered protein can be introduced and subsequently produce large quantities of foreign proteins. Typically, plasmids are between 5 and 20 kb pairs long and are of circular supercoiled conformation (Kriegler, 1990; Lasic and Templeton, 1996). In addition to the coding sequence for the particular protein, DNA plasmid also possesses the sequences which can enhance this synthesis in recipient cells or upon particular stimulus, as described elsewhere in this volume.

Figure 1 describes a typical expression system used in genosome preparations. The plasmid consists of two major domains. First, a prokaryotic cassette ensures propagation in *E. coli* host cells. This cassette codes for a selection factor such as Neomycin or Kanamycin resistance and an origin of replication which functions to replicate the plasmid in bacterial cultures. And second, a eukaryotic cassette which directs expression of a protein when introduced into mammalian cells by genosomes completes the plasmid (Kriegler, 1990). In the most widely used eukaryotic expression systems, the coding region of DNA is preceded by promoter and enhancer regions (and usually an intron) and then translation enhancing regions at the 5′ end of the messenger RNA. A polyadenylation signal at the 3′ end of the coding sequence stabilizes the mRNA and concludes the expression cassette. A selection marker (such as neomycin or dihydrofolate reductase) with its own promoter can be added to help prolong the longevity of the construct and its expression levels in mammalian cells grown in selective media. A eukaryotic origin of replication can be added along with appropriate factors to promote plasmid stability and retention. More sophisticated constructs can contain sequences to facilitate homologous recombination. Tissue-specific and inducible promoters may be utilized in order to regulate gene expression in response to various molecular cues. The whole plasmid must contain an origin of replication region which directs the replication process in bacteria. Many expression systems use the CMV-IE promoter. However with more demands being placed on tissue-specific approaches to gene expression, many genosomes are being created to direct expression within specific cells (such as Factor VIII, Greenberg *et al.*, 1995).

In vitro transfection assays are typically based on measuring the expression of reporter genes. Reporters are suitable for both *in vitro* and *in vivo* applications. The enzymes beta-galactosidase (β-gal), chloramphenicol acetyltransferase (CAT), luciferase and the green fluorescence protein (GFP) are widely used to quantify transfection efficiencies. These proteins are not found in any mammalian system. Measurements of enzymatic activity using these reporters generally give low background signal. However, stability of the exogenous proteins in mammalian cells and during the assay process are of paramount concern for reproducible quantitation. CAT and β-gal levels can also be quantified by immunoassays, thus providing an independent means to verify expression levels. Obviously, the expression of the gene of interest is more important than that of a reporter and advances in quantitative RT-PCR methodologies (reverse transcription polymerase chain reaction) that accurately measure gene expression levels have reduced the need to use reporters



Examples:

CMV-IE (high levels of expression in broad classes of cells)	CAT (Stable protein easily assayed)
SV40 (high levels of expression-works well when used in conjunction with SV40 large T-antigen)	Luciferase (very sensitive assay exists for detecting low levels of expression)
PGK (low levels of basal expression in almost all cell types)	Beta-Gal (colorometric assay-works well for *in situ* detection; can also be quantitated by immunoassay)
Factors VII/VIII (Liver specific)	Green Fluorescence Protein (GFP-in situ applications)

Figure 1 DNA plasmid construct (for details see text).

(Innis *et al*, 1990). *In vitro* and *in vivo* expression levels usually peak in about 6–24 hours after cationic liposome: DNA delivery to the cells, and then drop rapidly.

Extensive efforts to develop novel lipids and plasmids has resulted in 1000-fold improved transfection efficiency as compared to early results. Recent reports suggest that up to 5% of the total cellular protein can be synthesized from the plasmids giving rise to exogenous CAT protein levels of around 20 μg/million cells (Felgner, 1996). At present, however, obtaining long-term persistent expression remains an elusive goal for both *in vivo* and *in vitro* studies.

PHASE BEHAVIOR OF DNA

DNA is a macromolecule which exhibits very interesting colloidal properties. In aqueous suspension, DNA is a double-helix found mostly in the Watson-Crick B conformation and possesses a repeat unit of 10 base pairs from two interwound single strands to form a pitch of 3.4 nm and thickness around 2 nm (Watson and Crick, 1953). The interior of the helix contains the hydrophobic bases and the outside of the helix the deoxyribose and phosphate backbone groups. B-form DNA is well hydrated. DNA fragments of < 1000 bp are linear while longer molecules form wormlike semi-rigid random coils (Sinden, 1994). Supercoiled DNA has several twists which normally underwind the helix (Boles *et al.*, 1990; Bloomfield *et al.*, 1974). While there are no attractive interactions between DNA helices due to the negative charges of the phospate backbone (van der Waals attractive force is negligible), addition of polyvalent cations, external pressure, and higher concentrations, can concentrate DNA into a variety of completely or partially ordered phases (Leforestier and Livolant, 1994; Podgornik *et al.*, 1996a; Livolant and Leforestier, 1996). These phases can be crystalline (long range positional order in the direcion of the long axes of the molecules, long range positional order and long range bond orientational order in the plane perpendicular to the long axes of the molecules) hexatic (short range positional order in the direcion of the long axes of the molecules, short range positional order and long range bond orientational order in the plane perpendicular to the long axes of the molecules), hexagonal columnar (short range positional order in the direcion of the long axes of the molecules and long range cristalline order in the plane perpendicular to the long axes of the molecules) and twisted nematic (or cholesteric) arrays (long range orientational order perpendicular to the cholesteric axis) (see de Gennes and Prost (1993) for details). There is some evidence that at low concentrations DNA can also make blue phases (Livolant and Leforestier, 1996). In each of these phases the DNA molecules interact via effective molecular potentials specific for each phase (Strey, Parsegian and Podgornik (1997)).

For colloidal suspensions, the most important DNA phase transition is condensation in which random coils of DNA collapse upon shielding of negative phosphate charges into a small condensed particle (Bloomfield, 1991, 1996). Typically, these particles are of toroidal (60–90 nm sized doughnut with inner diameter of 30–50 nm) or elongated rod-like shape (diameter 50–80 nm, length 300 nm) in which DNA helices are packed hexagonally. Local DNA concentration can vary, in relation to the counterion concentration and charge or external pressure, from 30 to 60 mg/ml as does the equilibrium spacing between DNA helices from between 3.3 and 2 nm (Podgornik *et al.*, 1996b). In the condensed phase DNA is either in the hexegonal columnar or hexatic liquid cristalline phase.

To induce DNA condensation, multivalent cations (Co^{3+}, La^{3+}), polycations (spermine^{4+}, spermidine^{3+}, polylysine^{n+}, polyarginine^{n+}, polyhistidine^{n+} and other polyamines), cationic polyelectrolytes (polyethylineimide, polybrene, polylysine or dendrimers) and cationic lipids, or any combination of these, can be used.

CATIONIC LIPIDS AND THEIR PHASE BEHAVIOR

Diacyl cationic lipids form similar liquid crystalline phases as do other lipids (Luzzati *et al.*, 1960), with lamellar and inverse hexagonal being the most frequently formed.

