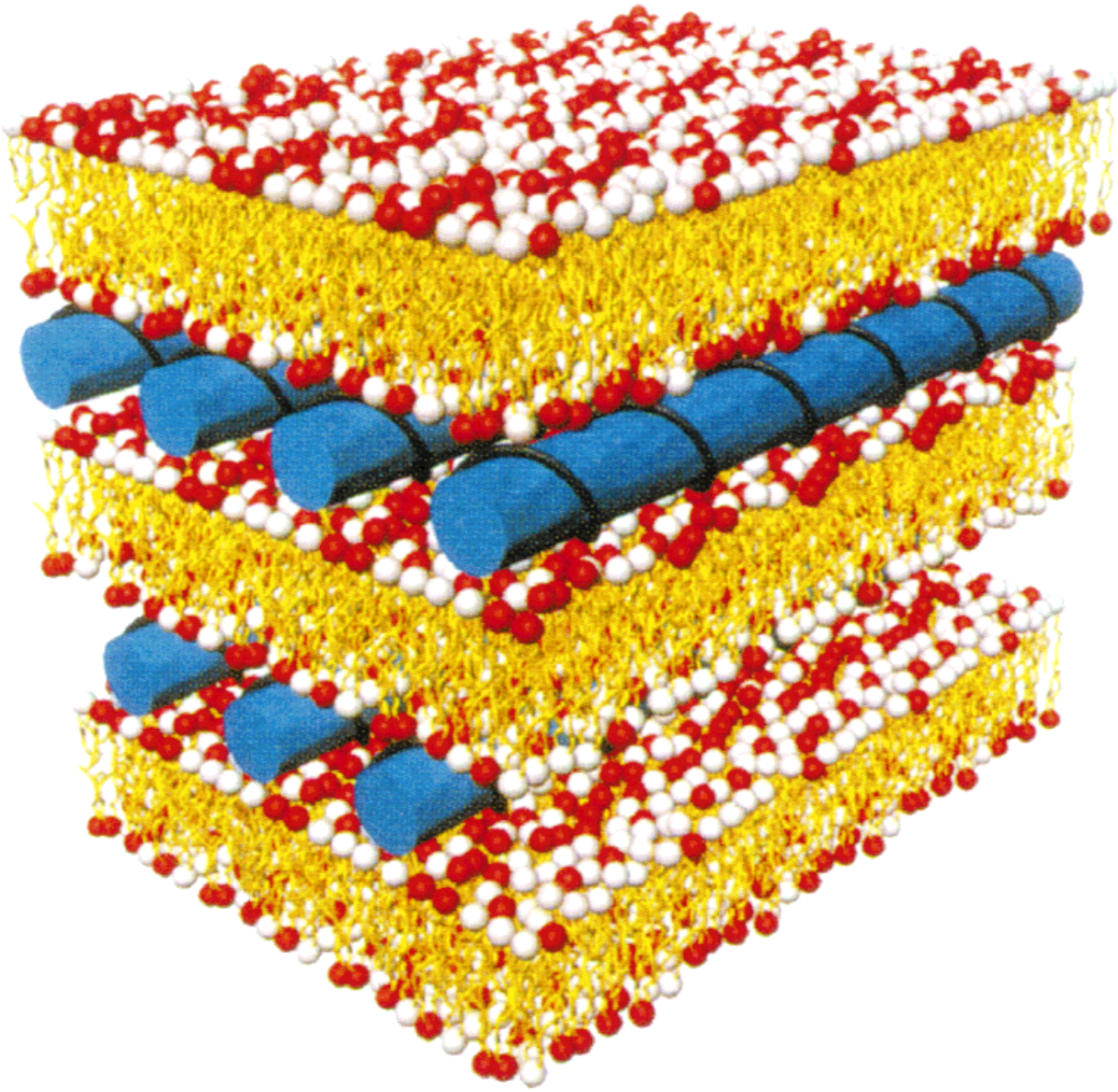
A model for the local arrangement of cationic liposome/DNA complexes—such complexes may be of great importance in the future for gene therapy.



The DNA molecules are represented as blue rods. The head groups of anionic/zwitterionic lipids are shown as white spheres, and those of cytofectins as red spheres (reproduced with permission from J. O. Rädler, I. Koltover, T. Salditt, and C. R. Safinya, *Science* **1997**, *275*, 810–814).

Cationic Liposomes for Gene Therapy**

Andrew D. Miller*

Cationic liposomes are now recognized as a potent means to assist the delivery of genes and other nucleic acids to cells. This holds out the tantalizing possibility that cationic liposomes could play a major role in the future as a core technology in the emerging field of gene therapy. In this area, cationic liposomes could function as vectors (i.e., gene-delivery vehicles) to deliver therapeutic genes and/or other nucleic acids into diseased cells and organs of patients in order to cure or treat disease. How realistic is this? Certainly, cationic liposome mediated delivery of nucleic acids for gene therapy is still fraught with problems. However, there have been some recent spectacular successes. In view of these successes and the relative youth of the field, there does not appear to be any reason why cationic liposomes cannot be refined both now and in the future for general use in gene therapy. What makes the process of cationic liposome refinement and development so exhilarating is the opportunities for partnership between chemistry and medicine. This is a field that requires a multidisciplinary approach with organic, physical-organic, and physical chemists in the vanguard of designing, synthesizing, formulating, and testing ever more potent cationic liposomes. This review illustrates this nascent partnership between chemistry and medicine by describing the chemical origins of cationic liposomes, their physical properties in complexes with nucleic acids, their biological efficacy, and their current limitations.

Keywords: gene therapy · lipids · liposomes · nucleic acids · vesicles

1. Introduction

With the rapid conclusion of the human genome sequencing project, more will be known about the genetic basis of human life than at any other time in history. In addition, the genetic codes of microorganisms, such as the bacterium *Methanococcus jannaschii* and yeast, have now been completely determined, and the genetic codes of further organisms will also shortly be eludicated.[1] All this new genetic information should offer the medical research community an unparalleled opportunity to understand the genetic basis of disease, a very possible consequence of which would be the realization of gene therapy as an integral part of stategies to cure diseases in the future.[2] Gene therapy is not yet a clinical reality, although proof of principle has now been established.[2] Nevertheless, this is a young science, and its proponents, including myself, are quite convinced that gene therapy could change the face of medicine in the next century, with substantial consequences for the way in which the pharmaceutical industry, in particular, conducts itself.

The principle of gene therapy is simple. If a patient is suffering from a disease caused by a known genetic defect, then the delivery of a correct copy of the defective gene to the diseased cells or body organ, by means of a specially designed vector, would be expected to correct directly for the genetic defect and hence cure the disease. Alternatively, if a patient is suffering from a disease for which there is no clear genetic cause but whose pathophysiology is well understood, then the vector might be used to deliver corrective gene(s) or some other nucleic acid agent (such as antisense oligonucleotides or mRNA) to the diseased cells and so disrupt the known disease pathophysiology in some other way. Hence, generally speaking, gene therapy may be defined either as "the use of genes as medicines to treat disease" or "the delivery of nucleic acid (with a vector) to patients for some therapeutic purpose."

