Gene Ther Mol Biol Vol 7, 273-289, 2003

Advances in cationic lipid-mediated gene delivery

Review Article

Benjamin Martin[1], Abderrahim Aissaoui[2], Matthieu Sainlos[1], Noufissa Oudrhiri[2], Michelle Hauchecorne[2], Jean-Pierre Vigneron[1], Jean-Marie Lehn[1] and Pierre Lehn[2*]

[1] Laboratoire de Chimie des Interactions Moléculaires, CNRS UPR 285, Collège-de-France, 11 Place Marcelin Berthelot, 75005 Paris, France.

[2] INSERM U458, Hôpital Robert Debré, 48 Boulevard Sérurier, 75019 Paris, France

---

***Correspondence**: Pierre Lehn, INSERM U458, Hôpital Robert Debré, 48 Boulevard Sérurier, 75019 Paris, France. Phone: 33(0)140031932, Fax: 33(0)140031903, E-mail: lehn@idf.inserm.fr

Key words: gene therapy, gene delivery, transfection, cationic lipid, synthetic vector, lipoplex

Abbreviations: N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethyl ammonium chloride, (DOTMA); dioctadecylamido-glycylspermine, (DOGS); 3,-[_N-(N',N'-dimethylaminoethyl)carbamoyl]-cholesterol, (DC-Chol); dioleoyl phosphatidylethanolamine, (DOPE); dimethyldioctadecyl ammonium bromide, (DDAB); 1,2-dioleoyloxy-3-[trimethylammonio]-propane, (DOTAP); N^1-[2-((1S)-1-[3-amino propyl)amino]-4-[di(3aminopropyl)amino]butylcarboxamido)ethyl]-3,4-di(oleyloxybenzamide), (MVL5); 3β–[6'-kanamycin-carbamoyl]cholesterol, (KanaChol); bis-guanidinium-spermidine-cholesterol, (BGSC); bis-guanidinium-tren-cholesterol, (BGTC); 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, (DMRIE); N'-octadecylsperminecarboxamide hydrofluoroacetate, ($C_{18}Sper^{3+}$); O-(2R-1,2-di-O-(1 Z, 9 Z-octadecadienyl)-glycerol)-3-N-(bis-2-aminoethyl)-carbamate, (BCAT); 1,2-dioleoyl-sn-glycero-3-succinyl-2-hydroxyethyl disulfide ornithine, (DOGSDSO); cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine, (CHDTAEA); gamma-interferon-inducible lysosomal thiol reductase, (GILT); small-angle x-ray scattering, (SAXS); dioleoyl phosphatidylcholine, (DOPC); polyethylenimine, (PEI); nuclear pore complexes, (NPCs); glucocorticoid receptors, (GRs); peptide nucleic acid, (PNA); polyethyleneglycol, (PEG)

Received: 3 November 2003; Accepted: 1 December 2003; electronically published: December 2003

Summary

Over previous years, problems associated with virus-mediated gene delivery have stimulated the synthesis and biological evaluation of non-viral vectors as a possible alternative for gene therapy applications. Of the various non-viral vectors, cationic lipids have come forward as effective gene delivery agents, although it is clear that their transfection efficiency must be increased in order for them to become of real therapeutic value. This can be achieved by overcoming both the intracellular and extracellular barriers they encounter while conveying the transgene towards the nucleus of the target cells. The purpose of this review is to highlight the advances made to date in facing these challenges by paying particular attention to the design of the cationic lipid itself and the complexes (termed lipoplexes) formed on interacting with DNA. Because the structures of all three parts of a cationic lipid – the cationic headgroup, the hydrophobic moiety and the connecting linker – are important determinants of transfection efficiency, each will be considered here in turn, with special attention focused on recent studies including our own work. In addition, the stability of the lipoplex in the extracellular medium and the features of its intracellular trafficking towards the cell nucleus will be assessed from both chemical and biological viewpoints. In conclusion, the future will probably see the development of sophisticated modular self-assembling gene delivery systems incorporating various functional elements to face the various biological barriers encountered. Such vectors can be envisaged as 'virus-like' systems which share the levels of gene delivery efficiency of their viral counterparts, but coupled with the safety of purpose-made organic molecules.

I. Introduction

The use of genes as medicines remains both a captivating goal and a formidable challenge. By deliberate introduction of either a functional gene or a sequence capable of interfering with the functioning of a cellular gene, a wide variety of diseases of inherited and acquired origin are open to treatment in a most fundamental sense (Mulligan, 1993; Anderson, 1998). Those working in the field of gene delivery have much to learn from viruses which achieve efficient levels of gene transduction commensurate both with their need to deliver their genetic material into host cells for the purpose of reproduction and

with the evolutionary time-scale upon which these abilities have been honed. Although it seemed therefore natural to harness viruses (among them adenoviruses, adeno-associated viruses and retroviruses) for therapeutic gene delivery (Mulligan, 1993; Kootstra and Verma, 2003), it can be contested that much of their inconveniences are yet to be discarded. Problems with immunogenicity and toxicity remain, added to the practical issues of large scale production and quality control.

Focus has therefore shifted to a *de novo* approach in vector design, where synthetic organic molecules are used to bind the transgene and facilitate its passage across the significant extracellular and intracellular barriers that separate it from the cell nucleus where expression takes place via the cellular transcription machinery (Crystal, 1995; Lehn et al, 1998). Such carriers are termed non-viral vectors and generally take the form of cationic lipids or cationic polymers. In addition to avoiding problems associated with the use of recombinant viruses, an advantage of using synthetic vectors is that there is no limit on the size of DNA to be delivered. A large number and wide variety of synthetic non-viral vectors have been prepared and their transfection efficiency assessed not only in *in vitro* and *in vivo* experimental studies, but further, into the clinical setting for treatment in particular of cancer (Roth and Cristiano, 1997; Hersh and Stopeck, 1998) and cystic fibrosis (Alton et al, 1999; Boucher, 1999; Griesenbach et al, 1999; Davies et al, 2001). An exhaustive list of clinical gene therapy trials is available at www.wiley.co.uk/genmed/clinical.

Despite some positive results, the overall outcome indicates that a critical requirement for successful gene therapy is the use of more efficient gene delivery systems, i.e. systems leading to a higher percentage of transfected cells or an increased amount of transgene protein in the transfected cells according to the given experimental or clinical situation (Crystal, 1995; Aissaoui et al, 2002; Miller, 2003). This review aims to highlight the recent advances in improving cationic lipid-mediated gene delivery in terms of overcoming both intracellular and extracellular barriers to gene transfer. This will be dealt with by firstly surveying the progress made in vector design at the molecular level. Structure and functionality of the cationic lipid/DNA complexes will then be described with special focus placed on our own work with novel lipids. Finally, the stability of the lipoplex in the extracellular medium and the features of its intracellular trafficking towards the cell nucleus will be discussed, as well as the proposal of creating sophisticated modular self-assembling gene delivery systems incorporating various functional elements to face the barriers encountered. In short, the goal is the development of 'virus-like' systems, which share the levels of gene delivery efficiency of viral counterparts, but coupled with the safety of purpose-made organic molecules.

II. Basic principles

The first stage in the preparation of particles suitable for gene delivery is the condensation of the large DNA molecules by the vectors. The general structure of a cationic lipid vector is shown in **Figure 1**. The cationic nature of the amphiphilic vector drives an electrostatic interaction in the presence of negatively charged DNA, spontaneously self-assembling into nanometric vector/DNA complexes termed lipoplexes (stage 1**, Figure 2)**. This initial compaction step enables protection of the DNA from nucleases which are found in the extracellular medium. Use of an excess of cationic vector (quantified by the lipid/DNA ratio resulting in a mean theoretical charge ratio of the lipoplex (+/-)) conveniently decorates the outer surface of the lipoplex with a net positive charge which is generally considered to facilitate subsequent cellular uptake by interaction with negative cell surface residues such as proteoglycans (Friend et al, 1996; Labat-Moleur et al, 1996). Non-specific endocytosis ensues, encapsulating the lipoplex in intracellular vesicular compartments (Zabner et al, 1995) (stage 2), though fusion-based uptake cannot be entirely ruled out (Gao and Huang, 1995). Internalisation achieved, the DNA must avoid degradation in the late endosome and lysosome (barred arrow) by escaping the endosome to the cytoplasm (stage 3) (Zabner et al, 1995; Mukherjee et al, 1997). Trafficking of the DNA to the perinuclear region precedes passage across the nuclear membrane (stage 4) and subsequent expression of the transgene (stage 5). When localised within the nucleus, the DNA is already separated from its vector (Hasegawa et al, 2001) and it has been shown by microinjection experiments (Zabner et al, 1995) that gene expression does not occur if the complex remains intact.

Cationic lipids designed to achieve the ambitious task of gene delivery were first introduced by Felgner et al, whose work was founded on initial attempts to transfer nucleic acids via encapsulation into classical liposomes (Nicolau and Sene, 1982; Nicolau et al, 1983).

The lipid DOTMA (N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethyl ammonium chloride) resulted, consisting of a quaternary amine connected to two unsaturated aliphatic hydrocarbon chains via ether groups (Felgner et al, 1987) (**Figure 3**).

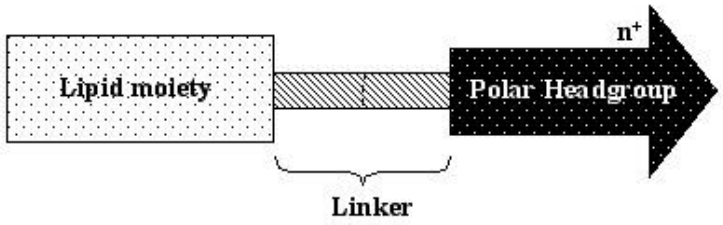
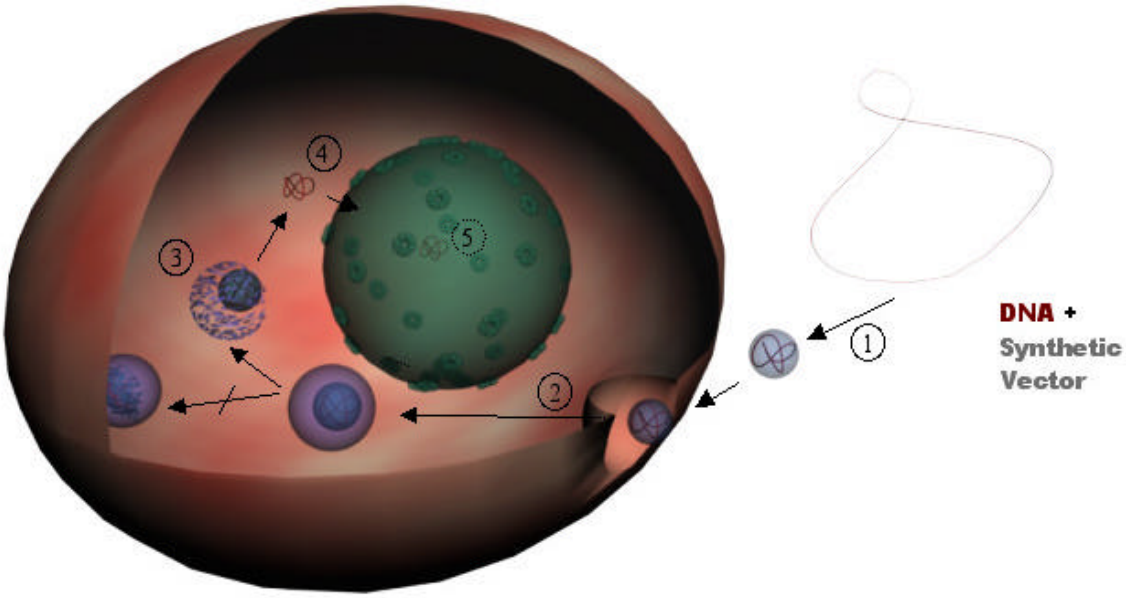


Figure 1: Schematic representation of a cationic lipid: lipid moiety, linker and cationic headgroup



1 Spontaneous self-assembly of vector/DNA complex (lipoplex)
2 Endocytosis – encapsulation of lipoplex into endosome
3 Endosomal escape before degradation of DNA
4 Trafficking of DNA to perinuclear region and nuclear ingress
5 Nuclear expression of the transfected DNA

Figure 2: Schematic representation of lipoplex formation and trafficking to the target cell nucleus.