When these stable phases are diluted during agitation they disperse into colloidal particles which can be very stable. Dispersal of lamellar phase results in the formation of liposomes (Bangham, 1983; Lasic and Barenholz, 1996). These are microscopical spherical structures in which a membrane, composed from lipid bilayer, encapsulates a fraction of solution, in which they are suspended, into their interior. With respect to geometry of liposomes, one distinguishes between large and small vesicles which can be uni-, oligo-, or multilamellar (Lasic, 1993). Lipids with several positive charges tend to form micellar phases in aqueous solutions, while in mixtures with neutral lipids they can form relatively small liposomes.

With respect to swelling and formation of colloidal lipid particles as well as liposome preparation, cationic lipids closely resemble anionic ones (Lasic, 1993; 1997). In aqueous solutions, dried lipid films exhibit infinite swelling behavior and such systems can gel at concentrations above 10–20 mM. Addition of neutral lipids and salt reduces the gel formation. Sonication, extrusion or homogenization of larger oligolamellar ("hand-shaken" or "vortexed") liposomes results in the formation of small unilamellar vesicles. Down-sizing is accompanied with a decrease of the solution viscosity. Cationic liposomes are typically composed from a mixture of charged and neutral lipid. Dioleoyl phosphatidyl ethanolamine (DOPE) and cholesterol are the most frequently used neutral lipids. These systems obey DLVO (Derjaguin-Lifshitz-Verwey-Overbeek) model of colloidal stability with exception of smaller liposomes which may be less stable than predicted due to the presence of hydrophobic defects at high membrane curvatures (Carmona Ribeiro, 1989). The shape of small liposomes depends on the lipid component but in many cases nonspherical liposomes are observed. DODAB, for instance, forms ellipsoidal, lens-like particles which may coexist with open fragments (if the sizes are reduced below 100 nm). Very small DODAB particles are not spherical. In the DOTAP system, invaginated liposomes have been observed (Frederik, unpublished, Lasic and Templeton, 1996). Similar structures were observed in DOTAP/cholesterol systems prepared by extrusion (Templeton *et al.*, 1997). The shape of mixtures of cationic lipids with polyvalent polar heads in mixtures with DOPE has not been published yet. It is possible that these particles form large anisotropic micelles.

At present, the shape of these liposomes is not fully understood. The observed deviations from sphericity can be due to several factors including partial phase segregation, lipid degradation and subsequent phase separation, uneven charge dissociation, or negative contributions of Gaussian curvature to the bending elasticity (Lasic, 1993 and 1997).

Heightened interest in developing cationic lipids as vehicles for gene therapy delivery systems has resulted in the synthesis of hundreds of cationic lipids and some of them are shown in Figure 2 (Felgner *et al.* 1994; Lee *et al*, 1996). Indeed, studies based on new lipid formulations in liposomes have shown that the use of different liposomes resulted in large differences in transfection efficiencies. However at present, little is known about structure activity relationships (SAR), physico-chemical (number of charges and their pK values, critical micelle concentration, miscibility with other lipids) and biological properties (safety, toxicity, expression capabilities in cells) of these novel lipids. pK values of cationic lipids which are weak bases can be determined by potentiometric titration, while lipids which are quaternary ammonium salts are expected to be fully ionized. The degree od ionization in colloidal structures, however, remains to be determined because zeta potential and surface force apparatus measurements do not follow Poisson Boltzmann equation

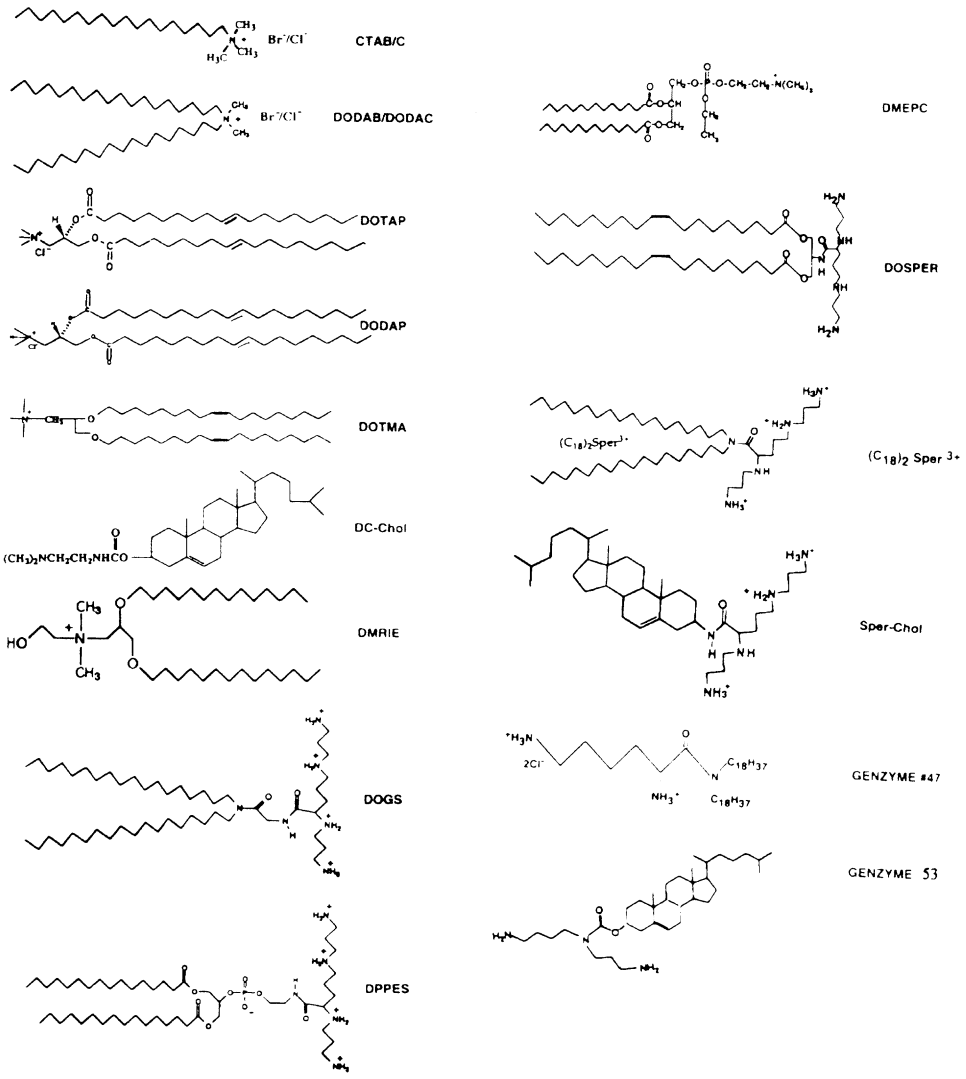


Figure 2 Chemical formulae of several cationic lipids: CTAB – cetyl trimethyl ammonium bromide; DODAB/C – dioctadecyl dimethyl ammonium bromide/chloride; DOTAP – dioleoyl trimethyl ammonium propane; DODAP – dioleoyldimethyl ammonium propane; DOTMA – dioleoyl propyl trimethylammonium chloride; DC-Chol – dimethylaminoethane – carbamoyl cholesterol; DMRIE – dimyristoyl oxypropyl dimethyl hydroxyethyl ammonium bromide; DOGS – dioctadecyl amido glycil spermine; DPPES – dipalmitoyl phosphatidylethanolamyl spermine; DMEPC – dimyristoyl ethyl phosphatidylcholine; DOSPER – dioleoyl oxy carboxy spermyl propylamide(four) acetate; diC18 Sper – distearoyl spermine; SperChol – spermine cholesterol.