It is important to make clear that gene therapy should not be seen as a technology designed to rid future generations of genetic and other more complex diseases by permanent manipulation of the human genome. Instead, genes or other nucleic acid agents should be seen as alternatives to small-molecule drugs which, once delivered to patients, offer the real possibility of curing the disease rather than just treating the symptoms, as is currently the case with all but bacterial infections. If genes or other nucleic acid agents are "drugs",

[*] Dr. A. D. Miller
Imperial College of Science, Technology & Medicine
Department of Chemistry
South Kensington, London, SW7 2AY (UK)
Fax: (+44) 171-594-5803
E-mail: a.miller@ic.ac.uk

[**] A list of frequently used abbreviations is provided in the Appendix.

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then the principle technological hurdle which faces the budding gene therapist is the now familiar challenge of drug delivery. In other words, how do we intend to transport or deliver our therapeutic gene or nucleic acid to the site of disease so that only the diseased cells are entered by the therapeutic agent? The answer will lie ultimately in the appropriate choice of the vector for nucleic acid delivery.

2. Vectors for Nucleic Acid Delivery

At this stage, vectors fall into two broad categories, namely viral or nonviral. Neither category may lay claim to being the universal panacea of choice for nucleic acid delivery since both categories have problems which will need to be solved before clinical gene therapy will become possible. That is, no one vector for nucleic acid delivery to date fulfills the criterion of a perfect vector (i.e., offer precise nucleic acid delivery characteristics with no toxic side effects following use). However, of the current range of nonviral vectors, cationic liposomes (which are the subject of this review) show particular promise and potential as clinically useful vector systems for the delivery of therapeutic nucleic acids to patients.[3, 4] This is a particularly exciting proposition for the organic, physical-organic, or physical chemist who is looking for a role to play at the interface between chemistry and medicine. The preparation and use of cationic liposomes is very much a "problem for chemists" at its heart, as I shall attempt to illustrate here.

2.1. Cationic Liposomes

Cationic liposomes are formed from either a single cationic amphiphile (known as a cytofectin; *cyto-* for cell and *-fectin* for transfection, that is, gene delivery and expression) or more commonly from a combination of a cytofectin and a neutral lipid. The way in which they mediate nucleic acid delivery is simple: Positively charged cationic liposomes interact electrostatically with negatively charged nucleic acid sequences to form complexes which are capable of entering a cell. Once there, nucleic acid sequences are slowly released either to be expressed in the cell nucleus or to control gene expression. This review will emphasize that this seductively simple principle is attended with many difficult problems which as yet have no clear solution. The consequence of these problems is that most of the current cationic liposome formulations are unlikely to have any ultimate clinical use in the future. However, that is not to say that the critical problems of nucleic acid delivery mediated by a cationic liposome cannot be solved. In fact, it is my belief that they will be, with the result that cationic liposome assisted gene therapy should be a clinical reality within the forseeable future.

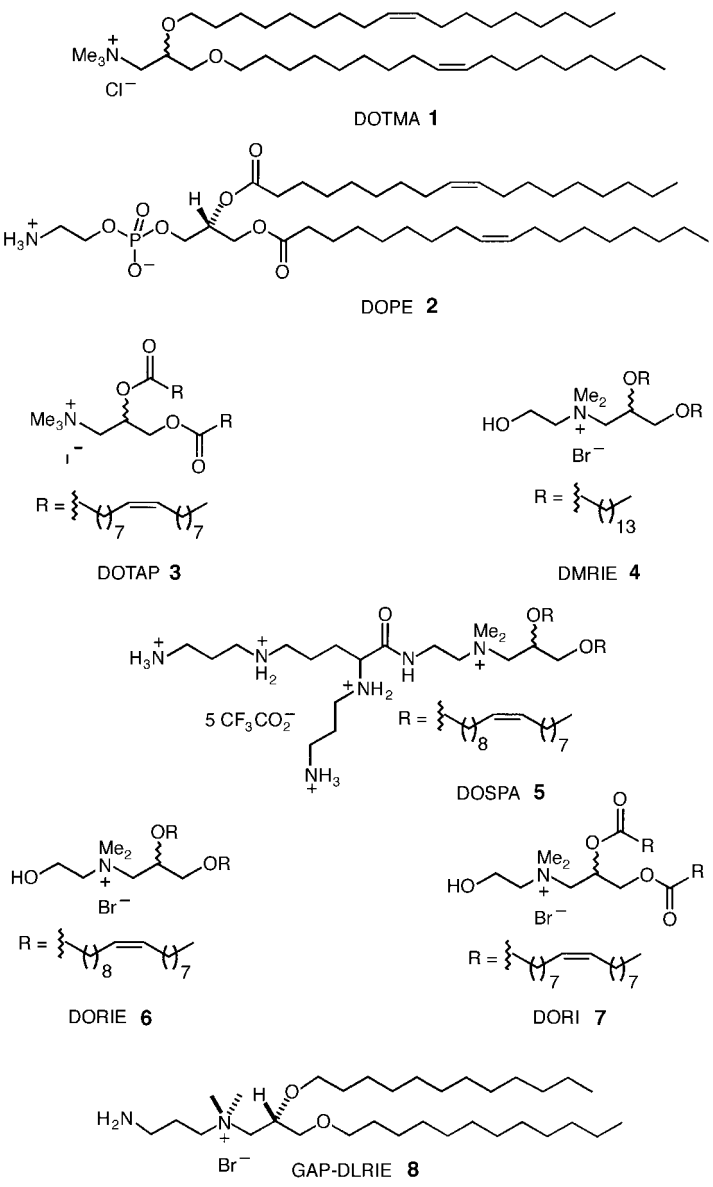
2.2. Other Vectors

A discussion of alternative nonviral vector systems to cationic liposomes, and of course viral vectors themselves, is beyond the scope of this review. Briefly, alternative chemical vectors to cationic liposomes include calcium phosphate precipitation, (diethylamino)ethyldextran, and polybrene (a copolymer from *N*,*N*,*N*′,*N*′-tetramethyl-1,6-hexanediamine and 1,3-dibromopropane; more sophisticated chemical vectors will be touched upon later in this review with respect to the development of cationic liposome systems). Physical methods such as microinjection, electroporation, and biobal-listics are effective, although perhaps of doubtful clinical use with the exception of the last. Finally, of the viral vectors, adeno-viruses (AV), adeno-associated virus (AAV), and retro-viruses have seen considerable use in the context of gene delivery. For more details on all these methods, the interested chemist is referred to general reviews on the use of these vector systems.[5–15]

3. Current Cationic Liposome Formulations

The use of cationic liposomes in nucleic acid delivery was originally pioneered by Felgner and co-workers, who developed the cationic liposome now available commercially as Lipofectin (GIBCO/BRL).[16] Lipofectin is made up of a 1:1 ratio of the cytofectin DOTMA (**1**) and the naturally available, neutral lipid DOPE (**2**). Since this key invention,