A multivalent lipid soon followed in the form of the lipopolyamine DOGS (dioctadecylamido-glycylspermine) (Behr et al, 1989) and use of cholesterol as the hydrophobic portion was subsequently validated by the vector DC-Chol (3,-[_N-(-(N',N'-dimethylaminoethyl)carbamoyl] cholesterol) (Gao and Huang, 1991) (**Figure 3**). It is of particular importance that the transfection efficiencies of many cationic lipids can be enhanced by their formulation as stable cationic liposomes (Farhood et al, 1995). This is achieved by mixing cationic lipids, especially those which are incapable of forming bilayers alone, with the neutral colipid DOPE (dioleoyl phosphatidylethanolamine) prior to complexation with DNA. For example, DOTMA/DOPE liposomes are commercially available as Lipofectin (Gibco BRL). Also, the structural analogues DDAB (dimethyldioctadecyl ammonium bromide) (Gibco BRL) and DOTAP (1,2-dioleoyloxy-3-[trimethylammonio]-propane, where ester groups replace ethers of DOTMA) (Boehringer Mannheim) (**Figure 4**), are commercially available alone, as well as formulated with DOPE.

In addition to stabilisation properties, DOPE is also thought to have fusogenic properties which are expected to play a role in endosomal membrane disruption and so enhance escape of the lipoplexes into the cytoplasm (Ellens et al, 1986; Farhood et al, 1995; Vidal and Hoekstra, 1995). However, because a selection of lipoplexes formed in the absense of DOPE are also able to escape the endosome (Behr et al, 1989; Vigneron et al, 1996), these cationic lipids are also credited with intrinsic membrane destabilisation properties.

Since the initial 'proof of principle' period which confirmed the ability of cationic lipids to protect, transfer and release DNA for cellular expression, a challenging period has followed. Indeed slow progress has been made in improving the level of transfection efficiency up to that required for the potential therapeutic use of non-viral vectors, and this is largely attributed to an unclear structure-activity relationship in vector design. Thus, the development of novel vectors is justified as the highly complex series of steps that connect the DNA outside the cell to its expression in the nucleus are not fully understood and so a novel cationic lipid may not just be a 'me too' addition to an already extended list, but rather open new possibilites for differently influencing those steps (Lehn, 1999). As a result, a diverse library of vectors exists, representing a wide variety in structures and thus numerous potential mechanisms by which better transfection levels might be obtained. Highlights of its contents will be discussed in the following section.

III. Design of the basic domains of a cationic lipid

All cationic lipids are positively charged amphiphiles containing the three following functional domains: i) a polar hydrophilic headgroup which is positively charged, generally via the protonation of one (monovalent lipid) or several (multivalent lipid) amino groups; ii) a linker whose length and nature may influence the stability and the biodegradability of the vector; and iii) a hydrophobic portion composed of alkyl chains (saturated or unsaturated) or of a steroid (**Figure 1**).

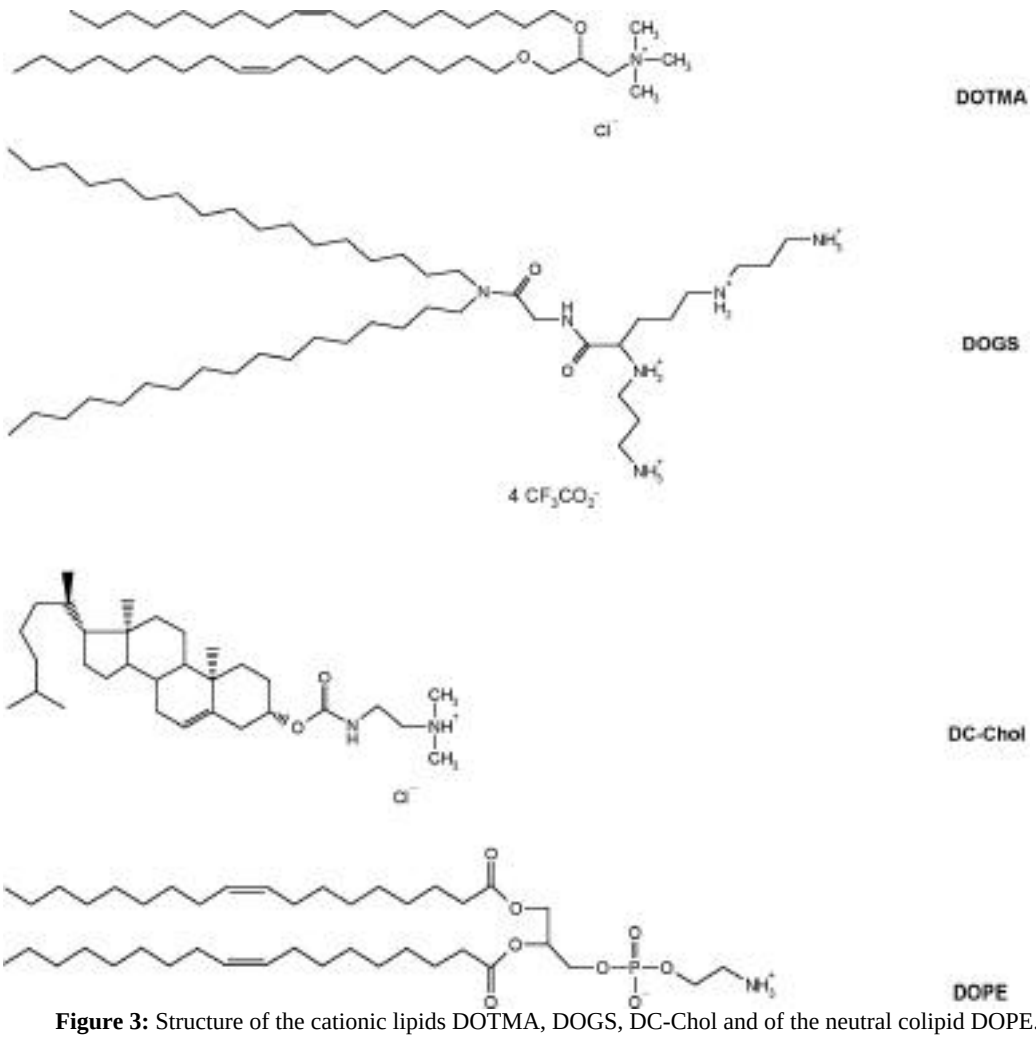


Figure 3: Structure of the cationic lipids DOTMA, DOGS, DC-Chol and of the neutral colipid DOPE.

Progress has been made in the design of each of these components. In particular, the choice of headgroup has expanded into the use of natural architectures and functional groups with recognised DNA binding modes. Linkers have been developed which are sensitive to biological stimuli, inducing DNA release from the lipoplex at defined time-points.

Finally, modifications of the hydrophobic portion have revealed that optimal vector design is often dependant on this moiety. Accordingly, the following sections will deal with the advances made to the three functional domains.

A. Headgroup design

1. Monovalent amine headgroups and the hydration issue

The purpose of the headgroup is to sustain a positive charge for binding of the DNA sequence to be transferred. The charge is often located on ammonium groups (exceptions include phosphonium and arsonium headgroups (Guenin et al, 2000)) and a relationship between the degree of hydration of the ammonium headgroup and the transfection efficiency has been elucidated (Bennett et al, 1997). According to this correlation, the greater the imbalance between the cross-sectional area of the headgroup (small end) and hydrophobic domain (large end) - that is the more cone-shaped the cationic lipid - the more unstable the resulting lipid assembly and so the greater the likeliness to undergo fusion with anionic vesicles. Instability of the lipoplex is thought to be related to improved transfection efficiencies because a fusion event between the cationic lipoplex and the endosomal membrane is associated with DNA release into the cytoplasm (Gao and Huang, 1995; Xu and Szoka, 1996; Hasegawa et al, 2002). A decrease in headgroup hydration can be achieved by incorporation of a hydroxyalkyl chain which is capable of hydrogen-bonding to neighbouring cationic headgroups and therefore reduces the space available to associated water and thus the cross-sectional area of the headgroup. Accordingly, gene delivery by the vectors DOTMA (**Figure 3**) and the ester-linked variant DOTAP (**Figure 4**) was improved by incorporation of a hydroxyethyl group into their structures to give vectors DORIE (1,2-dioleoyl-3-dimethyl-hydroxyethyl ammonium bromide) and DORI (1,2-dioleoyloxypropyl-3-dimethyl-hydroxyethyl ammonium chloride) respectively (**Figure 4**) ((Felgner et al, 1994; Bennett et al, 1997). In complement, the hydration of the hydrophobic domain of both vectors was increased by incorporating alkyl chains that contain cis-unsaturated bonds (e.g. oleoyl rather than myristoyl) which lead to reduced packing with neighbouring lipids, thereby leaving space for water molecules and increasing the cross-

sectional area of this end of the molecule (Bennett et al, 1997). Nakanishi and coworkers found that replacement of the dimethylamino headgroup of DC-Chol (**Figure 3**) by diethylamino and diisopropylamino groups led to reduced gene delivery (Takeuchi et al, 1996). This again is in accordance with the relationship between headgroup size and transfection efficiency as the alkyl chains are likely to cause steric repulsion between neighbouring vector headgroups. The same research group went on to show that lipid I (cholesteryl-3β-carboxyamido ethylene-N-hydroxyethylamine) (**Figure 4**) was better at gene delivery than its non-hydroxyethylated dimethyl tertiary amino homologue and demonstrated by means of FRET experiments that lipoplexes formulated from lipid I were particularly unstable in the presence of anionic liposomes (Hasegawa et al, 2002). It was therefore suggested that the ability of lipid I to transfect efficiently was related to lipoplex instability in the endosomes leading to subsequent DNA release.

However, other results have not confirmed the favourable effect of the incorporation of a hydroxyethyl group on transfection efficiency. An ether-linked cholesterol conjugate with a dimethyl hydroxyethyl headgroup (cholest-5-en-3β-oxyethane-N,N-dimethyl-N-2-hydroxyethyl ammonium bromide) was found to be less efficient at gene delivery than its trimethyl non-hydroxyethylated homologue (Ghosh et al, 2000). However, it should be taken into account that in another study, the transfection efficiency of the ester analogue lipid II (cholesteryl-3β-carboxy-ethylene-N,N-dimethyl-N-2-hydroxyethyl ammonium iodide) (**Figure 4**), was found to be dependant on the lipoplex charge ratio (which was not assessed for the ether analogue). At charge ratios up to 5, the hydroxyethylated vector lipid II was less efficient compared to its dimethyl tertiary amino and trimethyl quaternary amino homologues, but efficiency was seen to overtake the methylated homologues at ratios above 7 at which the dimethyl and trimethyl homologues were inefficient (Fichert et al, 2000; Ghosh et al, 2000).

Finally, the nature of the counter-ion has been identified as a determinant of transfection efficiency. By varying the counter-ion of DOTAP (**Figure 4**) using ion-exchange chromatography, it has been shown that the transfection efficiency *in vitro* and *in vivo* varies according to the known ability of the counter-ion to either structure water or shield the cationic charge (Aberle et al, 1996). Indeed, anions such as bisulphate and iodide were found to convey better transfection efficiencies than acetate and chloride anions. Again, the reduced hydration of the headgroups thought to occur when using bisulphate or iodide counter-ions is expected to lessen the distance between neighbouring headgroups, leading to liposomes or lipoplexes formed from more conical vectors and therefore prone to undergo the non-bilayer lipid reorganisations required for membrane fusion.