(Campbell, Lasic, Israelachvili, unpublished). Also, from anionic surfactants it is known that degree of ionization drops upon micellization.

INTERACTIONS OF CATIONIC LIPIDS AND DNA

The accurate theoretical description of DNA complexation with cationic lipids, liposomes or micelles is very difficult. The approach of choice is the Poisson Boltzmann theory, which is, however, at high surface charges and low ionic strengths, very difficult to implement exactly, because of ill defined boundary conditions. Linearized Poisson Boltzmann equation, however, can be solved in cylindrical geometry to describe DNA, in spherical geometry to describe small liposomes and in planar geometry for large liposomes, respectively. The interaction is proportional to the linear charge density of DNA and liposome surface charge. For low ionic strengths, interactions decays as logarithm of the separation while for higher ionic strengths interaction fades away exponentially with Debye decay length (Frank-Kamenetskii, 1989; Lasic, 1997). It is very likely that after relatively long-range electrostatic attraction brings DNA and liposomes close together, other attractive interactions, including van der Waals, ion correlation attraction, and hydrogen bonding play an important role. These interactions which are also responsible for the reversible decomplexation of genosomes, i.e. , dissociation of DNA-lipid complex and DNA decondensation upon cell entry, may reflect different activities of various lipids (Lasic, 1997; Xu and Szoka, 1996).

These interactions are capable of releasing enough energy to compensate for the structural changes. DNA can become condensed and liposomes can restructure. Often, especially in the case of small unilamellar vesicles, liposomes disintegrate and reassemble into stacks of rather flat bilayers in which an array of parallel DNA helices are sandwiched between. This lipid restructuring requires energy which is obtained from attractive interactions: each charge neutralization brings kT of energy at Bjerrum length (about 0.7 nm at physiological conditions, kT = thermal energy of particles/molecules at temperature T, k being Boltzmann constant) while DNA and liposome conformational and structural changes require tens to hundreds kT.

STRUCTURE OF THE COMPLEXES

In contrast to the large amount of data on the synthesis, and transfection properties of many novel lipids, not much data on the structure of DNA-lipid complexes (genosomes) exist. Early work established that complexes are heterogeneous with respect to the size and shape. After early stoichiometric models of complexes of liposomes and DNA (Felgner and Ringold, 1989), the picture of condensed DNA encapsulated into spherical (Behr, 1993) or elongated bilayered particles was shown (Gherson *et al.*, 1993; Minsky *et al.*, 1996). Subsequent freeze fracture (Sternberg *et al.*, 1994) and cryoelectron microscopy (Gusstafson *et al.*, 1995) investigations established that complexes resemble aggregated spherical particles surrounded by a halo of fibrilar structures, which were shown to be DNA encapsulated in a bilayered tube (Sternberg *et al.*, 1994; Sternberg, 1996). More detailed analyses show that in the tube there may be two interwound DNA helices (Sternberg, personal

communication). Hexagonal arrangement was also proposed (Felgner *et al.*, 1996). Studies of other complexes established sandwiched structure, in which DNA is locally adsorbed between lipid bilayers and alternate lipid bilayers and two dimensionally ordered DNA form stacks (Lasic *et al.*, 1996, 1997). On the edges such structures are much less ordered due to topological constraints of self-closed DNA plasmid. Between the bilayers, however, the supercoiled coils and knots of DNA helices are unwound and untwisted (Lasic *et al.*, 1996, 1997; Radler *et al.*, 1997). Similar two dimensional arrangement of DNA helices was also observed upon adsorption on cationic monolayers (Fang and Yie, 1997).

The aggregated structures surrounded by a halo of fibers and clusters of spherical particles, as observed in DC-Chol systems (Sternberg *et al.*, 1994; Gustafson *et al.*, 1995), are mostly random, amorphous aggregates. On the other hand, in some systems, such as DODAB/Chol or DOTMA:DOPE (Lasic *et al.*, 1997; Raedler *et al.*, 1997) aggregates are characterized by a short range lamellar order. The mechanism for complexation reactions giving rise to amorphous, disordered complexes, and others to locally well ordered complexes is still not known and has not been systematically studied. Obviously stronger electrostatic attraction (at low salt and at pH < pK-3) and higher concentrations of reactants favor short range order as does rapid mixing which assures more uniform bulk concentration of the charges. The genosome structure is simply a consequence of attractive van der Waals, attractive and repulsive electrostatic forces and repulsive steric and undulation interactions coupled with local concentration of reactants. An additional factor which determines their morphology is the energy needed to restructure lipid bilayers and energy associated with conformational changes of DNA (Lasic, 1997). We must also keep in mind that the ordered genosome structure is a consequence of the DNA stiffness. For instance, replacing DNA with polyglutamic acid gives rise to amorphous complexes and precipitates (Podgornik and Lasic, unpublished).

The above analysis implies that genosome structure is mostly a consequence of thermodynamic and kinetic factors. Thermodynamic factors are concentration of reactants, electrolytes, and free energy changes associated with conformational and structural changes. The kinetic component is apparent from the reaction dependence on the rate and sequence of mixing as well as the instability of the complexes. Therefore genosomes, such as liposomes, are kinetic traps rather than thermodynamically stable structures (Lasic, 1997). Their morphological changes in time simply reflect the fact that the system is reducing the free energy by sliding in lower energy minima in the local environment. Such structural changes occur on a molecular and colloidal level. The former accounts for liposome and bilayer restructuration and DNA conformational changes (typically time scale from a fraction of a second to minutes) and the latter include mostly aggregation of particles followed by flocculation and phase separation (precipitation) in longer time scales.

When small, homogeneous and well dispersed particles are formed, however, they can be rather stable due to a strong electrostatic repulsion in low ionic strength media. In addition to ubiquitous van der Waals and ion-correlation forces, possible attractive interactions can be electrostatic attraction of unevenly charged colloidal particles and bridging interactions of non-condensed DNA fibers. Indeed, it is well known that complexes containing particles with attached fibrillar DNA strands precipitate very quickly.

It was shown that the dense, intercalated lamellar genosomes are extremely stable (no change in size distribution for months) and may represent a thermodynamically

stable phase (within a particle) as will be discussed below. Namely, both reactants, DNA as well as lipids can form thermodynamically stable lyotropic liquid crystalline phases and it is therefore not unexpected that their product retains some of the long range symmetry of the constituents.