Andrew David Miller, born in 1963, began his chemistry education at the University of Bristol (UK), where he graduated in 1984 with a BSc degree. He finished his PhD thesis at the University of Cambridge (UK) in the research group of Professor Alan Battersby, following which he was tempted to stay a further year as a postdoctoral researcher before joining the group of Professor Jeremy Knowles at Harvard University (USA). Since 1990 he has been a lecturer in the Chemistry Department at Imperial College in London (UK), where he has been particularly developing active research interests in the chemistry of stress proteins and in the design of vectors for the delivery of nucleic acids to cells for gene therapy. He has received several awards and fellowships including the Thomas Malkin and Robert Garner prizes for chemistry (Bristol University), an Emmanuel College research scholarship (Emmanuel College, Cambridge), a Lindemann Trust Fellowship, and most recently a Leverhulme Trust Fellowship. This year he was appointed a founding director of the Imperial College Genetic Therapies Centre.



upwards of twenty new cationic liposome formulations have been reported to bring about nucleic acid delivery; a number of these have been commercialized (Table 1). These new formulations usually involve the combination of a novel, synthetic cytofectin with **2** or another neutral lipid. The choice of cytofectin and the manner of formulation are both critical to the success of any given cationic liposome formulation. Therefore, the structures and syntheses of several cytofectins will be described here together with the procedures used to formulate these amphiphiles into cationic liposomes.

3.1. Structures of Cytofectins

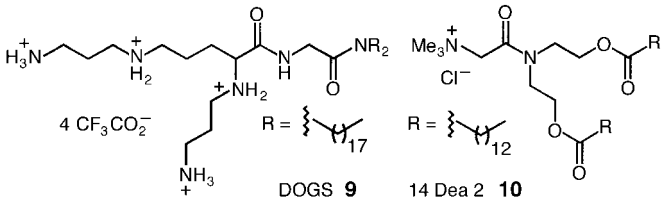
Cytofectins are made up of a cationic head group attached by a linker to a hydrophobic moiety. All cytofectins are therefore positively charged amphiphiles. This is the essential, unifying structural principle of what otherwise may be seen as a diverse family of structures. In this reveiw, the cytofectins are classified into various subgroups: DOTMA analogues, complex alkylamine/alkylamides, cholesterol derivatives, and synthetic derivatives of dipalmitoyl L-α-phosphatidyl-ethanolamine, glutamate, imidazole, and phosphonate.

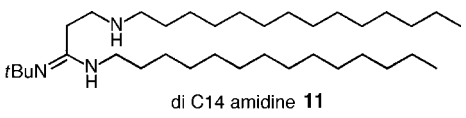
DOTMA analogues have achieved the most widespread use in cationic liposome formulations. The main analogues are DOTAP (**3**),[17] DMRIE (**4**),[18] and DOSPA (**5**). Other DOTMA analogue structures reported include DORIE (**6**)[18] and the corresponding ester analogue DORI (**7**).[18–20] Homologues of DMRIE, DORIE, and DORI are also known.[18, 20] The most recent and most effective addition to this subgroup of compounds has been GAP-DLRIE (**8**).[21] The main members of the complex alkylamine/alkylamide subgroup are DOGS (**9**),[22] 14 Dea 2 (**10**),[23, 24] di C 14 amidine (**11**),[25, 26] and DDAB (**12**).[27] Homologues of both 14 Dea 2 and di C 14 amidine have been reported.[23–26] Most recently, GS 2888 (**13**) was added to this subgroup.[28] Of these, only **9** and **12** have experienced widespread utility thus far.

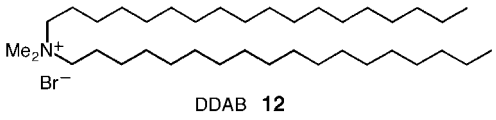
Table 1. The main cationic liposome formulations to date.[a]

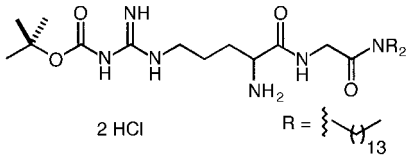
Commercialized		Not commercialized	
Cytofectin	Formulation (trade name/manufacturer)	Cytofectin	Formulation
DOTMA **1**[16]	DOTMA/DOPE 1:1[b] (Lipofectin/GIBCO BRL)	DMRIE **4**[18]	DMRIE/DOPE 1:1
		DORIE **6**[18]	DORIE/DOPE 1:1
DOTAP **3**[17]	DOTAP (DOTAP/Boehringer Mannheim)	DORI **7**[18, 20]	DORI/DOPE 1:1
		14 Dea 2 **10**[23, 24]	14 Dea 2
DOSPA **5**	DOSPA/DOPE 3:1[b] (LipofectAMINE/GIBCO BRL)	GS 2888 **13**[28]	G 2888/DOPE 1:1
		DC-Chol **14**[29]	DC-Chol/DOPE 6:4
DOGS **9**[22]	DOGS (Transfectam/Promega)	cholic acid hexamine **17**[31]	**17**/DOPE 1:1[b]
		lipid 67 **18**[32]	lipid 67/DOPE 1:2
di C 14 amidine **11**[25, 26]	di C 14 amidine/DOPE 1:1 (Clonfectin/Clontech)	CTAP **19**[33]	CTAP/DOPE 1:2
		BGTC **21**[36]	BGTC/DOPE 3:2
DDAB **12**[27]	DDAB/DOPE 1:2.5[b] (LipofectAce/GIBCO BRL)	DPPES **22**[22]	DPPES
		Lys-Pam_2-Gro*P*Etn **23**[37]	Lys-Pam_2-Gro*P*Etn/Chol/egg PC 1.5:3.0:5.5[b]
cholesterylspermidine **20**[35]	cholesterylspermidine (Transfectall/Apollon Inc.)	L-PE **23a**[38]	L-PE/CEβA **39** 6:4
		$2C_{12}$-L-Glu-ph-C_2-N^+Br^- **24**[39]	$2C_{12}$-L-Glu-ph-C_2-N^+
		$2C_{14}$-L-Glu-C_2-N^+Cl^- **25**[39]	$2C_{14}$-L-Glu-C_2-N^+
		DOTIM **26**[40]	DOTIM/Chol 1:1 or DOTIM/DOPE 1:1

[a] Abbreviations used: Chol: cholesterol (**38**), egg PC: egg phosphatidyl ethanolamine. Ratios given are molar ratios, unless indicated otherwise. [b] Weight ratio.