2. Novel headgroups with known nucleic acid binding modes

Multivalent cationic lipids are expected to form liposomes with a greater surface charge density than monovalent equivalents, and as such are generally considered better than the latter at DNA binding and delivery to the target cells. A logical step in moving from monovalent to multivalent species was the incorporation of natural polyamines such as spermidine and spermine, which have the further benefit of a pre-characterised ability to interact with the minor groove of B-DNA (Schmid and Behr, 1991). Incorporation of the triamine spermidine into cholesteryl-spermidine (Moradpour et al, 1996) (available commercially as Transfectall (Apollon Inc)) (**Figure 5**), and of the tetraamine spermine into the lipid DOGS (Behr et al, 1989) (**Figure 3**) are early examples. In addition, in a lipid such as DOGS, it is possible that the presence of protonation sites with different pKa values may lead to buffering of the endosomal acidification, thereby protecting the DNA from degradation and providing a possible endosome escape mechanism (Demeneix and Behr, 1996). More recently, the importance of the length of, and charge distribution on, the polyamine chain have been investigated. Ohwada and coworkers found that additions of amino groups separated by methylene portions to the end of a linear polyamine chain did not automatically enhance gene delivery by a series of polyamine-steroid conjugates, regardless of the extra protonation sites (Fujiwara et al, 2000). With reference to molecular modelling data, the authors highlight the importance of the flexibility of the polyamine chain which can adopt increasingly folded conformations on increasing length. It was suggested that the folded conformations may disfavour interactions with DNA. With the aim of designing a polyamine headgroup that had an optimised interaction specifically with DNA, Blagbrough and coworkers have shown that the central tetramethylene portion of the polyamine spermine is crucial in conferring high transfection activity in a series of cholesterol-polyamine conjugates (Geall et al, 1999). Indeed, the tetramethylene portion of spermine may be able to bridge between the complementary strands of DNA, whereas a polyamine with a trimethylene central spacer would only interact with adjacent phosphate groups on the same DNA strand. The central tetramethylene portion may equally be found in branched polyamino headgroups such as that of the multivalent lipid MVL5 (N^1-[2-((1S)-1-[3-amino propyl)amino]-4-[di(3aminopropyl)amino]butylcarbox amido)ethyl]-3,4-di[oleyloxybenzamide) (**Figure 5**) which can afford the inclusion of additional protonation sites as the problems of linear polyamine folding encountered by Ohwada and coworkers are conveniently avoided (Byk et al, 1998; Ewert et al, 2002). Thus the resulting lipoplexes can achieve the same charge density with lesser amounts of the cationic lipid in the formulation. Accordingly, the use of small quantities of multivalent cationic lipids is proposed as a simple solution to lessen the problem of cationic lipid-associated cytotoxicity.

The incorporation of natural moieties in headgroup design has recently been extended by ourselves to the family of aminoglycoside antibiotics. This group of natural compounds is characterised by oligosaccharides decorated with up to six amino groups as well as numerous hydroxyl groups, thus providing a versatile polycationic framework (Umezawa and Hooper, 1982).

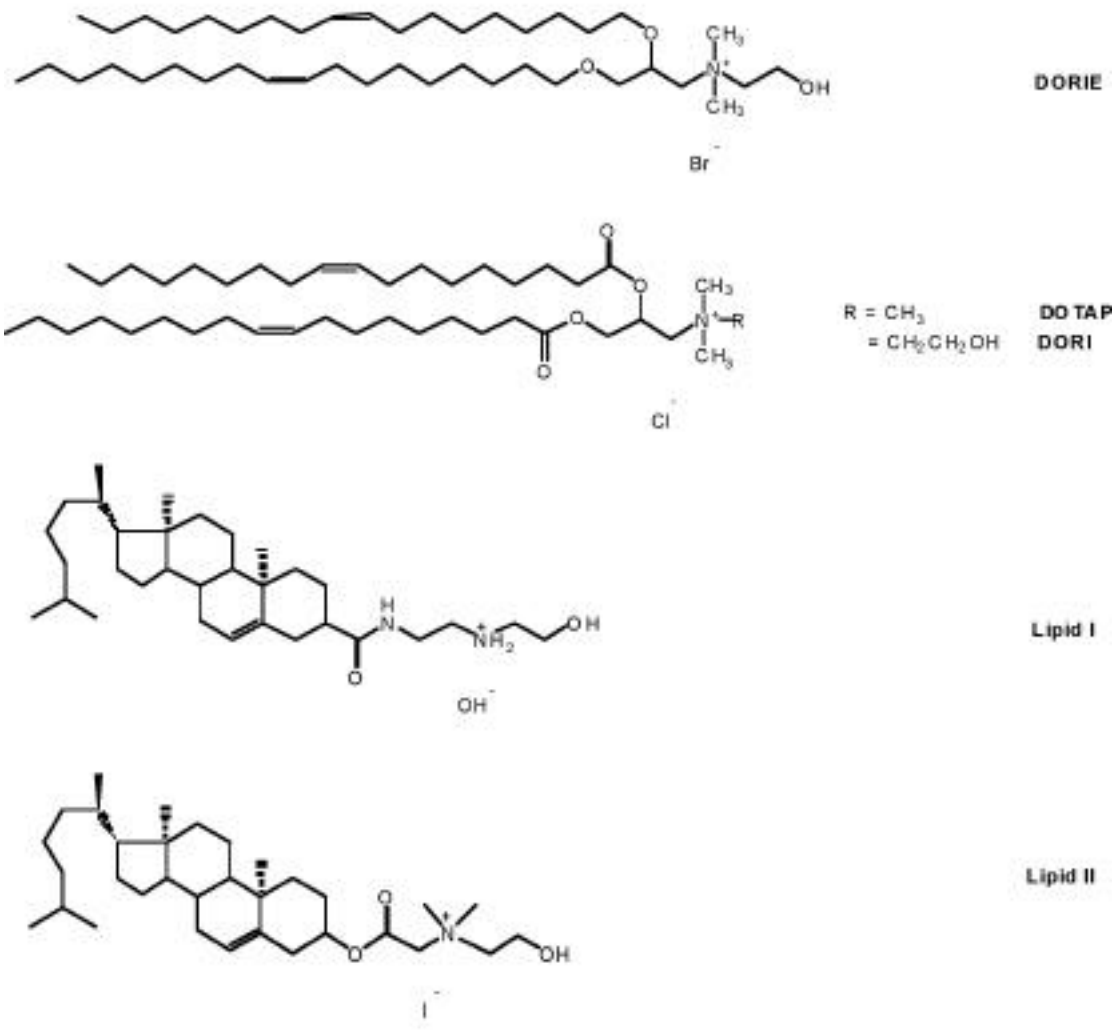


Figure 4: Modification to the headgroup moiety: inclusion of a hydroxyethyl group.

Aminoglycosides have the further advantage of a known interaction with rRNA sequences (Moazed and Noller, 1987; Hendrix et al, 1997). Thus we have recently reported acylation of the 6' amine of kanamycin A, resulting in a series of transfection vectors typified by KanaChol (3β–[6'-kanamycin-carbamoyl]cholesterol) whose structure is shown in **Figure 5** (Sainlos et al, 2003). KanaChol may be triply charged at physiological pH and was found to be highly effective for *in vitro* gene transfection into a variety of mammalian cell lines, especially when formulated with DOPE (Belmont et al, 2002). Gene transfer into the airway epithelium is an active area of research as it could offer treatment for lung diseases both inherited, such as cystic fibrosis, and acquired, such as asthma. Investigation into the usefulness of KanaChol/DOPE liposomes to deliver reporter genes into the mouse airways, confirmed its significant ability to transfect the respiratory epithelium. The applicability of lipid-derivatives of other members of the aminoglycoside family to gene delivery is currently under investigation.

Guanidinium groups are also able to bind DNA phosphate anions, the interaction in this case being characterised by a pair of parallel hydrogen bonds which provide binding strength by their structural organization. In addition, guanidinium groups are highly basic and evidence also exists for hydrogen-bonding to nucleic bases (in particular guanine). Their DNA binding ability should therefore be relatively insensitive to environmental modifications (pH, proximity of other positively charged groups). Further, the group is found naturally in arginine amino acid residues which play a key role in DNA-binding proteins such as histones and protamines. Two bis-guanidinium cholesterol derivatives have been synthesised and tested within our group: BGSC (bis-guanidinium-spermidine-cholesterol) and BGTC (bis-guanidinium-tren-cholesterol) (Vigneron et al, 1996). The structure of BGTC is shown in **Figure 5**. Both vectors were found to be highly efficient for gene transfection *in vitro* into a variety of mammalian cell lines when formulated as cationic liposomes with DOPE. In addition, as BGTC was found to be soluble in aqueous medium, it could be successfully used for transfection when formulated without DOPE. Further studies confirmed the ability of BGTC/DOPE liposomes to efficiently transfect airway epithelial cells *in vivo* (Oudrhiri et al, 1997). Interestingly however, per-guanylation of the amino groups of KanaChol caused a significant drop in transfection activity (Belmont et al, 2002).

Finally, the ability of the four natural cationic amino acids to function as headgroups has been assessed (Heyes et al, 2002). Tryptophan as headgroup caused aggregation during formulation and would not form liposomes, whereas the histidine headgroup yielded moderate levels of transfection, with the lysine and arginine headgroups leading to highly efficient gene delivery. However, not only poly-L-lysine (Gao and Huang, 1993; Monsigny et al, 1994; Wagner, 1998) but also histidine rich polymers and peptides have been identified as efficient gene delivery agents (Pichon et al, 2001; Ihm et al, 2003) and recent studies with divalent cationic lipids incorporating a single histidine residue suggest that their transfection efficiency is due to histidine-mediated membrane fusion activity within the endosome (Kumar et al, 2003). Because membrane fusion was only induced at acidic pH, a mechanism whereby protonation of the imidazole group of the histidine led to an increased interaction between the vector and the phospholipids of the anionic endosomal membrane was proposed.

B. Lipid moiety design

It has been shown that the length and type of aliphatic chain incorporated into cationic lipids can significantly affect transfection efficiency and so vectors are often prepared in a series differing in their hydrophobic portion. Results obtained with the vector DMRIE (Felgner et al, 1994) (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide, an analogue of DOTMA), glycine betaine derivatives (Floch et al, 1998), alkyl acyl carnitine esters (Wang et al, 1998), lactic acid derivatives (Laxmi et al, 2001) and bis-ether lipids

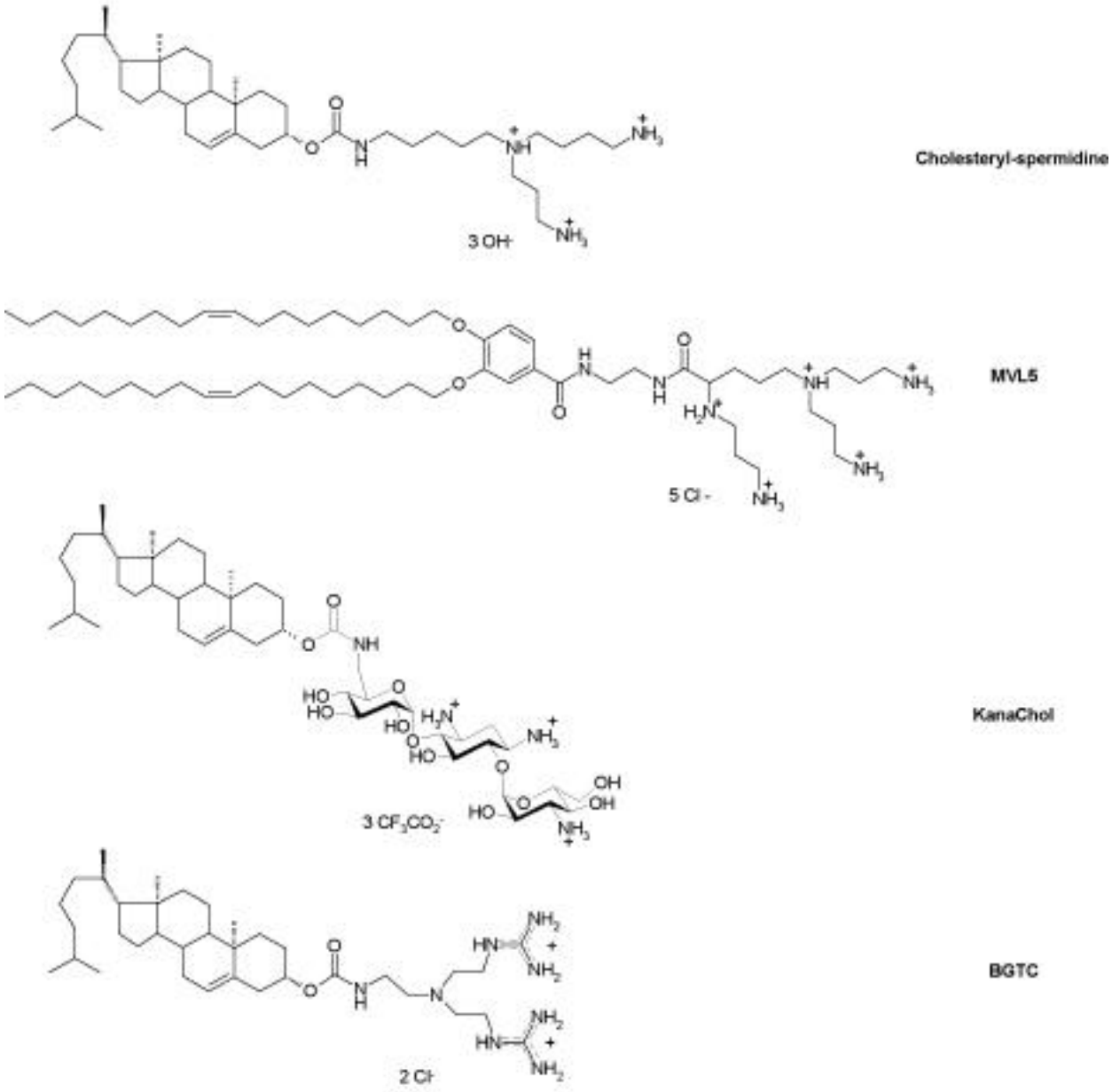


Figure 5: Advances made in headgroup design: multivalent lipids with recognised modes of nucleic acid binding.