Condensed phases of (cationic) lipid bilayers and DNA are distinguished by two different symmetry as well as dimension classes. Lipid mesophases with smectic (lamellar) symmetry are characterized by one dimensional quasicrystalline order in the direction perpendicular to the bilayer surfaces and a two dimensional liquid order within the smectic layers (deGennes and Prost, 1993). Lipid ordering tendencies are not compatible with the ordering tendencies of condensed DNA that preferrs local hexagonal symmetry. The result of these opposing ordering tendencies is the lowering of the effective dimensionality of the condensed DNA phase. Instead of remaining in a two dimensional locally hexagonally packed hexatic phase (Podgornik *et al.* 1996) DNA becomes intercalated between smectic planes of lipid bilayers, thus turning from a three dimensional line hexatic into something that shows roughly a two dimensional smectic symmetry, i.e., the intercalated DNAs are packed in domains where they remain essentially parallel. A two dimensional smectic as opposed to a three dimensional smectic, like in the case of lipid bilayers, would show a one dimensional quasicrystalline order perpendicular to the long axes of the DNA molecules. This line of thought is reasonably well born out by experiments, where the intercalated DNA contributes an additional peak to the scattering function of the DNA — lipid aggregates (Lasic *et al.*, 1997; Raedler *et al.*, 1997).

This effective lowering of dimensionality of the condensed DNA phase by the strong smectic order of the lipid array can be understood by energy arguments. Lipids that are oppositely charged from DNA, i.e., carrying positive charges, the symmetry consideration for the combined ordering of DNA as well as cationic lipids are connected to energy arguments. Intercalation of DNA in this perspective serves two basic purposes. First, as already alluded to, DNA remains in an ordered phase (however, with a lower effective dimensionality) just as before the complexation. Also its large negative charge can effectively neutralize the repulsive forces between positively charged lipid bilayers in the smectic array and thus stabilize the complex.

Furthermore, the width of the DNA diffraction peak in the complex as well as the conformational snapshots provided by strong adsorption of DNA to mica-immobilized cationic lipid bilayers (Fang and Yie, 1997) suggest that DNA intercalated between lipid bilayers undergoes pronounced elastic shape fluctuations. The balance of forces for the DNA is thus a bit more complicated. Elastic fluctuations of the DNA molecules within the intercalation planes contribute an additional repulsive force (of the same general fluctuation type as observed in the bulk DNA cholesteric phase) between them, trying to push them apart. This tendency to lower the density of intercalated DNA is however offset by the large electrostatic attraction between uncompensated charges on the lipid bilayers. It is unclear at this point whether Helfrich-like interactions between fluctuating lipid bilayers (Israelachvili, 1991) would contribute anything to the overall energy balance. It is more probable that lipid bilayers with intercalated oppositely charged DNA fluctuate only through collective undulations preserving the separation between the bilayers, set effectively by the diameter of the DNA.

Most of the experimental work has been done with cationic complexes. Anionic complexes (negative charge from DNA exceeds positive charge from lipids), which are sometimes used for intramuscular, subcutaneous, intratracheal, pulmonary or

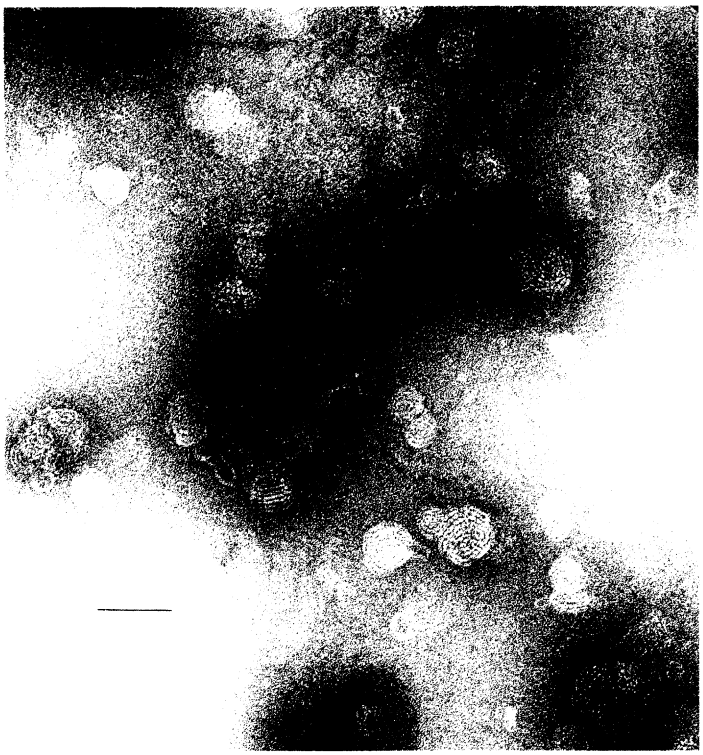


Figure 3 Negative stain electron microscopy of DOTMA/DOPE-DNA genosomes (negative/positive ratio = 1/2). Bar indicates 100 nm. Courtesy of Drs. Ron Cohn and John Caulfield from Roche BioSciences, Palo Alto, CA.

intratumoral DNA delivery, are much less characterized. Cryoelectron microscopy studies of these complexes either yielded a fraction of lamellar structures or snake-like particles. Zeta potentials vary from expected values (around −20 to −40 mV) to values identical to free DNA (−60 mV) in other cases.

The simple observation, that multilamellar vesicles disintegrate upon addition of excess DNA (turbid solution turns clear), however, may offer some clues. Most likely it indicates, that at higher values of ρ (negative/positive charge ratio) the structure must contain polymers coated by lipids. In turbid solutions at higher values of <rho> qualitative relations between size and turbidity are not obeyed, possibly indicating nonspherical structures. Even transparent solutions when sized by quasielastic ligth scattering give rise to diameters around 200–300 nm, indicating an artifact of the methodology. This phenomenon was also observed with long, flexible rodlike micelles.

Recent work has established that many different univalent cationic lipids can give rise to the intercalated lamellar phase and that thermodynamic factors (low ionic strength, concentration range 10—2 mM cationic lipid, negative/positive charge ratio < 1) and kinetics of mixing are more important than the nature of cationic and neutral lipid, if pK of the lipid is 2 units above the pH of the sample (Podgornik, Strey, Lasic, unpublished). Figure 3 shows a negative stain micrograph of DOTMA:DOPE – DNA cationic complex and the presence of a lamellar phase can be clearly observed.