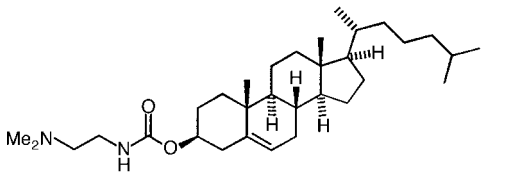


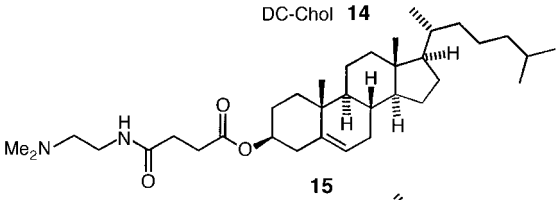


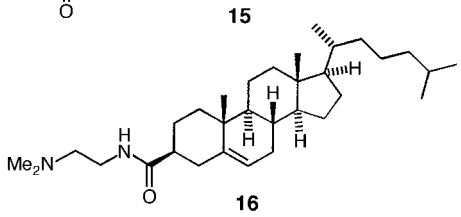


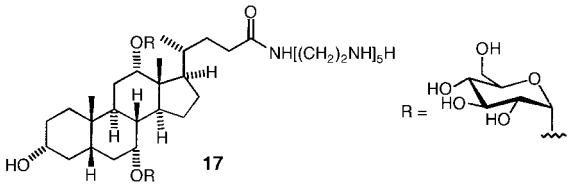


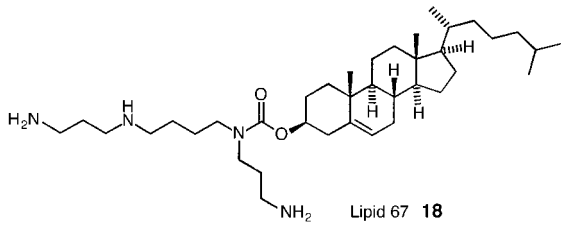
The first reported cholesterol derivative was DC-Chol (**14**),[29] and it is still widely used. Other derivatives were described soon after, including cholesteryl-3β-oxysuccinamidoethylenedimethylamine (**15**) and cholesteryl-3β-carboxyamidoethylenedimethylamine (**16**)[30] as well as ester analogues of both.[17] Recently, three unusual classes of polyamine cholesterol cationic "face" amphiphiles have been reported that are based on the three naturally occuring cholesterol-derived bile acids.[31] These are typified by the bisglycosylated cholic acid hexamine **17**. Two of the most efficaceous cytofectins reported to date are cholesterol derivatives; these are polyamine lipid 67 (**18**)[32] and CTAP (**19**).[33] Other polyamine derivatives have also been described,[34–36] including cholesterylspermidine (**20**),[35] and BGTC (**21**).[36]

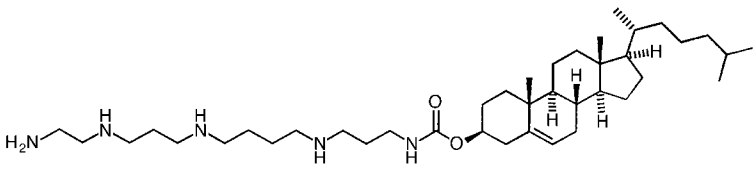


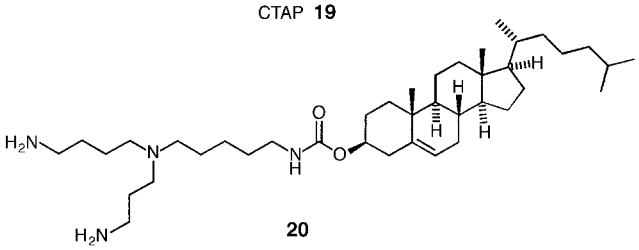


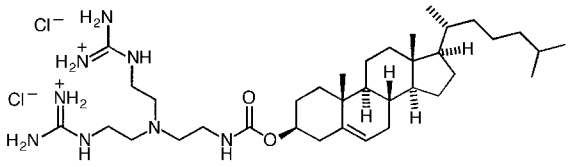




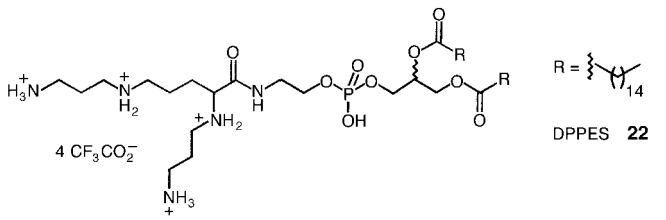


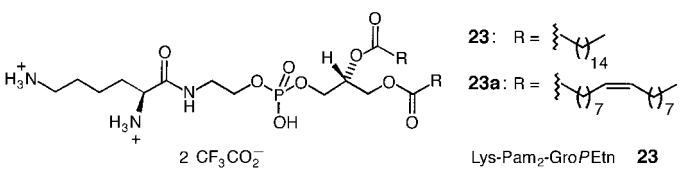




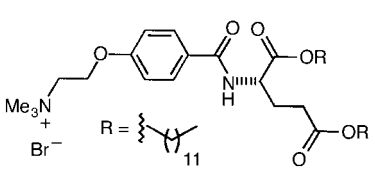


Of the remaining subgroups, there are two main dipalmitoyl L-α-phosphatidylethanolamine derivatives which have been reported, namely, DPPES (**22**)[22] and Lys-Pam$_2$-Gro*P*Etn

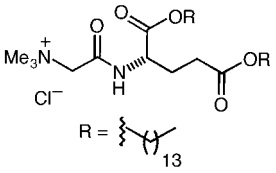




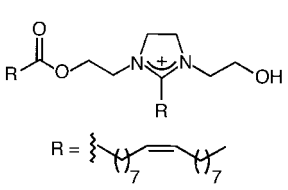
(**23**).[37] A third derivative, L-PE (**23a**), is known, but is apparently little more than an "oleoyl version" of **23**.[3, 38] $2C_{12}$-L-Glu-ph-C_2-N^+Br^- (**24**) and $2C_{14}$-L-Glu-C_2-N^+Cl^- (**25**) are reported to be the best glutamate-derived cytofectins.[24, 39] Finally, imidazole derivatives, typified by DOTIM (**26**), have recently been announced[40] as well as two phosphonate diester derivatives, GLB43 (**27**) and GLB90 (**28**).[41]