related to DOTAP (Heyes et al, 2002), have shown that a comparison of vectors based solely on the lengths of the two saturated aliphatic chains led to identify the order $C_{14}>C_{16}>C_{18}$ in transfection efficiency evaluated in terms of transgene expression. As explanation, it was suggested that a shorter chain length may facilitate intermembrane mixing, an important factor in endosomal escape (Felgner et al, 1994). A common modification is the use of cis-monounsaturated alkyl chains such as the oleoyl group (C_{18}) which appears to lead to higher levels of transfection than saturated stearyl derivatives (C_{18}) likely related to issues of hydrophobic moiety hydration (see above) (Felgner et al, 1994; Wang et al, 1998). It should be noted here that because of the wide variety of vectors that have been synthesized, it is difficult to make general assumptions based solely on a single aspect of the vector design. For example, the transfection efficiency of the lipopolyamines prepared by Behr and coworkers was seen to be independent of chain saturation (oleoyl *vs* stearyl lipopolyamines) (Remy et al, 1994), and when comparing saturated chains, Scherman and coworkers found C_{18} chains to be optimal with decreased efficiency on gradual shortening (Byk et al, 1998).

The use of two aliphatic chains of different length, has been assessed and suggests that C_{12}/stearyl or C_{12}/oleoyl combinations may convey the vector with a more fusogenic character advantageous for endosomal escape (Balasubramaniam et al, 1996; Heyes et al, 2002). Micelles are expected to form when using vectors containing either two short alkyl chains of equal length (such as the C_8 chain homologue of the C_{12} lipopolyamine DOGS (**Figure 3**)), or just a single aliphatic chain (such as the lipopolyamine $C_{18}Sper^{3+}$ (N'-octadecylsperminecarboxamide hydrofluoroacetate)) (Remy et al, 1994). However, such micellar vectors were not found to transfect mammalian cell lines. Ongoing research focuses on the use of single-chained cationic detergents capable of dimerisation via oxidation (Lleres et al, 2001; Zuber et al, 2003).

Finally, because of the rigidity of cholesterol, as well as its endogenous biodegradability and fusion activity, it has been often used as an alternative to aliphatic chains, especially when lipoplexes with a high degree of physical stability are required as for aerosol delivery. Covalently bound cholesterol was first included as the hydrophobic portion of the vector DC-Chol by the Huang group (Gao and Huang, 1991) (**Figure 3**), and then subsequently with our own lipids BGTC and KanaChol and their analogues (see above). Finally, cholesterol can be used as a neutral colipid alternative to DOPE in the formulation of cationic liposomes.

C. Linker design

Stable linking of the hydrophobic and hydrophilic portions of cationic lipids is commonly achieved using carbamate, amide, ester or ether bonds with no particular group emerging as consistently optimal in structure-activity studies across different vector types. A balanced choice must be made between the stability of the vectors mediated by the linking bond, and their toxicity which may be related to the half-life of the vector in the cell. Although inconsistencies exist, it is generally agreed that ether linked vectors (Ghosh et al, 2000) are particularly stable, but as such are expected to be more toxic than ester linked lipids which may be more easily cleaved within the cell and so correspondingly less toxic (Gao and Huang, 1995). Carbamates in particular are thought to achieve a suitable balance between stability and toxicity, and as such are often used in vector design (Gao and Huang, 1991; Vigneron et al, 1996; Aissaoui et al, 2002).

Of emerging interest however is the use of linkers incorporating functional groups that are cleavable on shorter time scales and under specific stimuli such that DNA release may be facilitated by a triggered decomplexation mechanism. Cleavable vectors have thus been designed that are sensitive to stimuli such as decrease in pH, change in redox potential and, recently, photo-sensitivity (Nagasaki et al, 2003). Clearly biological stimuli occurring post-internalisation of the lipoplexes are of special interest and therefore only the advances in linker design that incorporate pH and redox sensitivity will be discussed here. The incorporation of unstable linkers into the neutral colipid rather than the cationic (vectoring) lipid is a complementary approach that will not be covered, though a comprehensive review has recently been published which covers this topic (Guo and Szoka, 2003). Before detailing the methods by which cationic lipids can be made to facilitate intracellular release of DNA, it should be stressed that the use of degradable amphiphiles may be associated with reduced cellular toxicity when compared with lipids with a more chemically stable linker.

1. pH-sensitive linkers

It is generally agreed that lipoplexes are taken up by cells via an endosomal pathway (stage 2, **Figure 2**). Evidence suggests that the poor levels of transfection activity attained by non-viral vectors are in part due to an inefficiency in escaping the endosome (stage 3, **Figure 2**) before degradation of the DNA by nucleases in the late endosomes and lysosomes (barred arrow in **Figure 2**) (Zabner et al, 1995). Upon internalisation, the pH of the endosome, which is initially that of the extracellular fluids (pH 7.2-7.4), is lowered to approximately 5.0 by the action of ATP-dependent proton pumps located in the endosomal membrane (Mukherjee et al, 1997). By incorporating an acid-sensitive functional group into the linker between the hydrophobic and hydrophilic moieties, it may therefore be hoped that the pH drop will act as a trigger, cleaving hydrophobic and hydrophilic portions of the lipoplex, and thereby destabilising the lipoplex structure. Thus, if the resulting DNA decomplexation would be concomitant with endosomal membrane destabilisation (by remaining intact cationic lipids or a colipid such as DOPE), release of the DNA into the cytosol should be enhanced and consequently transfection efficiency might be improved.

Boomer et al, have reported the synthesis of the cationic lipid BCAT (O-(2R-1,2-di-O-(1 Z, 9Z-octadecadienyl)-glycerol) -3- N- (bis-2-aminoethyl) carbamate) incorporating acid-sensitive vinyl ether groups (Boomer et al, 2002) (**Figure 6**). The vector undergoes

complete hydrolysis in acidic solution and was found to effect higher levels of transgene expression than a non-hydrolysable control cationic lipid. The hydrolytically less stable ortho ester bond has also been integrated into the structure of a cationic lipid by Zhu et al, who reported the successful gene delivery by the hydrolysable trioxabicyclo[2,2,2]octane containing cationic lipid III of **Figure 6** (Zhu et al, 2000).

The acid-sensitive acylhydrazone group has been much used as a linker between antineoplastic drugs (doxorubicin, daunorubicin) and carriers (antibodies, serum albumin, transferrin, polyethylene glycol) with the aim of reducing unwanted drug toxicity (Mueller et al, 1990; Kaneko et al, 1991; Kratz et al, 1997). We have recently introduced an acylhydrazone group between a steroid moiety and a bis-guanidinium headgroup for the purposes of gene delivery. The vector undergoes hydrolytic cleavage in acidic solution and was found to be relatively stable at physiological pH. Transfection activity has been confirmed *in vitro* and the vector showed low cytotoxicity likely due to the unstable bond. Further, our acylhydrazone cationic lipid was found to be tolerant to serum and showed significant gene transfection efficiency into mouse lungs (Aissaoui et al, submitted for publication).

2. Redox-sensitive linkers

Another class of triggerable vectors are the redox potential-sensitive lipids. These vectors work on the principle that the lipoplex, once internalised into the cells, is presented with a relatively high concentration of reductive substances, such as glutathione present in concentrations of up to 10 mM (Meister and Anderson, 1983), and reducing enzymes including thioredoxin and glutaredoxin (Saito et al, 2003). On incorporation of disulphide bonds into the vector structure, cleavage of the group is expected to coincide with exposure of the lipoplex to the reducing environment of the cytoplasm, destabilising the complex and leading to DNA release in much the same way as pH-sensitive systems. Tang and Hughes reported the synthesis of the disulphide-containing ornithine conjugate DOGSDSO (1,2-dioleoyl-sn-glycero-3-succinyl-2-hydroxyethyl disulfide ornithine), shown in **Figure 7**, which can be cleaved by dithiothreitol with concomitant release of plasmid DNA (Tang and Hughes, 1998). *In vitro* testing demonstrated a 6 to 15-fold increase in transfection compared to DOTAP, depending on the cell line used, and up to 50-fold enhanced transfection compared to a non-cleavable analogue. Increased sensitivity of the disulphide linker was achieved using dithiodiglycolic acid to tether the polar and hydrophobic domains, such that the less reducing but endogenous glutathione could induce cleavage of CHDTAEA (cholesteryl hemidithiodiglycolyl tris(aminoethyl)amine) (**Figure 7**) (Tang and Hughes, 1999). Interestingly, the increased sensitivity to cleavage rendered the lipid non-cytotoxic. Redox-sensitive cationic lipids are a developing branch of triggerable non-viral vector as the mechanism of disulphide reduction is as yet not fully understood. For example, endosomal cleavage has only recently been recognised with the discovery of the reducing enzyme GILT (gamma-interferon-inducible lysosomal thiol reductase) which is the first to be primarily located in the endosomal pathway (Phan et al, 2000).

Environment sensitive or 'triggerable' cationic lipids represent the first generation of a new approach to gene delivery by non-viral vectors. With the incorporation of a 'triggerable' function, direct parallels can be drawn with viral vectors which themselves exploit the acidification of the endosome and the reducing environment of the cytoplasm (Goff, 2001; Meier and Greber, 2003). Connections are indeed being established between viral and non-viral gene therapy with the inclusion of the virus-derived EALA and GALA fusogenic peptides into multimodular formulations with cationic lipids. Endosomal membrane destabilisation by these systems has been seen to occur in response to the drop in pH, a conformational change from random coil to α-helix being induced in the protein (Parente et al, 1990; Parente et al, 1990; Gottschalk et al, 1996; Vogel et al, 1996). However, regardless of whether dealing with viral or non-viral mediated gene transfer, it is clear that the environment-responsive function must be sensitive enough to be triggered at the correct time during the trafficking process with avoidance of premature or late responses. If more than one trigger is included, as is certainly the case with viruses, then the sequence of transitions must take place chronologically (Lehn et al, 1998). Thus the development of highly 'sophisticated' cationic lipid-based gene delivery systems may be viewed as 'programmed supramolecular systems' obtained via a defined plan, the information necessary for the assembly process to take place and the

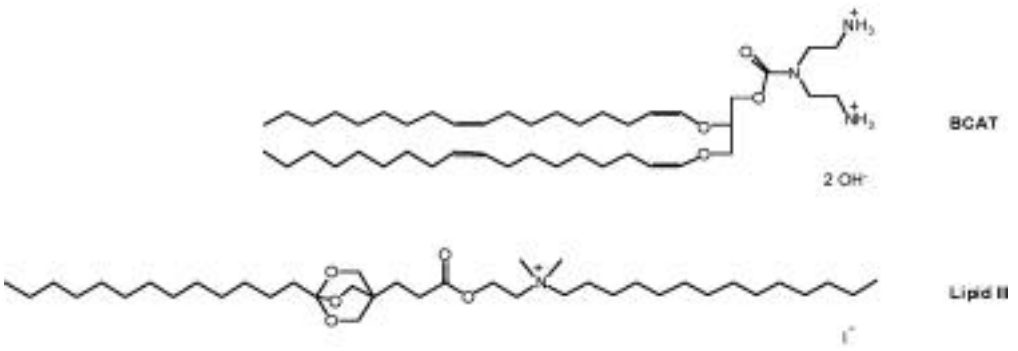


Figure 6: The use of acid-sensitive linkers in cationic lipid design.