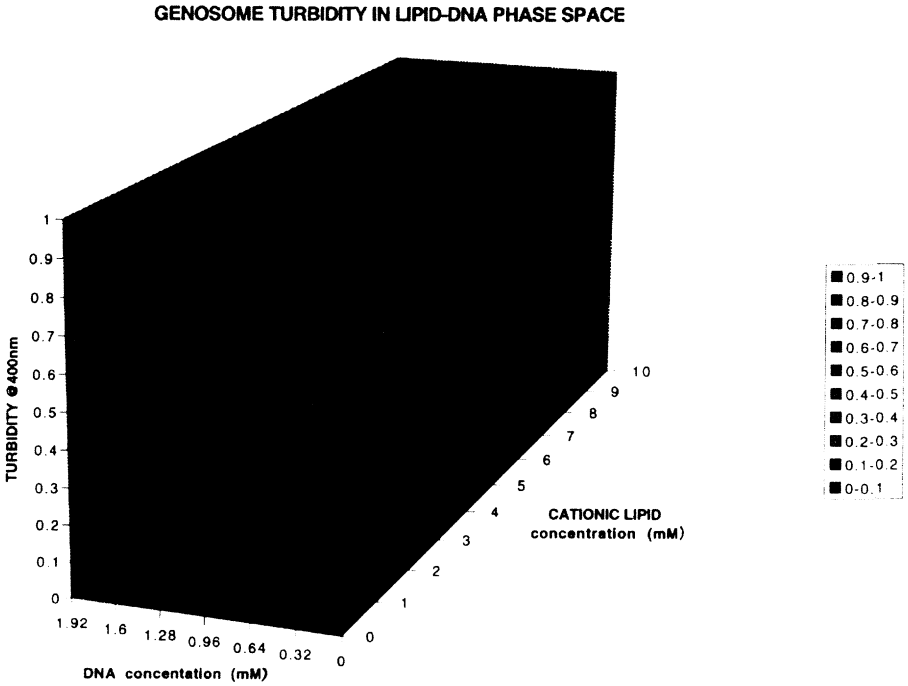


Figure 4 An approximate phase diagram of phase behavior of cationic lipid (equimolar DOTMA/DOPE) and DNA mixtures. Turbidity values are approximate and relative because at values above 0.35–0.4 mixtures (20 fold diluted with distilled water) partially flocculate and turbidity measurements are inaccurate. One can define 3 regions in htis phase diagram: stable colloidal suspension (relative turbidity, RT, < 0. 25), metastable region (0.3 < RT < 0.6) and phase separation (solubility gap, RT > 0.8). Around electrical neutrality immediate precipitation occurs which is indicated by the relative turbidity of 1. On the left of this diagonal anionic complexes are stable and on the right the cationic ones (in time scale of hours to days, depending on the proximity of the solubility gap (relative turbidity > 0.8). At higher lipid and DNA concentrations precipitation and phase separation are still more intense because either system is approching denser phases. If genosome size (as measured by dynamic light scattering in uni-modal distribution) is plotted, similarly shaped surface would be obtained. Complex size ranges from 80–120 nm in the soluble range (relative turbidity 0.1–0.2) to 150–300 nm in the regions close to the phase separation range (relative turbidity 0.2–0.4) and from 500 nm to > μm (measurement above μm are rather inaccurate by dynamic light scatttering. Such samples often also contain large flocculae which start to precipitate) for the relative turbidity in 0. 5 to 1 range.

GENOSOME PREPARATION

Early reports emphasized the importance of genosome preparation. Stability, reproducibility and activity of complexes was stated to depend on the exact nature of mixing of DNA and liposomes. Often transfection activity critically depends on the preparation protocol. Recent work has shown, however, that genosome formation is both, a thermodynamic and kinetic phenomenon and by controlling the two, stable and reproducible genosomes can be produced. These genosomes enable reproducible experiments to be achieved *in vitro* and *in vivo*.

Figure 4 shows a schematic phase diagram of DNA and cationic liposomes. In a phase space of DNA and cationic lipid concentrations one can define stable colloidal suspension (relative turbidity, RT < 0.25), metastable region (0.3 < RT < 0.6) and two phase system (precipitation, RTN > 0. 8). Many univalent and polyvalent liposomes or intact lipid particles give rise to similar phase behavior which tends to be independent of the DNA length in the range from few to 20–40 kb pairs. In the case of identical preparation procedure (see kinetic effects below), the complex size distribution, extent of DNA and lipid association and the resulting stability are influenced by thermodynamic parameters, such as concentrations of the reactants and solution ionic strength. Temperature does not appear to have a significant effect on the colloidal properties of genosomes formed. While one can find some quantitative relations between genosome characteristics and thermodynamic parameters, the explanation of the kinetic effects is qualitative at best.

Mixing rate of DNA — cationic lipid reaction and sequence of mixing (DNA into liposomes or vice versa) are the most frequently manipulated variables used to control kinetics of genosome formation and to minimize precipitation, i.e., suspend DNA at maximal concentration. The influence of sequence of mixing on the properties of genosomes formed (precipitation, stability and size distribution) can be easily explained by phase diagram which indicates that complexes should be prepared in such a way that the solubility gap should never be broached. The importance of quick mixing has been attributed to the fact that quick dispersal of reactants is essential to give rise to small and uniform complexes (Lasic *et al.*, 1997). In the case of slow mixing, large local spots exist where local concentration markedly deviates from the bulk and locally large volumes with electrical neutrality are created. These give rise to very large flocculae if not dispersed by rapid mixing or vortexing (Lasic, 1997).

The kinetic origin of complex formation can also explain their stability. Genosomes are, similarly to liposomes, in general thermodynamically unstable states. These states are often referred to as kinetic traps and such systems are inherently unstable because the free energy is constantly decreasing because the system can be continuously changing and drifting into local free energy minima. When complexes are prepared by a slow mixing/incubation process, possibly from large, heterogeneous (vortexed and not sized down) liposomes, a very heterogeneous sample results which can, during time, change its morphology. Typically they grow and precipitate. On the contrary, when genosomes are prepared in a well controlled process (from homogeneous vesicles in a quick mixing process), structures closer to thermodynamic equilibrium are formed. Typically such structures are characterized by intercalated lamellar short range symmetry and can be stable in liquid or freeze dried form for months. Along these lines, many studies published on the structure of genosomes are artefacts of measuring random, irreproducible and transient states of metastable systems which are constantly changing. Obviously, experimental observation itself, such as sample dilution/concentration, heating/cooling, centrifugation and similar may also cause structural and colloidal changes.

TRANSFECTION AND GENE EXPRESSION *IN VITRO* AND *IN VIVO*

Gene delivery *in vitro* can rely on more than a dozen commercially available liposome kits for transfection (Lasic, 1997). These kits are typically suspensions of small

unilamellar liposomes containing DOPE at various fractions and cationic lipids vary from univalent DOTMA or DODAB to DOSPA which has 5 positive charges. Some cationic lipids, such as DOTAP and DOGS are formulated without neutral lipid. Using such liposome kits, many different cell types have been successfully transfected.

Systematic approaches to genosome delivery in vitro started in 1987 (Felgner *et al.*) when it was shown that a hundred-fold improvement in the transfection of JZ-1 cell line (tenfold in COS-7 cells) was achieved when DNA was complexed to DOTMA:DOPE liposomes (as compared to the use of standard polyelectrolyte – DEAE). Practically all cell cultures have been successfully transfected. According to numerous reports, however, there is no general recipe for successful and lasting gene expression. Normally, different liposome kits give rise to different transfection activities in different cell lines and transfection of each cell line must be optimized with respect to the liposome formulation as well as DNA to lipid ratio. No comprehensive review on the transfection of different cell lines with different liposomes exist but an interested reader may gather a lot of information in various data bases. In other experiments, plant cells were also transfected (Ballas *et al.*, 1988). Because cytotoxicity was observed, an effort was initiated to synthesize less toxic and more efficient lipids. Towards these goals, structure-activity studies were used to design novel cationic lipids, as will be described below.

Transfection *in vivo* is much more difficult to achieve. Low levels of expression were observed in the lung after intravenous injection and intratracheal instillation of DOTMA:DOPE complexed to DNA plasmids containing a marker gene in mice (Brigham *et al.*, 1989) and rats (Hazinski *et al.*, 1991). Since then several groups reported measurable expression in animals. Upon intravenous administration the largest gene expression was observed in lungs (Zhu *et al.*, 1993; Mahato *et al.*, 1997) and some expression was found in the spleen, liver and heart. Typically levels up to around 0.1–0.2 ng of CAT protein per mg of lung protein were reported while luciferase typically gives rise to larger numbers.