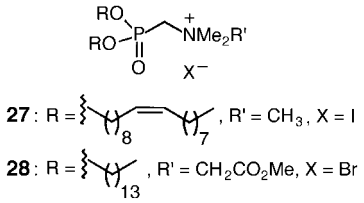
$2C_{12}$-L-Glu-ph-C_2-N^+Br^- **24**



$2C_{14}$-L-Glu-C_2-N^+Cl^- **25**



DOTIM **26**

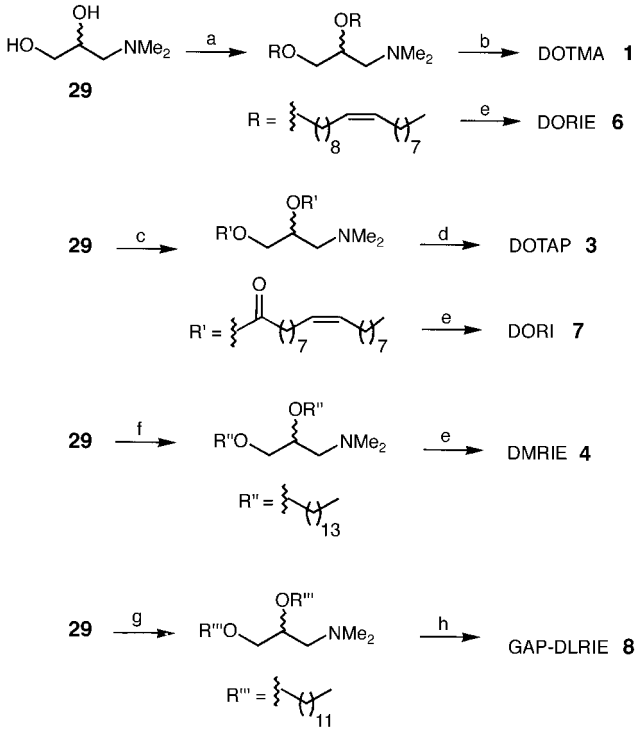


GLB43 **27**
GLB90 **28**

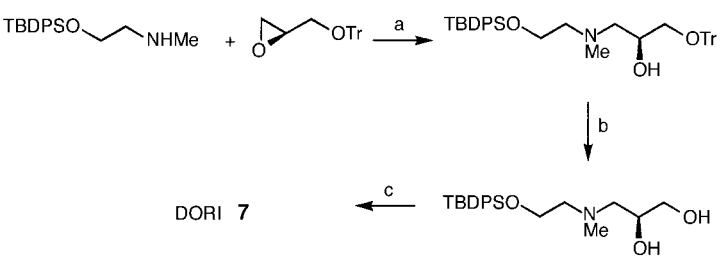
3.2. Syntheses of Cytofectins

The organic syntheses of the main cytofectins are in general short and high-yielding, a fact which has certainly aided their utility. The synthesis of DOTMA (**1**) is shown in Scheme 1 together with the syntheses of DOTMA analogues DOTAP (**3**), DMRIE (**4**), DORIE (**6**), and DORI (**7**). DOTMA is prepared in a two-step procedure from 3-(dimethylamino)-1,2-propanediol (**29**).[16] Both **3** and **7** are made in a similar way by oleoylation of propanediol **29** followed by alkylation of the resulting tertiary amine with methyl iodide or 2-bromoethanol, respectively.[17, 18] Alternatively, alkylation of **29** with myristyl or oleyl mesylate, followed by N-alkylation with 2-bromoethanol, gives **4** and **6**, respectively.[18] GAP-DLRIE (**8**) is synthesized in an analogous manner to **4** and **6**.[21] More complex syntheses of DOTMA analogues have also been reported, including an elegant synthesis of **7** (Scheme 2),[19] which proved flexible enough to allow the synthesis of a range of fatty acid analogues of **7**.[20] The synthesis of the polyamine DOSPA (**5**) appears not to have been reported.

The complex alkylamine/alkylamide subgroup of cytofectins have been prepared in a variety of different ways consistent with their more diverse structures. DOGS (**9**) is synthesized by a multistep, convergent procedure culminating in the coupling of L-5-carboxytetrabutoxycarbonylspermine (**30**) with glycyldioctadecylamide (**31**, Scheme 3),[22] cytofectin 14 Dea 2 (**10**) and its homologues by N-acylation and amine substitution (Scheme 4),[24] and di C 14 amidine (**11**) and its



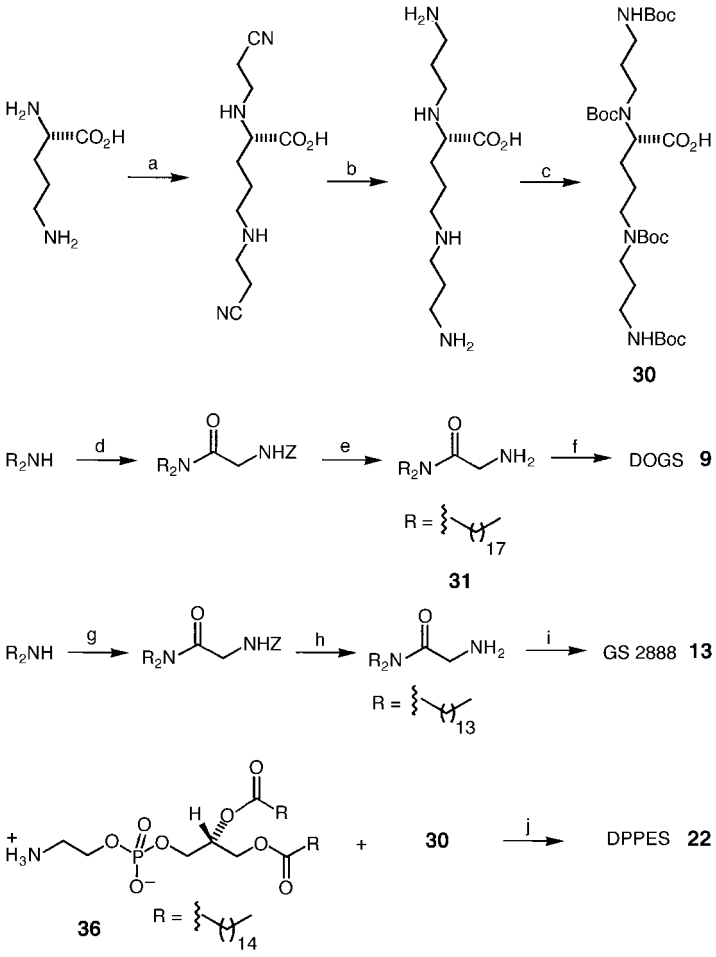
Scheme 1. Reagents and conditions: a) xylene, *t*BuOK (3 equiv), oleyl toluenesulfonate (3 equiv) or oleyl mesylate (3 equiv), 140 °C, 3 h; b) MeCl, 70 °C, 48 h; c) Et_2O, pyridine, oleoyl chloride, 40 h, 78 %; d) $CHCl_3$/DMSO (1/1), MeI, 63 %; e) $Br(CH_2)_2OH$; f) xylene, *t*BuOK (3 equiv), myristyl mesylate (3 equiv), 70 °C; g) 1. THF (anhydrous), NaH, reflux, 24 h; 2. THF (anhydrous), dodecyl mesylate (3 equiv), reflux, 72 h, 65 %; h) 1. DMF (anhydrous), *N*-(3-bromopropyl)phthalimide (2 equiv), 105 °C, 72 h, 61 %; 2. EtOH, hydrazine (30 equiv), room temperature (RT), 18 h, 75 %.