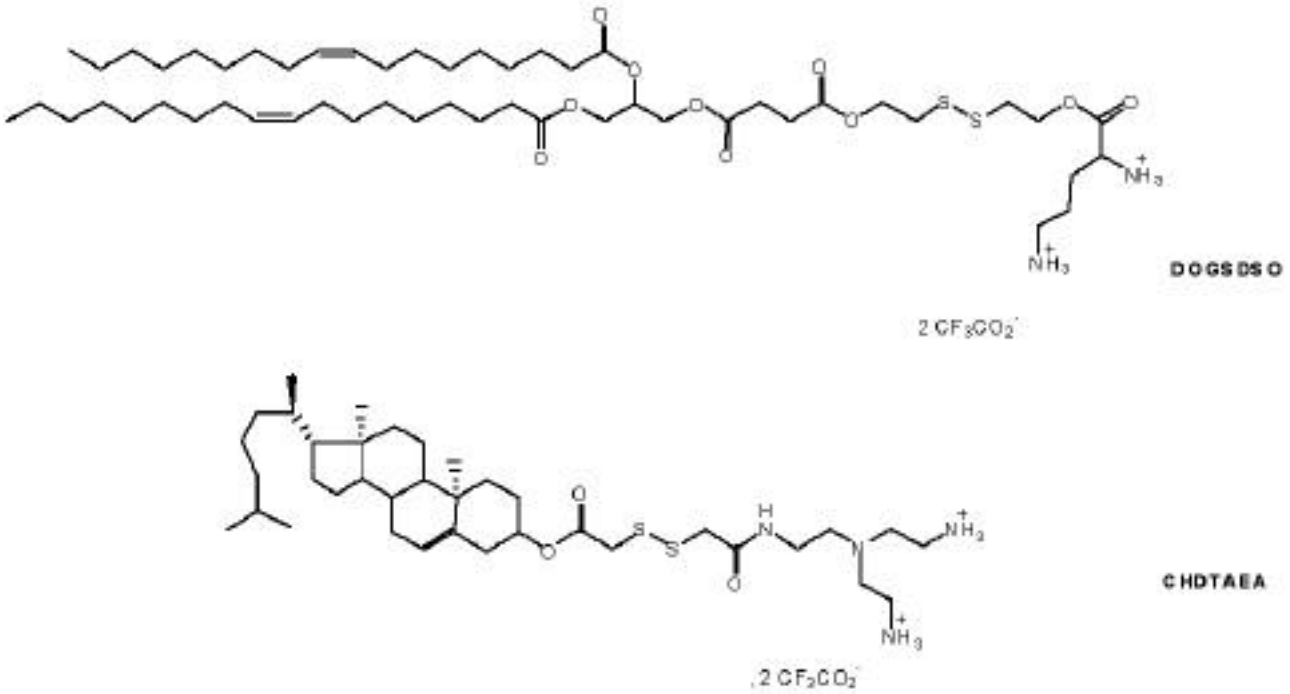


Figure 7: The use of redox-sensitive linkers in cationic lipid design.

algorithm that it follows being stored in the components and operating via selective molecular recognition events (Lehn, 1993).

IV. Structural features of supramolecular assemblies formed by lipoplexes

As gene transfection by cationic lipids involves the spontaneous formation of discrete lipid/DNA particles, it may be expected that the transfection efficiency of a given cationic lipid system depends not only on the properties (at the molecular level) of the cationic lipid itself, but also on the structural and functional characteristics of the self-assembled supramolecular assemblies formed by the lipoplexes. Because of the extensive characterisation that we have undertaken on the BGTC-based lipoplexes prepared by our group (Pitard et al, 1999), the structural features of these assemblies will be summarised here and compared with the results obtained by others when using other cationic lipid systems.

The structural features of BGTC-based lipoplexes were visualised by cryotransmission electron microscopy (cryo-TEM) which has the advantage of imaging bioassemblies close to their native state. With the additional perspective given by data from synchrotron small-angle x-ray scattering (SAXS), precise structural details could be resolved. The results obtained indicated the presence of highly ordered multilamellar domains with a regular spacing of 70 _ and 68 _ in BGTC/DOPE/DNA and BGTC/DNA lipoplexes, respectively (Pitard et al, 1999). It can be assumed from this data that the DNA is intercalated between the lipid bilayers. Interestingly, DNA lipoplexes with similar lamellar patterns were also detected inside transfected HeLa cells by conventional electron microscopy and as such may be the "active" lipoplexes in the process of trafficking. Further, these lamellar lipoplexes were mainly detected in intracellular vesicles, a finding suggesting that endocytosis was the major route of uptake.

Characterisation of the structural features of lipoplexes used for gene transfection has always been an active area of research. A hypothetical "bead on the string" model in which unmodified cationic liposomes were distinctly attached to the DNA was originally proposed (Felgner and Ringold, 1989). Over the years, various electron microscopy techniques have then been used to visualise the structures of the lipoplexes formed by different cationic lipids. These studies essentially suggested that the DNA was entrapped in condensed structures formed by interrelated lipid fusion and DNA collapse for which thermodynamic models have been proposed in order to facilitate analysis and understanding of the process of particle formation (Gershon et al, 1993; Ahearn and Malone, 1999). These condensed structures were found to exhibit various ordered patterns of supramolecular organisation, including multilamellar structures and direct or inverted hexagonal packing (Gustafsson et al, 1995; Labat-Moleur et al, 1996; Lasic et al, 1997; Lasic et al, 1998). Of particular note is the study of DC-Chol/DOPE/DNA lipoplexes where in addition to concentric lamellar structures, tubular "spaghetti-like" structures consisting of DNA rods coated by a single lipid bilayer were observed (Sternberg et al, 1994). As concerns x-ray diffraction studies, lamellar domains with a periodicity similar to that found in BGTC lipoplexes were observed with DOTAP/DOPC (dioleoyl phosphatidylcholine, a structural analogue of DOPE), DDAB and lipopolyamines (Lasic et al, 1997; Pitard et al, 1997; Radler et al, 1997; Safinya and Koltover, 1999). However, a study has shown that DOTAP/DOPE/DNA

lipoplexes have an inverted hexagonal organization which was attributed to the presence of DOPE (as a multilamellar ordering was found when DOPC was used instead of DOPE) and it was further suggested that such an inverted hexagonal arrangement favoured endosomal escape of the lipoplexes via its bilayer-destabilising activity (Koltover et al, 1998).

In conclusion, it should be emphasised here that elucidation of the structural and functional features of the "active" lipoplexes may require the determination not only of their initial features but also of the structural changes that may be induced in the lipoplex by the environments that are encountered during trafficking to the nucleus. For example, it has been demonstrated that an interaction with the microtubules is involved in the cellular trafficking of the lipoplex. Fluorescently tagged lipid I/DOPE lipoplexes (**Figure 4**) were found to be co-localised with microtubules using laser scanning confocal microscopy in cells expressing fluorescently tagged tubulin (Hasegawa et al, 2001). Equally, live-cell real-time multiple particle tracking has been used to observe the trafficking of the cationic polymer polyethylenimine (PEI) (Boussif et al, 1995) through the cell, presumably by the same process (Suh et al, 2003). These results provide a striking parallel with virus-mediated uptake which is known to be 'directed' through the cytoplasm (Tang et al, 1999; Goff, 2001; Seisenberger et al, 2001) rather than relying on random diffusion, which was expected to be the only mode of transport available to non-viral vector/DNA complexes.

V. Inclusion of ligands for nuclear uptake

As vectors have become increasingly sophisticated, it has become clearer that significant advances can only be made if cationic lipid design also addresses the most considerable barrier to gene delivery: the passage across the nuclear membrane. Indeed, experiments comparing transgene expression after microinjection of plasmid DNA either into the cytoplasm or directly into the nucleus have identified the nuclear membrane as being a severely limiting step in transgene delivery (Capecchi, 1980; Zabner et al, 1995; Escriou et al, 1998). Accordingly, the success of the current non-viral vectors may at least partly rely on the disappearance of the nuclear membrane during mitosis, as transfection is generally found to depend on the proliferation status of the cells (Fasbender et al, 1997; Oudrhiri et al, 1997; Mortimer et al, 1999; Escriou et al, 2001). As such it is suggested that gene delivery during interphase is likely to be low. When adding to this the relatively instability of plasmid DNA in the cytoplasm (probably due to degradation by Ca^{2+}-sensitive nucleases (Lechardeur et al, 1999; Pollard et al, 2001), the need for an efficient method of crossing the nuclear membrane becomes evident.

The nuclear membrane consists of two concentric lipid bilayers which make contact at numerous points, forming aqueous pores through the two membranes, which are termed the nuclear pore complexes (NPCs) and have an internal diameter of roughly 9 nm (Mazzanti et al, 2001). These are the only known route for direct exchange of substances between the cytoplasm and the intranuclear compartment. Consequently, the transport of large molecules such as plasmid DNA is non-passive and requires signal mediation by shuttling molecules (importins) (Boulikas, 1997; Escriou et al, 2003). The basic peptide from the SV40 large tumor antigen characterised by a PKKKRL amino acid sequence acts as a nuclear localisation signal (NLS) and has been used in attempts to induce nuclear uptake of plasmid DNA. Binding of multiple NLSs to plasmid DNA (Collas et al, 1996; Ciolina et al, 1999; Neves et al, 1999) was found to be bettered by attachment of just a single NLS peptide to one end of a capped linear DNA fragment (Zanta et al, 1999). However, although transfection (mediated by PEI or DOGS) was enhanced by an impressive 10-1000 fold with the single NLS-DNA conjugate, the technique remains only a proof of principle as preparation of such a DNA fragment requires complex engineering and is therefore relatively impractical for gene therapy applications.

An alternative approach to the use of NLSs for nuclear uptake of plasmids is steroid-mediated gene delivery. This technique relies on binding of the transfected plasmid to the glucocorticoid receptors (GRs), which thus stimulated, actively transport the bound plasmid into the nucleus. The steroid dexamethasone was used as a GR-binding motif and so attached to a DNA binder (psoralen) via a short spacer (Rebuffat et al, 2001). The small increase in transfection activity seen in comparison to lipofection of unmodified DNA in dividing cells, became more pronounced (15-40 fold) in non-dividing cells. Importantly, increasing the number of GR targeting molecules attached to the DNA (via psoralen coupling and thus without regiospecificity) led to loss of transgene expression probably due to covalent damage to the reporter gene sequence in the plasmid. These studies were therefore extended, and the preparation of a peptide nucleic acid (PNA) 'clamp' followed, which was demonstrated to link dexamethasone to the DNA at a defined position where transgene expression remained unhindered (Rebuffat et al, 2002).

VI. Extracellular barriers and requirements of *in vivo* lipofection

The applicability of cationic lipids for *in vivo* gene delivery was investigated following their proven efficiency for *in vitro* gene transfection into a great diversity of established cell lines and primary cell cultures. Although some promising results have been reported, such as the transfection of the airway epithelium via the airway passages, numerous studies in animals yielded much less satisfactory results, especially as regards systemic administration (Gao and Huang, 1995; Miller, 1998). Accordingly, in the first gene therapy clinical trials in man with cationic lipids, the lipoplexes were applied to the patients via *in situ* administration such as intranasal instillation or direct intratumoral injection (Hersh and

Stopeck, 1998; Alton et al, 1999; Boucher, 1999; Griesenbach et al, 1999; Davies et al, 2001).

Identifying the numerous environments that the lipoplex is to encounter on passage to the target cells and highlighting the transfection-limiting barriers therein has been a particular priority. Firstly, serum-associated inhibition of lipofection has been reported (Li and Huang, 1996). Indeed, binding of the positive lipoplexes to the negatively charged molecules found in the serum may lead to their neutralisation, thereby hindering the non-specific electrostatic interaction of the lipoplex with the plasma membrane of the target cells. However, serum sensitivity of lipofection was found to be dependent on the type of cationic lipid, as different observations were noted by different investigators including serum resistance and lipoplex charge ratio and size dependence of the inhibitory effect of serum (Brunette et al, 1992; Yang and Huang, 1997; Turek et al, 2000; Almofti et al, 2003). Our own results with guanidinium-based cationic lipids have shown for example that the *in vitro* transfection activity of lipid BGTC, of a bisguanidinylated diacetylene lipid and of an acylhydrazone linked bisguanylated lipid were not decreased in the presence of serum (Patel et al, 2001; Aissaoui et al, submitted for publication).