A decade of research on novel plasmids, novel lipids or structurally novel genosomes has resulted in much higher levels of the synthesized protein (Felgner, 1996). CAT levels in the lung of up to approximately 40 ng/mg of protein were observed upon intratracheal administration of CAT plasmids complexed with a series of novel cationic lipids (Lee *et al.*, 1996). These lipids increased transfection efficiency up to 1000-fold over some other lipids. Expression of up to 200 ng/mg of protein in the lung were found upon changing colloidal properties of the complexes following intravenous administration by using DOTAP, a simple cationic lipid which has not been associated with enhanced expression levels before, in combination with cholesterol as a neutral lipid (Templeton *et al.*, 1997). In addition to highest expression and the broadest biodistribution of gene expression, targeting of these complexes to the liver by surface attached ligands has been demonstrated.

Parallel cryo-electron microscopy and small angle x-ray scattering studies have revealed unique structure of these complexes — two dimensional condensed DNA is encapsulated in the middle of spherical liposomes. It is hypothesized that this particular structure protects DNA and allows better biodistribution than other complexes which are characterized by either stacks of lamellae with adsorbed DNA plasmid or lipid aggregates surrounded by a halo of fibrilar DNA coated by lipid tubules. Indeed, complexes were found to be stable in 50–100% plasma for at least one day. The unusual "self-encapsulation" of DNA was attributed to the use of

specially prepared, invaginated liposomes which resemble spherical vase-like structures and have a large excess of free surface area. Upon DNA adsorption, such liposome can undergo inversion, resulting in complete DNA encapsulation. Recent theoretical analysis of strongly adsorbed polymer on an elastic membrane surface has also showed that orientational ordering of the polymers promotes curving of the supporting lipid bilayer leading eventually to a complete encapsulation of the adsorbed polymers (Podgornik, 1997). Because such an interaction neutralizes charges only on one side of DNA, often a second liposome adsorbs on the adsorbed DNA. It seems that, the liposomes composed from mechanically very strong and cohesive (cholesterol, as opposed to DOPE, improves mechanical stability of the membrane), but fluid, lipid bilayer with a large excess of free surface area allow much better DNA organization and better condensation than regular liposomes. Condensation, packing and lipid restructuring processes have more degrees of freedom and time to self-assemble and self-organize than in the case of small unilamellar liposomes which can only break, or large multi/oligo lamellar vesicles which inevitably give rise to large complexes. As a consequence of effective DNA condensation, these liposomes can also colloidally suspend higher DNA concentrations than other systems. This is very important, because gene expression is dose-dependent. An important observation was also that optimal size distribution of the complexes was between 200 and 450 nm.

The duration of expression was longer than in similar experiments reported in the literature. For instance, gene expression dropped twofold in a week and 5-fold after three weeks. In addition, safety of these formulations, which typically contain several-fold less cationic lipid per amount of DNA, was carefully studied. Detailed pathologies of all the tissues showed no tissue damage at plasmid concentrations up to 0. 75 mg DNA/ml and DOTAP concentration of 5 mM. Toxicity was observed only at very high lipid (and DNA) doses and was expression plasmid dependent, possibly indicating the toxicity of synthesized reporter proteins (Templeton *et al.*, in preparation).

Following exhaustive efforts in chemical synthesis which resulted in several optimal lipids for transfection and showed that different lipids have different transfection potential, at present, we still do not know what optimization is required with respect to the colloidal properties of these lipids complexed to DNA. It is possible that different lipid chemical structures, having different geometries, values of pK and critical micelle concentration, and tendency to form hydrogen bonds, may simply form complexes with different structures and different stabilities. Unfortunately, the differences in structure, DNA compaction, size and stability (in the presence of electrolytes, plasma, etc) of complexes made from different lipids have not been compared in parallel to their transfection activity. For intravenous administration preliminary data have shown that small (150–300 nm) and dense complexes yielded much higher gene expression as opposed to large complexes consisting of aggregated liposomes with surface and interstitially adsorbed DNA (Lasic, 1997). Former complexes offer better protection to DNA and can achieve larger biodistribution and likely can be internalized more easily (Lasic, 1997). On the other hand, we still do not know what is the optimal size, neutral lipid and charge ratio for genosomes which are administered topically (intratracheally, as an aerosol, percutaneously) or locally (intraperitoneally, subcutaneously, intratumorally).

This is paralleled by the poor knowledge of the mechanisms involved in transfection and gene expression.

STRUCTURE ACTIVITY RELATIONSHIPS

In cell culture transfection studies only minute amounts of DNA are needed and also precipitated complexes can be used. Therefore the problem of sample precipitation does not abolish transfection. Often, commercially available liposomes are mixed with DNA in electrolyte solutions, resulting in large aggregated complexes. While these systems work satisfactorily *in vitro*, such genosomes yield very low level of transfection *in vivo*. One reason is the size and morphology of such complexes and the other is the low dose of applied DNA because transfection, if experiment is performed properly, is dose dependent. Concentrations of liposomes in commercially available transfection kits are simply too low to colloidally suspend DNA at sufficient concentrations. Also, many studies have shown that cholesterol can be a more active neutral lipid than DOPE for systemic transfection *in vivo*.

With respect to some published results and many data presented at conferences we must also warn that many artefactual results have been presented. Sometimes, high expression levels reported may be due to non-lethal and reversible physical damage to cellular structures and processes which can enhance transfection.

Structure activity studies have been performed mostly *in vitro* (Leventis and Silvius, 1990; Felgner *et al.*, 1994; Farhood and Huang, 1996). When studying cationic cholesterol derivatives it was noted that tertiary amine gave the best transfection at lowest toxicity (Farhood *et al.*, 1992). For multivalent cholesterol derivatives it was shown that the site and the angle of the attachment of the polyamine was important. Molecules having perpendicular arrangement between long axis of sterol and direction of polyamine were found to be more active than the parallel ones (Lee *et al.*, 1996). Sterol hydrophobic anchor was found to be more effective for in vivo gene delivery while for in vitro systems diacyl chains were found to give rise to higher gene expression. In the case of diacyl lipids it was discovered that dioleoyl and dimyristoyl chains give rise to the highest expression (Felgner *et al.*, 1994; Lee *et al.*, 1996). This is hardly surprising, because for both interaction of DNA with liposome as well as of genosomes with cells, fluid membranes are necessary. With respect to polar heads and number of charges no clear conclusions have been reported. Studies of DOTMA like molecules have shown that decorating polar head with hydroxyethyl group and with beta amines increased transfection efficiencies *in vitro* (Felgner *et al.*, 1994; Wheeler *et al.*, 1996).

The importance of neutral lipid was also carefully studied. While for *in vitro* transfection DOPE seems to be a superior choice (Farhood and Huang, 1992 and 1996; Felgner *et al.*, 1994), many recent experiments show that for systemic delivery the use of cholesterol can result in much higher transfection (Zhu *et al.*, 1993; Templeton *et al.*, 1997).