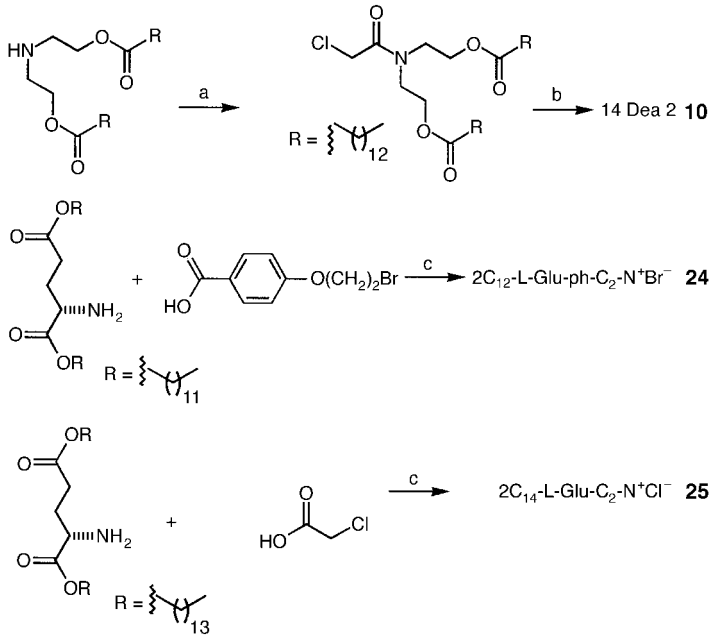
Scheme 2. Reagents and conditions: a) EtOH, $LiClO_4$ (2 equiv), 65 °C, 72 %; b) Et_2O, 85 % HCO_2H, 71 – 75 %; c) 1. CH_2Cl_2, oleoyl chloride (2.2 equiv), Et_3N, DMAP (cat.), 0 °C, 92 %; 2. THF, TBAF (3 equiv), 0 °C, 88 – 91 %; 3. MeI. TBDPS = *tert*-butyldiphenylsilyl, Tr = triphenylmethyl, DMAP = 4-dimethylaminopyridine, TBAF = tetrabutylammonium fluoride.

homologues by aminolysis of ethyl *N*-*tert*-butylacrylimidate (**32**, Scheme 5).[26] DDAB (**12**) is prepared by dialkylation of dimethylamine.[27] The recently reported GS 2888 (**13**) was prepared (Scheme 3) in a very similar way to **9**.[28]

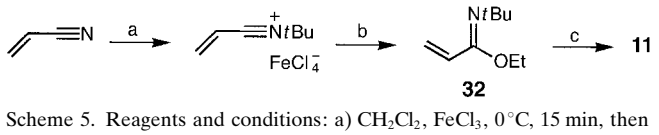
A single aminolysis reaction is sufficient to prepare DC-Chol (**14**) from cholesterol chloroformate (**33**),[29] and to prepare **15** and **16** from cholesterol hemisuccinate (**34**) and cholesteryl-3β-carboxylic acid (**35**), respectively (Scheme 6).[30] Aminolysis of **33** followed by guanidinylation of the product was sufficient to synthesize BGTC (**21**) as well (Scheme 6).[36] Rather surprisingly, workers in the field have often used a similar one-step aminolysis procedure to prepare



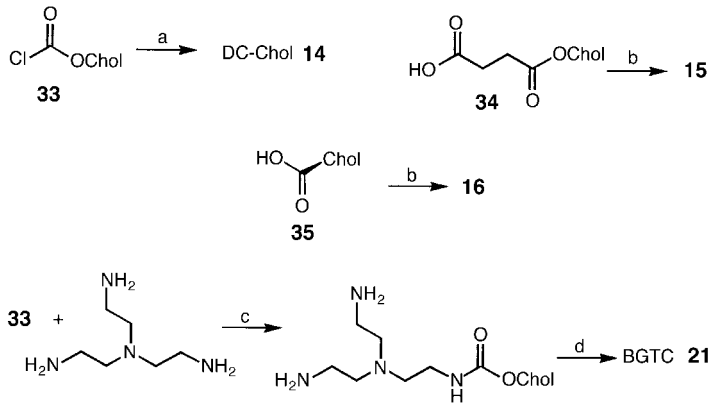
Scheme 3. Reagents and conditions: a) DMF, CH_2CHCN; b) EtOH, Raney Ni, H_2; c) THF, Boc-ON; d) CH_2Cl_2, ZGly*p*NP, Et_3N (1.1 equiv), 5 h; e) CH_2Cl_2/EtOH, 10% Pd/C, H_2, 1 h, 87%; f) 1. CH_2Cl_2, **30**, DCC (1.1 equiv), 12 h, 90%; 2. TFA, 10 min; g) 1. THF, Z-glycine, NHSu (1 equiv), DCC (1.1 equiv), RT, 16 h; 2. CH_2Cl_2, Et_3N (1.4 equiv), RT, 5 h, 18%; h) CH_2Cl_2/EtOH, 10% Pd/C, H_2, 8 h, 94%; i) 1. CH_2Cl_2, L-Boc-Arg(Boc)$_2$-OH, DIC (1.1 equiv), RT, 16 h, 37.5%; 2. dioxane, 4 M HCl, RT, 2.5 h; j) 1. CH_2Cl_2/THF, NHSu (1.1 equiv), DCC (1.1 equiv), 12 h; 2. $CHCl_3$/EtOH, Et_3N, 40 °C, 12 h, 55%; 3. CH_2Cl_2, TFA; k) 1. $CHCl_3$/EtOH (1/1), Et_3N (0.1 mol%), 50 °C, 12 h; 2. $CHCl_3$, TFA, 3 h. Boc = *tert*-butoxycarbonyl, Boc-ON = 2-(*tert*-butoxycarbonyloximino)-2-phenylacetonitrile, Z = benzyloxycarbonyl, *p*NP = *para*-nitrophenyl, DCC = dicyclohexylcarbodiimide, TFA = trifluoroacetic acid, NHSu = *N*-hydroxysuccinimide, DIC = diisopropylcarbodiimide.



Scheme 4. Reagents and conditions: a) $CHCl_3$, DCC (1.1 equiv), $ClCH_2CO_2H$; b) Me_3N; c) 1. CH_2Cl_2, DCC (1.1 equiv); 2. Me_3N.

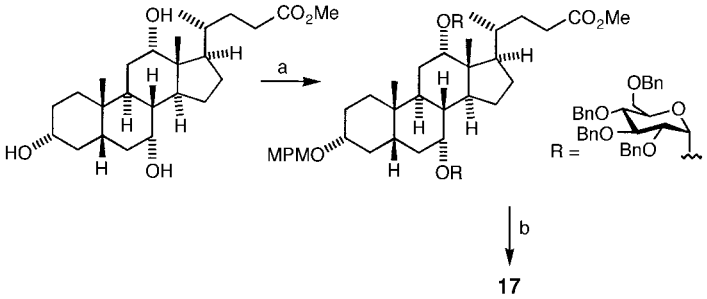


Scheme 5. Reagents and conditions: a) CH_2Cl_2, $FeCl_3$, 0 °C, 15 min, then *t*BuCl, 30 min; b) EtOH; c) $CH_3(CH_2)_{13}NH_2$ (2 equiv).