The second barrier to lipofection *in vivo* is the presence of opsonins in the blood stream which may bind to the positive lipoplexes and trigger their rapid clearance from the blood via uptake by the macrophages of the reticuloendothelial system, thereby leading to a decreased circulation of the lipoplexes and hindering DNA uptake by the target tissues (Gao and Huang, 1995; Li and Huang, 1996; Aissaoui et al, 2002). The components of the complement system are likely to be involved in opsonisation of intravenously administered lipoplexes (Plank et al, 1996). The degree of complement activation has been shown to depend on the type of cationic lipid used, with monovalent lipids being weak activators. However, incubation of the cationic vectors with DNA to form complexes was found to reduce complement activation, as was coating the DNA complexes with polyethyleneglycol (PEG) (Plank et al, 1996). Thus by limiting the half-life and the targetability of the lipoplexes, complement activation appears to be a potential barrier for intravenous gene delivery, although it may be minimised by appropriate vector formulation.

A third barrier to *in vivo* lipofection is the extracellular matrix which is present between cells and protects the plasma membrane of the target cells (Felgner, 1999). The extracellular matrix contains negatively charged glycosaminoglycans capable of interacting with, and limiting the diffusion of, positive lipoplexes. It is also noteworthy that DNases present in the serum and the extracellular space could rapidly degrade unprotected DNA.

Finally, it should be stressed here that *in vivo* lipofection usually necessitates the administration of highly concentrated solutions of lipoplexes which should ideally be electrically neutral and carry a specific ligand for receptor-mediated uptake when cell targeting is required. Unfortunately, it is common knowledge that the preparation of such lipoplexes is problematic, as high DNA concentrations and electroneutrality lead to colloidal instability resulting in the flocculation and precipitation of the lipoplexes (Lee and Huang, 1997; Lasic et al, 1998). We and others have reported the use of lipophilic PEG derivatives to sterically stabilise lipoplexes formed at high DNA concentration (Hong et al, 1997; Wheeler et al, 1999; Pitard et al, 2001). Indeed, it has been reported that incorporation of PEG derivatives into liposomes (thereby creating so-called 'stealth' liposomes) resulted in prolonged circulation times in blood, the PEG polymer forming an exclusion barrier around the liposome which hinders its aggregation as well as opsonisation in the presence of serum components (Klibanov et al, 1990; Lasic et al, 1991; Hong et al, 1997; Martin and Boulikas, 1998; Zhang et al, 1999). However, although PEG coatings have been undoubtably useful for the preparation of stabilised particles, recent reports state that the protection offered by the polymer can inhibit both binding to the target cell and, upon internalisation, release of the lipoplex from the endosomal compartment causing PEG concentration-dependent decreases in levels of transfection (Harvie et al, 2000; Shi et al, 2002; Song et al, 2002; Keller et al, 2003). Although careful choice of PEG length is likely to help avoid such inhibition (Mori et al, 1991), alternative options are also being sought such as the preparation of stabilised liposomes which are able to lose their protective coat, in particular after their accumulation at tumor sites (Martin and Boulikas, 1998). Disuphide-linked PEG chains have been reported, which, once cleaved intracellularly, have been shown to rapidly release the liposomal contents and re-enable membrane fusion properties (Kirpotin et al, 1996). Recovered transfection activity has also been achieved by varying the length of the acyl chain connecting a PEG polymer to a ceramide group used to anchor it to the lipsosome, short acyl chains being found to cause rapid dissociation of the PEG coating (Wheeler et al, 1999). In addition, entirely different stabilising agents are proposed based on a single carbohydrate linked to cholesterol. These neoglycolipids have been reported to confer the stabilisation properties of PEGylated systems, but without impairing transfection efficiency (Perouzel et al, 2003).

Finally, targeting ligands such as monoclonal antibodies (Trubetskoy et al, 1992), the iron-carrying protein transferrin (Zenke et al, 1990) and sugars (Wagner, 1998; Fajac et al, 1999) have been shown to confer selective delivery to target cells. For example, lipopolyamine-condensed DNA particles decorated with a triantennary galactose ligand have allowed targeted gene transfer into hepatoma cells (Remy et al, 1995). An interesting concept is use of the PEG coating as a linker to equip the lipoplexes with specific ligands for targeted receptor-mediated gene delivery. It has been shown that coupling of plasminogen to the ends of PEG chains led to long circulation times and effective target binding of the PEG-modified liposomes (Blume et al, 1993). In an elegant study, Behr and coworkers introduce, as an outer envelope to a nanometric pre-condensed lipoplex, a PEG chain which acts as linker between the targeting ligand folic acid at one end and DNA binding agents at the other (Zuber et al, 2003). However, although stability and folate

targeting was confirmed, this supramolecular assembly was not efficient. The inclusion of endosomolytic and nuclear uptake agents to the lipid-bearing nanoparticles is expected to be required for optimisation of such a 'virus-like' gene delivery system (Zuber et al, 2001).

VII. Conclusions

Cationic lipid-mediated gene delivery has now passed from the *in vitro* stage of validation onto the *in vivo* stage, and this with early clinical data emerging. It is clear though that the future of non-viral systems will require an increase in efficiency of gene delivery such that therapeutic levels of gene expression can be attained (Li and Huang, 2000). Because the multiple barriers to non-viral gene delivery are only just becoming clear at the molecular level, the advances in vector design, formulation and modular assembly are at present focused on surmounting single, at most a few, of these barriers in any distinct study. This article was intended to alert the reader to research efforts which have included stabilisation of the lipoplex in the extracellular medium, targeting of a particular cell type by pendant motifs, decomplexation of the transgene in response to the drop in endosomal pH or the reducing environment of the cytosol, and finally trafficking of the transgene to the perinuclear region with ultimately active passage across the nuclear membrane. The future will require the difficult task of incorporating the functions capable of conferring the series of actions described above into a single system, capable of working in a chronological order with unwanted inter-reactivity of the individual functions avoided. Certainly such a multimodular assembly is imaginable as viruses form such highly complex systems. For example, the human adenovirus is of about 90 nm in diameter and infects respiratory epithelial cells. Pendant targeting ligands in the knob of the viral fiber bind to cellular CAR receptors and locally activate α_V integrins which trigger endocytosis. The virus is then delivered to an intracellular compartment, whereupon it rapidly escapes on drop in pH *via* interactions including the binding of the penton base protein to $\alpha_V\beta_5$ integrin. The virus is subsequently transported to the perinuclear region by exploitation of microtubule- and dynein/dynactin-dependent mechanisms, upon which it docks with the nuclear pore receptor CAN/Nup214 and disassembles to allow entry of the 36-kb linear DNA into the nucleus (Meier and Greber, 2003). However, reasonably, the required non-viral gene delivery system is more likely to resemble something between a lipoplex and such a virus, together in structure, complexity and efficacy. The system will be designed to avoid the immunological and toxicological responses which impede virus-mediated transfer and the gap in efficiencies between the two methods may be further narrowed by using a greater number of non-viral transfecting particles of somewhat lesser efficiency. Clearly this does not obviate the advances that need to be made to bring non-viral gene transfer into the therapeutic mainstream, a gradual combination of the current approaches being an indispensable first step. Finally, one might also predict there will be no ultimate 'all purpose' vector, rather each vector having to be individualised according to the clinical setting.

Acknowledgements

This work was supported by the Marie Curie Individual Fellowship Program and grants from Vaincre la Mucoviscidose (Paris, France) and the Association Française contre les Myopathies (Evry, France).

References

Aberle AM, Bennett MJ, Malone RW, Nantz MH (**1996**) The counterion influence on cationic lipid-mediated transfection of plasmid DNA. **Biochim Biophys Acta** 1299, 281-283

Ahearn A, Malone R (**1999**) Models of cationic liposome mediated transfection. **Gene Ther Mol Biol** 4, 159-170

Aissaoui A, Martin B, Kan E, Oudrhiri N, Hauchecorne M, Vigneron J-P, Lehn J-M, Lehn P Novel cationic lipids incorporating an acid-sensitive acylhydrazone linker: synthesis and transfection properties, *submitted for publication*.

Aissaoui A, Oudrhiri N, Petit L, Hauchecorne M, Kan E, Sainlos M, Julia S, Navarro J, Vigneron JP, Lehn JM, Lehn P (**2002**) Progress in gene delivery by cationic lipids: Guanidinium-cholesterol-based systems as an example. **Curr Drug Targets** 3, 1-16

Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H (**2003**) Lipoplex size determines lipofection efficiency with or without serum. **Mol Membr Biol** 20, 35-43

Alton EWFW, Stern M, Farley R, Jaffe A, Chadwick SL, Phillips J, Davies J, Smith SN, Browning J, Davies MG, Hodson ME, Durham SR, Li D, Jeffery PK, Scallan M, Balfour R, Eastman SJ, Cheng SH, Smith AE, Meeker D, Geddes DM (**1999**) Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. **Lancet** 353, 947-954

Anderson WF (**1998**) Human gene therapy. **Nature** 392, 25-30

Balasubramaniam RP, Bennett MJ, Aberle AM, Malone JG, Nantz MH, Malone RW (**1996**) Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. **Gene Ther** 3, 163-172

Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J (**1989**) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. **Proc Natl Acad Sci U S A** 86, 6982-6986

Belmont P, Aissaoui A, Hauchecorne M, Oudrhiri N, Petit L, Vigneron J-P, Lehn J-M, Lehn P (**2002**) Aminoglycoside-derived cationic lipids as efficient vectors for gene transfection in vitro and in vivo. **J Gene Med** 4, 517-526

Bennett MJ, Aberle AM, Balasubramaniam RP, Malone JG, Malone RW, Nantz MH (**1997**) Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. **J Med Chem** 40, 4069-4078

Blume G, Cevc G, Crommelin MDJA, Bakker-Woudenberg IAJM, Kluft C, Storm G (**1993**) Specific targeting with polyethylene glycol-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. **Biochim Biophys Acta** 1149, 180-184

Boomer JA, Thompson DH, Sullivan SM (**2002**) Formation of Plasmid-Based Transfection Complexes with an Acid-Labile Cationic Lipid: Characterization of in Vitro and in Vivo Gene Transfer. **Pharm Res** 19, 1292-1301

Boucher RC (**1999**) Status of gene therapy for cystic fibrosis lung disease. **J Clin Invest** 103, 441-445

Boulikas T (**1997**) Nuclear localization signal peptides for the import of plasmid DNA in gene therapy (Review). **Int J Oncol** 10, 301-309

Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP (**1995**) A Versatile Vector for Gene and Oligonucleotide Transfer into Cells in Culture and in-Vivo - Polyethylenimine. **Proc Natl Acad Sci U S A** 92, 7297-7301

Brunette E, Stribling R, Debs R (**1992**) Lipofection does not require the removal of serum. **Nucleic Acids Res** 20, 1151

Byk G, Dubertret C, Escriou V, Frederic M, Jaslin G, Rangara R, Pitard B, Crouzet J, Wils P, Schwartz B, Scherman D (**1998**) Synthesis, Activity, and Structure-Activity Relationship Studies of Novel Cationic Lipids for DNA Transfer. **J Med Chem** 41, 224-235

Capecchi MR (**1980**) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. **Cell** 22, 479-488

Ciolina C, Byk G, Blanche F, Thuillier V, Scherman D, Wils P (**1999**) Coupling of Nuclear Localization Signals to Plasmid DNA and Specific Interaction of the Conjugates with Importin a. **Bioconjug Chem** 10, 49-55

Collas P, Husebye H, Alestrom P (**1996**) The nuclear localization sequence of the SV40 T antigen promotes transgene uptake and expression in zebrafish embryo nuclei. **Transgenic Res** 5, 451-458

Crystal RG (**1995**) Transfer of genes to humans: early lessons and obstacles to success. **Science** 270, 404-410

Davies JC, Geddes DM, Alton EW (**2001**) Gene therapy for cystic fibrosis. **J Gene Med** 3, 409-417

Demeneix BA, Behr J-P. The Proton Sponge: A Trick the Viruses Did Not Exploit. In: Felgner PL, Heller, H.J., Lehn, P., Behr, J.-P., Szoka, Jr.F.C. Eds (ed). *Artificial Self-Assembling Systems for Gene Delivery*. American Chemical Society, Washington, DC, USA, 1996, pp. 146-151.