Most of the *in vitro* experiments are also performed in the absence of serum, the presence of which typically reduces or eliminates gene expression. Several studies have shown, however, that neutralizing effects of plasma can be overcome by higher overall positive charges. Excess of cationic charge in the formulation typically results

in unreacted liposomes, i.e., liposomes which did not change topologically upon DNA introduction in the system. The excess of lipid and/or liposomes may have an effect in permeabilizing cells in culture and/or inactivating plasma components which neutralize genosomes *in vitro* and *in vivo*.

A conclusive observation of these *in vitro* transfection studies is that increased amount of cationic lipids increases transfection as well as cytotoxicity. The balance between activity and toxicity therefore determines transfection efficiency. Unfortunately, not much is known with respect to colloidal structure — transfection activity relationships. For instance, important information needs to be gained about activity as a function of genosome size at specified charge ratios.

Such simple mechanism, however, cannot be applicable for *in vivo* cases, where numerous other interactions take place, especially after systemic delivery.

At present, it is still often stated that in general there is no, or little, correlation between activity *in vitro* and *in vivo*. Activity in vivo obviously depends on the route of administration. For instance, intravenous administration presents very different conditions and gene expression depends on pharmacokinetics, biodistribution and stability of genosomes in biological environment to a much greater extent than in other administration routes. Intramuscular and subcutaneous injection may lay in between with respect to biological stress of the environment on the DNA-lipid complex. Therefore one has to determine these correlations in order to improve efficiency of gene expression. It is likely that each administration route has different optimization characteristics. For instance, for pulmonary delivery it is not known whether smaller or larger genosomes are preferred. Only recently scientists at Genzyme and GeneMedicine have shown that larger complexes lead to higher expression levels (Rolland, personal communication). Because lung instillation may be similar to *in vitro* tests, DOPE may be a superior neutral lipid over cholesterol. The situation was partially explained for intravenous administration where it was shown that complexes have to be smaller than 500–1000 nm and preferably tightly packed in order to express and produce optimal gene expression. This ensures protection of DNA in the bloodstream and allows an increased volume of biodistribution. In contrast, large, noncompact genosomes are very likely broken in blood in milliseconds and the exposed DNA is degraded in seconds (Lasic, 1997). For systemic administration cholesterol was shown to be in many cases much more effective neutral lipid than DOPE. Because physico-chemical characteristics of genosomes containing either neutral lipid are rather similar, this may imply that it is the stability of the structures in plasma which causes the difference and also eliminates the early endosomal release induced by DOPE as the main mechanism of transfection. Cholesterol is known as a membrane stabilizer while DOPE, on ther other side, destabilizes lipid bilayers (Lasic, 1993). This also may indicate that the major cell entry mechanism is independent of endocytotic processes but might be simply a cationic lipid induced transient cell membrane permeation/poration.

This analysis implies that the correlation between *in vitro* and *in vivo* experiments accounts for genosome stability in plasma or biological environment, pharmacokinetics and biodistribution. Cells in culture are also known to exhibit rather large differences in phagocytic activity upon subtle changes in the environment which further complicates comparisons (Lasic, 1997).

CONCLUSION

Using cationic lipids, polymers or their combination a number of different cell cultures can be transfected. Gene expression was also observed upon systemic and topical DNA delivery of genosomes. With improvements in plasmid and lipid design, transfection efficiencies have increased over thousand fold from early experiments. However, the duration of protein synthesis is still rather short and selectivity of transfection is poor. Further improvements may include preparation of colloidally optimal particles from the most potent cationic and neutral lipids, possibly in mixtures with polymers (Kabanov and Kabanov, 1995) and in the novel design of the plasmids, especially in their targeting of the nucleus and persistance therein. Additionally, we may speculate that by co-delivering DNA binding proteins possessing nuclear localization sequences, nuclear transport of plasmids would be facilitated.

REFERENCES

Ballas, N., Zakai, N., Sela, I. and Loyter, A. (1988) Liposomes bearing a quaternary ammonium detergent as an effective vehicels for functional transfer of TMV-RNA into plant protoplasts, *Biochim. Biophys. Acta*, **939**, 8–18.

Bangham, A.D. (Ed.) Liposome Letters, Acad. Press., New York, 1983.

Behr, J.P. (1993) Synthetic gene transfer vectors, *Acc. Chem. Res.*, **26**, 274–278.

Bloomfield, V.A. (1996) DNA condensation, *Curr. Op. Struct. Biol.*, **6**, 334–341.

Bloomfield, V.A. (1991) Condensation of DNA by multivalent cations, *Biopolymers*, **31**, 1471–1481.

Bloomfield, V.A., Crothers, D.M., Tinoco, I. Physical Chemistry of Nucleic Acids (1974), Harper and Row, New York.

Boles, T.C., White, J.H. and Cozzarelli, N.R. (1990) Structure of plectonemically supercoiled DNA, *J. Mol. Biol.*, **213**, 931–51.

Brigham, K.L., Meyrich, B., Christman, B., Magnusson, M. and Berry, L. (1989) *In vivo* transfection of murine lungs with functioning prokaryotic gene using a liposome vehicle, *Am. J. Med. Sci.*, **298**, 278–281.

Carmona-Ribeiro, A.M. (1989) Theoretical and experimental stabilities of amphiphilic vesicles in the presence of NaCl, *J. Phys. Chem.*, **93**, 2630–2634.

Current Protocols in Molecular Biology, F.M. Ausubel *et al.*, John Wiley & Sons, 1997.

De Gennes, P.G. and Prost, J. (1993) *The Physics of Liquid Crystals*, Clarendon Press, Oxford.

Fang, Y. and Jie, Y. (1997) Two dimensional condensation of DNA molecules in cationic lipid membrane, *J. Phys. Chem. B*, **101**, 441–449.

Farhood, H., Bottega, R., Epand, R.M. and Huang, L. (1992) Effect of cholesterol derivatives on gene transfer and protein kinase C activity, *Biochim. Biophys. Acta*, **1111**, 239–246.

Farhood, H. and Huang, L. (1996) Delivery of DNA, RNA and proteins by cationic liposomes, in *Liposomes: from Gene Therapy to Diagnostics and Ecology*, Eds. Lasic, D.D., Barenholz, Y., CRC Press, Boca Raton, pp. 31–42.

Felgner, P.L. (1996) Improvement in cationic liposomes for in vivo gene delivery, *Human Gene Ther.*, **7**, 1791–1793.

Felgner, J.H., Kumar, R., Sridhar, R., Wheeler, C., Tsai, Y.J., Border, R., Ramsay, P., Martin, M. and Felgner, P.L. (1994) Enhanced gene delivery and mechanism studies with novel series of cationic lipid formulations, *J. Biol. Chem.*, **269**, 2550–2561.

Felgner, P.L. and Ringold, R.G. (1989) Cationic liposome mediated transfection, *Nature*, **337**, 387–388.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.S., Wenz, M., Northrop, J.P., Ringold, M. and Danielsen, H. (1987) Lipofection: a highly efficient lipid-mediated DNA transfection procedure, *Proc. Natl. Acad. Sci. USA*, **84**, 7413–7417.