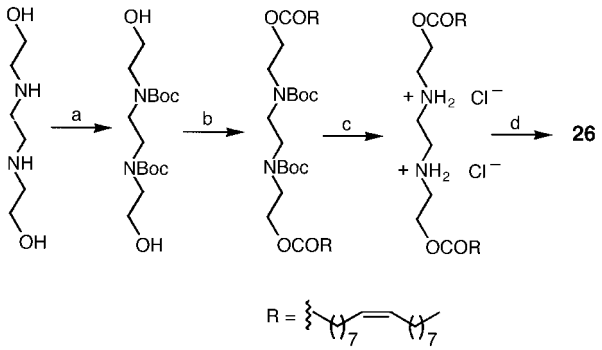
Scheme 6. Reagents and conditions: a) $CHCl_3$, $Me_2N(CH_2)_2NH_2$ (3.6 equiv), 0 °C, 22%; b) CH_2Cl_2, $Me_2N(CH_2)_2NH_2$, CDI; c) CH_2Cl_2, TREN; d) THF/MeOH, 1*H*-pyrazole-1-carboxamidine (2 equiv), *i*Pr_2NH (2 equiv), RT, 18 h, 60%. CDI = carbonyldiimidazole, Chol = 3-cholesteryl, TREN = tris(2-aminoethyl)amine.

cholesterol polyamine derivatives leading to mixtures of polyamine regioisomers rather than pure compounds.[34] For instance, cholic acid hexamine **17** has been prepared in several steps from cholic acid, but the addition of the hexamine was also accomplished by an uncontrolled aminolysis step that left **17** as a mixture of polyamine regioisomers (Scheme 7).[31] By contrast, polyamine CTAP (**19**) has recently been synthesized in a high-yielding, multistep procedure designed to avoid regioisomer formation.[33] The synthetic route devised proved to be flexible enough to allow the syntheses of a host of triamine, tetramine, and pentamine variants of cholesterol-derived cytofectins. The synthetic routes to lipid 67 (**18**) and cholesterylspermidine (**20**) have not thus far been reported.



Scheme 7. Reagents and conditions: a) 1. THF, NaH, MPMCl; 2. PhMe, Tf_2O (4 equiv), 2,6-di-*tert*-butyl-4-methylpyridine, $-78\,^{\circ}C$, then RS(O)Ph (4 equiv), 30 min, $\rightarrow$RT; b) 1. NaOH/EtOH/THF, 2–48 h, reflux; 2. CH_2Cl_2, NHSu, DCC, 3 h; 3. H_2O/Et_3N, hexamine, 48 h; 4. EtOH, $Pd(OH)_2/C$, H_2. MPM = *p*-methoxybenzyl, Tf_2O = trifluoromethylsulfonic anhydride.

Of the remaining subgroups of cytofectins, the two main dipalmitoyl-phosphatidylethanolamine derivatives, DPPES (**22**) and Lys-Pam_2-Gro*P*Etn (**23**), were synthesized analogously by N-acylation of the parent lipid **36** with *N*-hydroxysuccinimide-activated **30** or Boc-Lys(Boc)-OSu (**37**), respectively (Scheme 3).[22, 37] N-Acylation followed by amine substitution sufficed for the syntheses of both main glutamate diester derivatives $2C_{12}$-L-Glu-ph-C_2-N^+Br^- (**24**) and $2C_{14}$-L-Glu-C_2-N^+Cl^- (**25**) from glutamate esters (Scheme 4).[24] Finally, imidazole derivative DOTIM (**26**) and its homologues have been prepared by a four-step procedure culminating in a novel cyclization to form the imidazolinium ring (Scheme 8).[40]



Scheme 8. Reagents and conditions: a) $CHCl_3$, Boc_2O (2.1 equiv), $NaHCO_3$, 5 h, 80%; b) CH_2Cl_2, oleoyl chloride (2.1 equiv), Et_3N (2.4 equiv), 45 min, 94%; c) dioxane, HCl, 2 h, 95%; d) ethylene glycol, 110 °C, 10 min, 75%.

3.3. Methods for Cationic Liposome Formulation

The formulation of cytofectins into cationic liposomes, with or without a neutral colipid, has involved a number of different possible procedures. Broadly speaking these are sonication and/or vortex-mixing of an aqueous solution, reverse-phase evaporation, or aqueous dilution of an ethanolic stock solution. The method of formulation is undoubtedly important, but the extent to which this affects the efficiency of nucleic acid transfer has yet to be properly evaluated.

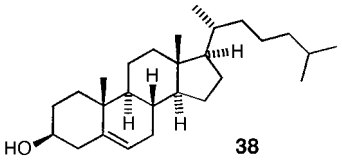
DOTMA (**1**) and its main analogues (**4**–**8**) are usually formulated into cationic liposomes with the neutral colipid DOPE (**2**). Liposomes are prepared by vortex-mixing of equimolar amounts of cytofectin and **2** at room temperature to produce large multilamellar vesicles (MLVs), which are then sonicated at 10 °C to produce small unilamellar vesicles (SUVs) suitable for gene transfection.[18, 20, 21] Cationic liposomes with DOSPA (**5**) are available commercially as LipofectAMINE (GIBCO/BRL) and contain **5** and **2** in a 3:1 ratio. Cationic liposomes containing DOTAP (**3**) are also available (Boehringer Mannheim) without neutral colipid; however, prior to commercialization, DOTAP cationic liposomes were also customarily formulated with **2**. In that instance, DOPE/DOTAP liposomes were prepared either by sonication[17] or by reverse-phase evaporation.[17, 42]

Cationic liposomes of the complex alkylamine/alkylamide cationic amphiphile DOGS (**9**) may be obtained commercially as Transfectam (Promega). Such liposomes are formulated simply by tenfold dilution of an aqueous ethanol stock solution of **9** into an aqueous buffer.[22] By contrast, cationic liposomes containing DDAB (**12**) are usually formulated with DOPE (**2**). Indeed, the commercial product LipofectAce (GIBCO/BRL) is a formulation of **12** with **2** in a 1:2.5 ratio. Di C 14 amidine (**11**) liposomes are now available commercially as Clonfectin (Clontech). These are mainly formulated by dilution of an ethanolic stock solution into neutral buffer,[25] a method analogous to the formulation of DOGS liposomes;[22] a second method has also been used in which **11** was combined with **2**, in 1:1 molar ratio, and liposomes formed by vortex-mixing and sonication.[25] This second method has also been used to form liposomes from GS 2888 (**13**), although the best formulation involved vortex mixing followed by freeze-thawing of a mixture of **13** and **2** in a 2:1 molar ratio.[28] Finally, sonication of a 10 mM suspension in water of cytofectin 14 Dea 2 (**10**) or its homologues is apparently sufficient to form active liposomes.[23]