Ellens H, Bentz J, Szoka FC (**1986**) Fusion of phosphatidylethanolamine-containing liposomes and mechanism of La-HII phase transition. **Biochemistry** 25, 4141-4147

Escriou V, Carriere M, Bussone F, Wils P, Scherman D (**2001**) Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. **J Gene Med** 3, 179-187

Escriou V, Carriere M, Scherman D, Wils P (**2003**) NLS bioconjugates for targeting therapeutic genes to the nucleus. **Adv Drug Deliv Rev** 55, 295-306

Escriou V, Ciolina C, Helbling-Leclerc A, Wils P, Scherman D (**1998**) Cationic lipid-mediated gene transfer: analysis of cellular uptake and nuclear import of plasmid DNA. **Cell Biol Toxicol** 14, 95-104

Ewert K, Ahmad A, Evans HM, Schmidt H-W, Safinya CR (**2002**) Efficient synthesis and cell-transfection properties of a new multivalent cationic lipid for nonviral gene delivery. **J Med Chem** 45, 5023-5029

Fajac I, Briand P, Monsigny M, Midoux P (**1999**) Sugar-mediated uptake of glycosylated polylysines and gene transfer into normal and cystic fibrosis airway epithelial cells. **Hum Gene Ther** 10, 395-406

Farhood H, Serbina N, Huang L (**1995**) The Role of Dioleoyl Phosphatidylethanolamine in Cationic Liposome-Mediated Gene-Transfer. **Biochim Biophys Acta** 1235, 289-295

Fasbender A, Zabner J, Zeiher BG, Welsh MJ (**1997**) A low rate of cell proliferation and reduced DNA uptake limit cationic lipid-mediated gene transfer to primary cultures of ciliated human airway epithelia. **Gene Ther** 4, 1173-1180

Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, Ramsey P, Martin M, Felgner PL (**1994**) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. **J Biol Chem** 269, 2550-2561

Felgner PL. Progress in gene delivery research and development. In: Huang L, Hung, M.C., Wagner, E., Eds (ed). *Nonviral Vectors for Gene Therapy*. Academic Press, San Diego, USA, 1999, pp. 25-38.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (**1987**) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. **Proc Natl Acad Sci U S A** 84, 7413-7417

Felgner PL, Ringold GM (**1989**) Cationic liposome-mediated transfection. **Nature** 337, 387-388

Fichert T, Regelin A, Massing U (**2000**) Synthesis and transfection properties of novel non-toxic monocationic lipids. Variation of lipid anchor, spacer and head group structure. **Bioorg Med Chem Lett** 10, 787-791

Floch V, Legros N, Loisel S, Guillaume C, Guilbot J, Benvegnu T, Ferrieres V, Plusquellec D, Ferec C (**1998**) New biocompatible cationic amphiphiles derivative from glycine betaine: a novel family of efficient nonviral gene transfer agents. **Biochem Biophys Res Commun** 251, 360-365

Friend DS, Papahadjopoulos D, Debs RJ (**1996**) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. **Biochim Biophys Acta** 1278, 41-50

Fujiwara T, Hasegawa S, Hirashima N, Nakanishi M, Ohwada T (**2000**) Gene transfection activities of amphiphilic steroid-polyamine conjugates. **Biochim Biophys Acta** 1468, 396-402

Gao X, Huang L (**1991**) A novel cationic liposome reagent for efficient transfection of mammalian cells. **Biochem Biophys Res Commun** 179, 280-285

Gao X, Huang L (**1993**) Cationic liposomes and polymers for gene transfer. **J Lip Res** 3, 17-30

Gao X, Huang L (**1995**) Cationic liposome-mediated gene transfer. **Gene Ther** 2, 710-722

Geall AJ, Eaton MAW, Baker T, Catterall C, Blagbrough IS (**1999**) The regiochemical distribution of positive charges along cholesterol polyamine carbamates plays significant roles in modulating DNA binding affinity and lipofection. **FEBS Lett** 459, 337-342

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

Ghosh YK, Visweswariah SS, Bhattacharya S (**2000**) Nature of linkage between the cationic headgroup and cholesteryl skeleton controls gene transfection efficiency. **FEBS Lett** 473, 341-344

Goff SP (**2001**) Intracellular trafficking of retroviral genomes during the early phase of infection: viral exploitation of cellular pathways. **J Gene Med** 3, 517-528

Gottschalk S, Sparrow JT, Hauer J, Mims MP, Leland FE, Woo SLC, Smith LC (**1996**) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. **Gene Ther** 3, 448-457

Griesenbach U, Geddes DM, Alton EWFW. Cystic fibrosis gene therapy. In: Huang L, Hung, M.C., Wagner, E., Eds (ed). *Nonviral Vectors for Gene Therapy*. Academic Press, San Diego, USA, 1999, pp. 337-356.

Guenin E, Herve A-C, Floch V, Loisel S, Yaouanc J-J, Clement J-C, Ferec C, Des Abbayes H (**2000**) Cationic phosphonolipids containing quaternary phosphonium and arsonium groups for DNA transfection with good efficiency and low cellular toxicity. **Angew Chem Int Ed** 39, 629-631

Guo X, Szoka FC, Jr. (**2003**) Chemical Approaches to Triggerable Lipid Vesicles for Drug and Gene Delivery. **Acc Chem Res** 36, 335-341

Gustafsson J, Arvidson G, Karlsson G, Almgren M (**1995**) Complexes between cationic liposomes and DNA visualized by cryo-TEM. **Biochim Biophys Acta** 1235, 305-312

Harvie P, Wong FMP, Bally MB (**2000**) Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. **J Pharm Sci** 89, 652-663

Hasegawa S, Hirashima N, Nakanishi M (**2001**) Microtubule involvement in the intracellular dynamics for gene transfection mediated by cationic liposomes. **Gene Ther** 8, 1669-1673

Hasegawa S, Hirashima N, Nakanishi M (**2002**) Comparative study of transfection efficiency of cationic cholesterols mediated by liposomes-based gene delivery. **Bioorg Med Chem Lett** 12, 1299-1302

Hendrix M, Alper PB, Priestley ES, Wong C-H (**1997**) Hydroxyamines as a new motif for the molecular recognition of phosphodiesters: implications for aminoglycoside-RNA interactions. **Angew Chem Int Ed** 36, 95-98

Hersh EM, Stopeck AT. Cancer gene therapy using nonviral vectors: preclinical and clinical observations. In: Kabanov AV, Felgner P.L., Seymour, L.W. Eds (ed). *Self-assembling Complexes for Gene Delivery*. John Wiley and Sons, Chichester, UK, 1998, pp. 421-436.

Heyes JA, Niculescu-Duvaz D, Cooper RG, Springer CJ (**2002**) Synthesis of Novel Cationic Lipids: Effect of Structural Modification on the Efficiency of Gene Transfer. **J Med Chem** 45, 99-114

Hong K, Zheng W, Baker A, Papahadjopoulos D (**1997**) Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. **FEBS Lett** 400, 233-237

Ihm J-E, Han K-O, Han I-K, Ahn K-D, Han D-K, Cho C-S (**2003**) High Transfection Efficiency of Poly(4-vinylimidazole) as a New Gene Carrier. **Bioconjug Chem** 14, 707-708

Kaneko T, Willner D, Monkovic I, Knipe JO, Braslawsky GR, Greenfield RS, Vyas DM (**1991**) New hydrazone derivatives of Adriamycin and their immunoconjugates - a correlation between acid stability and cytotoxicity. **Bioconjug Chem** 2, 133-141

Keller M, Harbottle RP, Perouzel E, Colin M, Shah I, Rahim A, Vaysse L, Bergau A, Moritz S, Brahimi-Horn C, Coutelle C, Miller AD (**2003**) Nuclear localisation sequence templated nonviral gene delivery vectors: Investigation of intracellular trafficking events of LMD and LD vector systems. **Chembiochem** 4, 286-298

Kirpotin D, Hong K, Mullah N, Papahadjopoulos D, Zalipsky S (**1996**) Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). **FEBS Lett** 388, 115-118

Klibanov AL, Maruyama K, Torchilin VP, Huang L (**1990**) Amphipathic polyethylene glycols effectively prolong the circulation time of liposomes. **FEBS Lett** 268, 235-237

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

Kootstra NA, Verma IM (**2003**) Gene therapy with viral vectors. **Annu Rev Pharmacol Toxicol** 43, 413-439

Kratz F, Beyer U, Schumacher P, Kruger M, Zahn H, Roth T, Fiebig HH, Unger C (**1997**) Synthesis of new maleimide derivatives of daunorubicin and biological activity of acid labile transferrin conjugates. **Bioorg Med Chem Lett** 7, 617-622

Kumar VV, Pichon C, Refregiers M, Guerin B, Midoux P, Chaudhuri A (**2003**) Single histidine residue in head-group region is sufficient to impart remarkable gene transfection properties to cationic lipids: evidence for histidine-mediated membrane fusion at acidic pH. **Gene Ther** 10, 1206-1215

Labat-Moleur F, Steffan AM, Brisson C, Perron H, Feugeas O, Furstenberger P, Oberling F, Brambilla E, Behr JP (**1996**) An electron microscopy study into the mechanism of gene transfer with lipopolyamines. **Gene Ther** 3, 1010-1017

Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D (**1991**) Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. **Biochim Biophys Acta** 1070, 187-192

Lasic DD, Papahadjopoulos D, Podgornik R. Polymorphism of lipids, nucleic acids and their interactions. In: Kabanov AV, Felgner P.L., Seymour, L.W. Eds (ed). *Self-assembling Complexes for Gene Delivery*. John Wiley and Sons, Chichester, UK, 1998, pp. 3-26.

Lasic DD, Strey H, Stuart MCA, Podgornik R, Frederik PM (**1997**) The Structure of DNA-Liposome Complexes. **J Am Chem Soc** 119, 832-833

Laxmi AA, Vijayalakshmi P, Balagopala Kaimal TN, Chaudhuri A, Ramadas Y, Rao NM (**2001**) Novel Non-Glycerol-Based Cytofectins with Lactic Acid-Derived Head Groups. **Biochem Biophys Res Commun** 289, 1057-1062

Lechardeur D, Sohn KJ, Haardt M, Joshi PB, Monck M, Graham RW, Beatty B, Squire J, O'Brodovich H, Lukacs GL (**1999**) Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. **Gene Ther** 6, 482-497

Lee RJ, Huang L (**1997**) Lipidic vector systems for gene transfer. **Crit Rev Ther Drug Carrier Syst** 14, 173-206

Lehn J-M (**1993**) Supramolecular chemistry - molecular information and the design of supramolecular materials. **Makromol Chem, Macromol Symp** 69, 1-17

Lehn J-M. Preface. In: Huang L, Hung, M.C., Wagner, E., Eds (ed). *Nonviral Vectors for Gene Therapy*. Academic Press, San Diego, USA, 1999, pp. xxi.