Felgner, P.L., Tsai, Y.J. and Felgner, J.H. (1996) Advances in the design and application of cytofectin formulations, in *Liposomes: from Gene Therapy to Diagnostics and Ecology*, Eds. Lasic, D.D., Barenholz, Y. CRC Press, Boca Raton, pp. 43–56.

Frank-Kamenitskii, M.D., Anshelevich, V.V. and Lukashin, P. (1987) Polyelectrolyte model of DNA, *Sov. Phys. Usp.*, **30**, 317–330.

Gerhson, H., Ghirlando, R., Guttman, S.B. and Minsky, A. (1993) Mode of formation and structural features of DNA-cationic liposome complexes used for transfection, *Biochemistry*, **32**, 7143–7151.

Greenberg, D. (1995) Liver-specific expression of the human factor VII gene, *Proc. Natl. Acad. Sci. USA*, **92**, 12347–12351.

Gustaffson, J., Almgrem, M., Karlsson, G. and Arvidson, G. (1995) Complexes between cationic liposomes and DNA visualized by cryoTEM, *Biochim. Biophys. Acta*, **1235**, 305–317.

Hazinski, T.A., Laddm P.A. and DeMAteo, C.A. (1991) Localization and induced expression of fusion genes in the rat lung, *Am. J. Resp. Cell Mol. Biol.*, **2**, 206–209.

Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (1990) "PCR Protocols: A Guide to methods and applications" Academic Press, Inc, New York.

Israelachvili, J.N. (1991) *Intermolecular and Surface Forces*, 2nd. Ed. Academic Press, London.

Kabanov, A.V. and Kabanov, V.A. (1995) DNA complexes with polycations for delivery of genetic material into cells, *Bioconj. Chem.*, **6**, 7–20.

Kreigler, M. (1990) Gene Transfer and Expression: A Laboratory Manual, Stockton Press.

Lasic, D.D., Strey, H.H., Podgornik, R. and Frederik, P. (1996) Recent developments in medical applications of liposomes: sterically stabilized and cationic liposomes, *4th European Symp. Control. Drug Del., Book of Abstracts*, pp. 61–65, Nordwijk aan Zee, April 1996. This lecture is published in full in *J. Control. Rel.* (1997), same title, **48**, 203–222.

Lasic, D.D. (1997) *Liposomes in Gene Delivery*, CRC Press, Boca Raton.

Lasic, D.D. (1993) *Liposomes: from Physics to Applications*, Elsevier, Amsterdam.

Lasic, D.D. and Barenholz, Y. (1996) Eds., *Liposomes: Theory and Basic Science*, CRC Press, Boca Raton.

Lasic, D.D., Strey, H.H., Podgornik, R. and Frederik, P.M. (1997) The structure of DNA-liposome complexes, *J. Am. Chem. Soc.*, **119**, 832–833.

Lasic, D.D. and Barenholz, Y. (1996b) Eds., *Liposomes: from Gene Therapy, Diagnostics to Ecology*, CRC Press, Boca Raton.

Lasic, D.D. and Templeton, N. (1996) Liposomes in gene therapy, *Adv. Drug Del. Rev.*, **20**, 221–266.

Lee, E.R., Marshall, J.M., Siegel, C.S., Jiang, C., Yew, N.S., Nichols, M.R., Nietupski, J.B., Ziegler, J.R., Lane, M.B., Wang, K.X., Wan, N.C., Scheule, R.K., Harris, D.J., Smith, A.E. and Cheng, S.H. (1996) Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung, *Human Gene Ther.*, **7**, 1701–1717.

Leforestier, A. and Livolant, F. (1994) Supramolecular ordering of DNA in cholesteric liquid crystalline phases, *Liquid Crystals*, **17**, 651–658.

Leventis, R. and Silvius, J.R. (1990) Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles, *Biochim. Biophys. Acta*, **1023**, 124–132.

Luzzati, V., Mustacchi, F., Skoulios, A. and Husson, R.E. (1960) La structure des phases d'association, *Acta Cryst.*, **13**, 660–667.

Mahato, R., Rolland, A. and Tomlinson, E. (1997) Cationic lipid based gene delivery systems:pharmaceutical perspectives, *Pharm. Res.*, **14**, 853–859.

Miller, N. and Wheelan, J. (1997) Progress in Transcriptionally Targeted and Regulatable vectors for gene therapy, *Human Gene Ther.*, **8**, 803–815.

Minsky, A., Ghirlando, R. and Gerhson, H. (1996) Features of DNA cationic liposome complexes and their implication for transfection, in *Liposomes: from Gene Therapy to Diagnostics and Ecology*, Eds. Lasic, D.D., Barenholz, Y. CRC Press, Boca Raton, pp. 7–30.

Podgornik, R., Strey, H.H., Rau, D.C. and Parsegian, V.A. (1996a) Watching molecules crowd: DNA double helix under osmotic srtess, *Biophys. Chem.*, **57**, 111–121.

Podgornik, R., Strey, H.H., Gawrisch, K., Rau, D.C., Rupprecht, A. and Parsegian, V.A. (1996) Bond orientational order, molecular motion and free energy of high-density DNA mesophases, *Proc. Nat. Acad. Sci. USA*, **93**, 4261–4266.

Podgornik, R. (1997) Supporting membrane shape instability — the presence of strongly adsorbed flexible polymers, *Langmuir*, **13**, 4791–4794.

Raedler, J.O., Koltover, I., Sadditt, T. and Safinya, C. (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, *Science*, **275**, 810–814.

Scheule, R.K., George, J., Bagley, R.G., Marshall, J., Kaplan, J.M., Akita, G.Y., Wang, K., Lee, E., Harris, D.J., Jiang, C., Yew, N., Smith, A.E. and Cheng, S.H. (1997) Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung, *Human Gene Ther.*, **8**, 689–707.

Sinden, R.R. (1994) *DNA Structure and Function*, Academic Press, San Diego.

Sternberg, B., Sorgi, F. and Huang, L. (1994) New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy, *FEBS Letters*, **356**, 361–6.

Sterneberg, B. (1996) Liposomes as models for membrane structures and structural transformations, in *Liposomes: from Gene Therapy to Diagnostics and Ecology*, Eds. Lasic, D.D., Barenholz, Y. CRC Press, Boca Raton, 271–298.

Templeton, N.S., Lasic, D.D., Frederik, P.M., Strey, H.H., Roberts, D.D. and Pavlakis, G. (1997) Improved DNA-liposome complexs for increased systemic delivery and gene expression, *Nature Biotech.*, **15**, 647–652.

Watson, J.D. and Crick, F.H.C. (1953) A structure for deoxyribose nucleic acid. *Nature*, **171**, 737–738.

Watson, J.D., Toose, J. and Kurtz, D.T. (1983) *Recombinant DNA*, Scientific American Books, New York.

Wheeler, C.J., Sukhu, L., Yang, G., Tsai, Y., Bustamante, C., Felgner, P., Norman, J. and Manthorpe, M. (1996) Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytofectin complexes, *Biochim. Biophys. Acta*, **1280**, 1–11.

Xu, Y. and Szoka, F.C. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection, *Biochemistry*, **35**, 5616–5623.

Zhu, N., Liggitt, D., Liu, Y. and Debs, R. (1993) Systemic gene expression after intravenous DNA delivery in adult mice, *Science*, **261**, 209–211.