Lehn P, Fabrega S, Oudrhiri N, Navarro J (**1998**) Gene delivery systems: Bridging the gap between recombinant viruses and artificial vectors. **Adv Drug Deliv Rev** 30, 5-11

Li S, Huang L (**1996**) Lipidic supramolecular assemblies for gene transfer. **J Lip Res** 6, 589-608

Li S, Huang L (**2000**) Nonviral gene therapy: promises and challenges. **Gene Ther** 7, 31-34

Lleres D, Dauty E, Behr JP, Mely Y, Duportail G (**2001**) DNA condensation by an oxidizable cationic detergent. Interactions with lipid vesicles. **Chem Phys Lipids** 111, 59-71

Martin F, Boulikas T (**1998**) The challenge of liposomes in gene therapy. **Gene Ther Mol Biol** 1, 173-214

Mazzanti M, Bustamante JO, Oberleithner H (**2001**) Electrical dimension of the nuclear envelope. **Physiol Rev** 81, 1-19

Meier O, Greber UF (**2003**) Adenovirus endocytosis. **J Gene Med** 5, 451-462

Meister A, Anderson ME (**1983**) Glutathione. **Annu Rev Biochem** 52, 711-760

Miller AD (**1998**) Cationic liposomes for gene therapy. **Angew Chem Int Ed** 37, 1769-1785

Miller AD (**2003**) The problem with cationic liposome/micelle-based non-viral vector systems for gene therapy. **Curr Med Chem** 10, 1195-1211

Moazed D, Noller HF (**1987**) Interaction of antibiotics with functional sites in 16S ribosomal RNA. **Nature** 327, 389-394

Monsigny M, Roche A-C, Midoux P, Mayer R (**1994**) Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. **Adv Drug Deliv Rev** 14, 1-24

Moradpour D, Schauer JI, Zurawski J, Vincent R., Wands JR, Boutin RH (**1996**) Efficient Gene Transfer into Mammalian Cells with Cholesteryl-Spermidine. **Biochem Biophys Res Commun** 221, 82-88

Mori A, Klibanov AL, Torchilin VP, Huang L (**1991**) Influence of the steric barrier activity of amphipathic polyethylene glycol and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. **FEBS Lett** 284, 263-266

Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB (**1999**) Cationic lipid-mediated transfection of cells in culture requires mitotic activity. **Gene Ther** 6, 403-411

Mueller BM, Wrasildo WA, Reisfeld RA (**1990**) Antibody conjugates with morpholinodoxorubicin and acid-cleavable linkers. **Bioconjug Chem** 1, 325-330

Mukherjee S, Ghosh RN, Maxfield FR (**1997**) Endocytosis. **Physiol Rev** 77, 759-803

Mulligan RC (**1993**) The basic science of gene therapy. **Science** 260, 926-932

Nagasaki T, Taniguchi A, Tamagaki S (**2003**) Photoenhancement of Transfection Efficiency Using Novel Cationic Lipids Having a Photocleavable Spacer. **Bioconjug Chem** 14, 513-516

Neves C, Byk G, Scherman D, Wils P (**1999**) Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. **FEBS Lett** 453, 41-45

Nicolau C, Le Pape A, Soriano P, Fargette F, Juhel MF (**1983**) In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I. **Proc Natl Acad Sci U S A** 80, 1068-1072

Nicolau C, Sene C (**1982**) Liposome-mediated DNA transfer in eukaryotic cells. Dependence of the transfer efficiency upon the type of liposomes used and the host cell cycle stage. **Biochim Biophys Acta** 721, 185-190

Oudrhiri N, Vigneron JP, Peuchmaur M, Leclerc T, Lehn JM, Lehn P (**1997**) Gene transfer by guanidinium-cholesterol cationic lipids into airway epithelial cells in vitro and in vivo. **Proc Natl Acad Sci U S A** 94, 1651-1656

Parente RA, Nadasdi L, Subbarao NK, Szoka FC, Jr. (**1990**) Association of a pH-sensitive peptide with membrane vesicles: role of amino acid sequence. **Biochemistry** 29, 8713-8719

Parente RA, Nir S, Szoka FC, Jr. (**1990**) Mechanism of leakage of phospholipid vesicle contents induced by the peptide GALA. **Biochemistry** 29, 8720-8728

Patel M, Vivien E, Hauchecorne M, Oudrhiri N, Ramasawmy R, Vigneron JP, Lehn P, Lehn JM (**2001**) Efficient gene transfection by bisguanylated diacetylene lipid formulations. **Biochem Biophys Res Commun** 281, 536-543

Perouzel E, Jorgensen MR, Keller M, Miller AD (**2003**) Synthesis and Formulation of Neoglycolipids for the Functionalization of Liposomes and Lipoplexes. **Bioconjug Chem** 14, 884-898

Phan UT, Arunachalam B, Cresswell P (**2000**) Gamma-interferon-inducible lysosomal thiol reductase (GILT). Maturation, activity, and mechanism of action. **J Biol Chem** 275, 25907-25914

Pichon C, Goncalves C, Midoux P (**2001**) Histidine-rich peptides and polymers for nucleic acids delivery. **Adv Drug Deliv Rev** 53, 75-94

Pitard B, Aguerre O, Airiau m, Lachages A-M, Boukhnikachvili T, Byk G, Dubertret C, Herviou C, Scherman D, Mayaux J-F, Crouzet J (**1997**) Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer. **Proc Natl Acad Sci U S A** 94, 14412-14417

Pitard B, Oudrhiri N, Lambert O, Vivien E, Masson C, Wetzer B, Hauchecorne M, Scherman D, Rigaud JL, Vigneron JP, Lehn JM, Lehn P (**2001**) Sterically stabilized BGTC-based lipoplexes: structural features and gene transfection into the mouse airways in vivo. **J Gene Med** 3, 478-487

Pitard B, Oudrhiri N, Vigneron JP, Hauchecorne M, Aguerre O, Toury R, Airiau M, Ramasawmy R, Scherman D, Crouzet J, Lehn JM, Lehn P (**1999**) Structural characteristics of supramolecular assemblies formed by guanidinium-cholesterol reagents for gene transfection. **Proc Natl Acad Sci U S A** 96, 2621-2626

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

Pollard H, Toumaniantz G, Amos JL, Avet-Loiseau H, Guihard G, Behr JP, Escande D (**2001**) Ca2+-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. **J Gene Med** 3, 153-164

Radler JO, Koltover I, Salditt T, Safinya CR (**1997**) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. **Science** 275, 810-814

Rebuffat A, Bernasconi A, Ceppi M, Wehrli H, Verca SB, Ibrahim M, Frey BM, Frey FJ, Rusconi S (**2001**) Selective enhancement of gene transfer by steroid-mediated gene delivery. **Nat Biotechnol** 19, 1155-1161

Rebuffat AG, Nawrocki AR, Nielsen PE, Bernasconi AG, Bernal-Mendez E, Frey BM, Frey FJ (**2002**) Gene delivery by a steroid-peptide nucleic acid conjugate. **FASEB J** 16, 1426-1428, 1410.1096/fj.1401-0706fje

Remy J-S, Kichler A, Mordvinov V, Schuber F, Behr J-P (**1995**) Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses. **Proc Natl Acad Sci U S A** 92, 1744-1748

Remy J-S, Sirlin C, Vierling P, Behr J-P (**1994**) Gene Transfer with a Series of Lipophilic DNA-Binding Molecules. **Bioconjug Chem** 5, 647-654

Roth JA, Cristiano RJ (**1997**) Gene therapy for cancer: what have we done and where are we going? **J Natl Cancer Inst** 89, 21-39

Safinya CR, Koltover I. I. Self-assembled structures of lipid/DNA nonviral gene delivery systems from synchrotron X-ray diffraction. In: Huang L, Hung, M.C., Wagner, E., Eds (ed). *Nonviral Vectors for Gene Therapy*. Academic Press, San Diego, USA, 1999, pp. 91-117.

Sainlos M, Belmont P, Vigneron J-P, Lehn P, Lehn J-M (**2003**) Aminoglycoside-Derived Cationic Lipids for Gene Transfection: Synthesis of Kanamycin A Derivatives. **Eur J Org Chem** 15, 2764-2774

Saito G, Swanson JA, Lee K-D (**2003**) Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. **Adv Drug Deliv Rev** 55, 199-215

Schmid N, Behr JP (**1991**) Location of spermine and other polyamines on DNA as revealed by photoaffinity cleavage with poly(amino)benzenediazonium salts. **Biochemistry** 30, 4357-4361

Seisenberger G, Ried MU, Endress T, Buening H, Hallek M, Brauchle C (**2001**) Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. **Science** 294, 1929-1932

Shi F, Wasungu L, Nomden A, Stuart Marc CA, Polushkin E, Engberts Jan BFN, Hoekstra D (**2002**) Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. **Biochem J** 366, 333-341

Song LY, Ahkong QF, Rong Q, Wang Z, Ansell S, Hope MJ, Mui B (**2002**) Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes. **Biochim Biophys Acta** 1558, 1-13

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

Suh J, Wirtz D, Hanes J (**2003**) Efficient active transport of gene nanocarriers to the cell nucleus. **Proc Natl Acad Sci U S A** 100, 3878-3882

Takeuchi K, Ishihara M, Kawaura C, Noji M, Furuno T, Nakanishi M (**1996**) Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. **FEBS Lett** 397, 207-209

Tang F, Hughes JA (**1998**) Introduction of a disulfide bond into a cationic lipid enhances transgene expression of plasmid DNA. **Biochem Biophys Res Commun** 242, 141-145

Tang F, Hughes JA (**1999**) Use of Dithiodiglycolic Acid as a Tether for Cationic Lipids Decreases the Cytotoxicity and Increases Transgene Expression of Plasmid DNA in Vitro. **Bioconjug Chem** 10, 791-796

Tang Y, Winkler U, Freed EO, Torrey TA, Kim W, Li H, Goff SP, Morse HC, III (**1999**) Cellular motor protein KIF-4 associates with retroviral gag. **J Virol** 73, 10508-10513

Trubetskoy VS, Torchilin VP, Kennel SJ, Huang L (**1992**) Use of N-terminal modified poly(L-lysine)-antibody conjugate as a carrier for targeted gene delivery in mouse lung endothelial cells. **Bioconjug Chem** 3, 323-327

Turek J, Dubertret C, Jaslin G, Antonakis K, Scherman D, Pitard B (**2000**) Formulations which increase the size of lipoplexes prevent serum-associated inhibition of transfection. **J Gene Med** 2, 32-40

Umezawa H, Hooper IR. *Aminoglycoside Antibiotics.* Springer-Verlag: New York, Heidelburg, **1982**.

Vidal M, Hoekstra D (**1995**) In vitro fusion of reticulocyte endocytic vesicles with liposomes. **J Biol Chem** 270, 17823-17829

Vigneron JP, Oudrhiri N, Fauquet M, Vergely L, Bradley JC, Basseville M, Lehn P, Lehn JM (**1996**) Guanidinium-cholesterol cationic lipids: Efficient vectors for the transfection of eukaryotic cells. **Proc Natl Acad Sci U S A** 93, 9682-9686

Vogel K, Wang S, Lee RJ, Low PS, Chmielewski J (**1996**) Peptide-mediated release of folate-targeted liposome contents from endosomal compartments. **J Am Chem Soc** 118, 1581-1586

Wagner E. Polylysine-conjugate based DNA delivery. In: Kabanov AV, Felgner P.L., Seymour, L.W. Eds (ed). *Self-assembling Complexes for Gene Delivery*. John Wiley and Sons, Chichester, UK, 1998, pp. 309-322.

Wang J, Guo X, Xu Y, Barron L, Szoka FC, Jr. (**1998**) Synthesis and Characterization of Long Chain Alkyl Acyl Carnitine Esters. Potentially Biodegradable Cationic Lipids for Use in Gene Delivery. **J Med Chem** 41, 2207-2215

Wheeler JJ, Palmer L, Ossanlou M, MacLachlan I, Graham RW, Zhang YP, Hope MJ, Scherrer P, Cullis PR (**1999**) Stabilized plasmid-lipid particles: construction and characterization. **Gene Ther** 6, 271-281

Xu YH, Szoka FC (**1996**) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. **Biochemistry** 35, 5616-5623

Yang JP, Huang L (**1997**) Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposome to DNA. **Gene Ther** 4, 950-960

Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ (**1995**) Cellular and Molecular Barriers to Gene-Transfer by a Cationic Lipid. **J Biol Chem** 270, 18997-19007

Zanta MA, Belguise-Valladier P, Behr JP (**1999**) Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. **Proc Natl Acad Sci U S A** 96, 91-96

Zenke M, Steinlein P, Wagner E, Cotten M, Beug H, Birnstiel ML (**1990**) Receptor-Mediated Endocytosis of Transferrin Polycation Conjugates - an Efficient Way to Introduce DNA into Hematopoietic-Cells. **Proc Natl Acad Sci U S A** 87, 3655-3659

Zhang YP, Sekirov L, Saravolac EG, Wheeler JJ, Tardi P, Clow K, Leng E, Sun R, Cullis PR, Scherrer P (**1999**) Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. **Gene Ther** 6, 1438-1449

Zhu J, Munn RJ, Nantz MH (**2000**) Self-Cleaving Ortho Ester Lipids: A New Class of pH-Vulnerable Amphiphiles. **J Am Chem Soc** 122, 2645-2646

Zuber G, Dauty E, Nothisen M, Belguise P, Behr JP (**2001**) Towards synthetic viruses. **Adv Drug Deliv Rev** 52, 245-253

Zuber G, Zammut-Italiano L, Dauty E, Behr J-P (**2003**) Targeted gene delivery to cancer cells: Directed assembly of nanometric DNA particles coated with folic acid. **Angew Chem Int Ed** 42, 2666-2669

Dr. Benjamin Martin