Cholesterol derivatives are usually formulated into liposomes with DOPE (**2**), but in a number of different ways. For instance, DC-Chol (**14**) is usually combined with **2** in a 6:4 molar ratio, and liposomes are generated either by sonication alone,[29] vortex-mixing followed by sonication,[43] or reverse-phase evaporation followed by sonication.[44] Liposomes containing cytofectins **15** and **16** have also been formulated with **2** in a 1:1 molar ratio by sonication or by vortex-mixing followed by sonication.[29, 43] The cholic acid hexamine **17** and related amphiphiles are combined with **2** in a 1:1 ratio, and liposomes formed by sonication.[31] Polyamines lipid 67 (**18**) and CTAP (**19**) have so far been transformed into liposomes, in combination with **2** in a 1:2 molar ratio, by vortex-mixing alone.[33] Out of the remaining cholesterol polyamine cytofectins, BGTC (**21**) has been reported to form liposomes, in a 3:2 molar ratio with **2**, by vortex-mixing and then sonication,[36] whilst cholesterylspermidine (**20**), now marketed as Transfectall (Apollon Inc), appears to be formulated without colipid.[35]

The remaining subgroups of cytofectins are frequently formulated either without colipid or with neutral colipids other than DOPE (**2**). For instance, liposomes of DPPES (**22**) are prepared without colipid, in the same way as DOGS (**9**),[22]

whilst those of Lys-Pam$_2$-Gro*P*Etn (**23**) are formed by sonication of mixture of **23** with cholesterol **38** and egg



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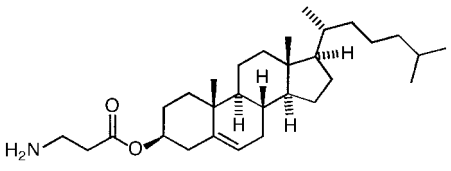
phosphatidylcholine in a ratio of 1.5:3.0:5.5.[37] L-PE (**23a**) is formulated into liposomes in combination (6:4 molar ratio) with a second cationic amphiphile, namely the cholesterol ester of β-alanine (CEβA, **39**).[38] In contrast, liposomes of



CEβA **39**

DOTIM (**26**) and its homologues are prepared by sonication of a mixture of the cytofectin with a 1:1 molar ratio of either cholesterol **38** or **2**.[40] Finally, the two main glutamate diester derivatives, 2C$_{12}$-L-Glu-ph-C$_2$-N$^+$Br$^-$ (**24**) and 2C$_{14}$-L-Glu-C$_2$-N$^+$Cl$^-$ (**25**), are formulated on their own by simple sonication of a suspension in neutral buffer.[39]

4. Mechanism of Nucleic Acid Delivery

The mechanism of cationic liposome mediated nucleic acid delivery is complicated. Therefore, to characterize the present state of knowledge, this process will be discussed in three separate stages. Firstly, the structures of complexes between cationic liposomes and nucleic acids will be described. Heterogeneous complexes are formed rapidly following the combination of nucleic acids with a suspension of cationic liposomes. It is these complexes which enter the cells. Nucleic acid delivery appears to be most efficient when complexes are formed under conditions where the ratio of liposome positive charges to nucleic acid negative charges lies around 1 and above. For gene delivery in vitro, the optimal charge ratio is frequently greater than 1 (i.e., complexes are cationic),[22] whilst the optimal ratio tends to be nearer to 1 for efficient in vivo gene delivery.[45] Therefore, the main focus of our attention in the next section will be on the special character of cationic liposome/nucleic acid complexes formulated with charge ratios of around 1 and above.

Second, the mechanism(s) involved in the entry of cationic liposome/nucleic acid complexes into cells will be discussed. The predominant mechanism now appears to be endocytosis, irrespective of the type of cationic liposome involved. Therefore, the best evidence for this will be presented together with some other possible mechanisms for cell entry. Finally, the fate of the nucleic acids will be described with special emphasis on the trafficking of nucleic acids to the nucleus, where their therapeutic site of action would be in most instances.

4.1. Structures of Cationic Liposome/DNA Complexes

The original, stylized depiction of Felgner and Ringold shows cationic liposomes binding to DNA electrostatically without changing their size and shape.[46] Experimental evidence now shows that cationic liposome/DNA complexes are in fact very heterogenous and dynamic, varying in size and shape depending upon the molar ratio of cationic liposomes to DNA. The most dramatic changes in structure appear to occur when the positive/negative charge ratio is around 1.

The first key experiments were carried out by Gershon et al.[47] Using ethidium bromide as a fluorescent probe for exposed DNA base pairs, they titrated fixed concentrations of DNA with DOTMA or DOPE/DOTMA liposomes (1:1 molar ratio) and observed the effects. They observed that DNA was generally accessible until the positive/negative charge ratio approached 1, at which point extensive DNA masking was diagnosed by a rapid decline in fluorescence intensity (Figure 1a). Sharp changes in behavior at the 1:1

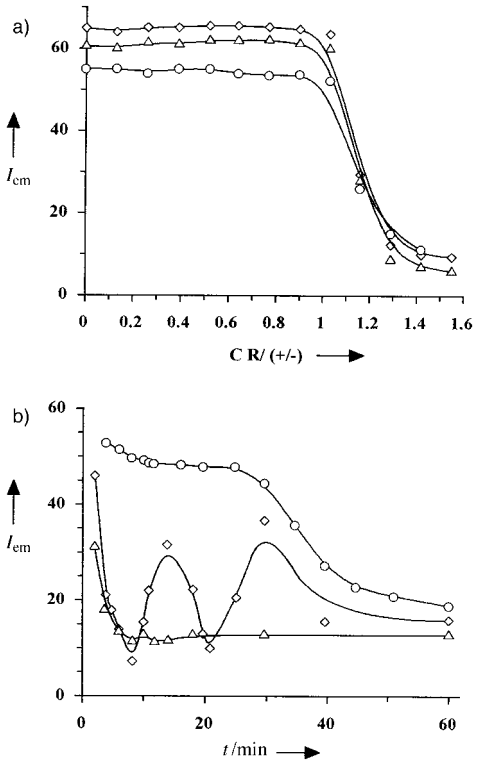


Figure 1. Effects of DNA length and liposome-to-DNA ratio (in terms of the positive/negative charge ratio) on the fluorescence intensity of ethidium bromide. a) A fixed concentration of DNA (nucleotide concentration) was titrated with DOPE/DOTMA liposomes (1:1 molar ratio) in the presence of ethidium bromide. Increasing liposome concentrations are reflected by the increasing liposome-to-DNA [positive/negative charge ratio: CR/(+/−)]. The intensity of fluorescence emission (I_{em}) for ethidium bromide is given in arbitrary units. Lengths of DNA segments were 100–300 (○), 500–8000 (△), and 23000 base pairs (◇). b) Three different concentrations of DOPE/DOTMA liposomes (1:1 molar ratio) were combined with DNA in the presence of ethidium bromide, and the change in fluorescence intensity was measured over time t. The liposome-to-DNA charge ratios (+/−) were 1.0 (○), 1.1 (◇), and 1.2 (△). Throughout, DNA concentration was normalized at 25 μM (nucleotide concentration); ethidium bromide (1:50 molar ratio of fluorescence probe to nucleotides) was added immediately prior to measurement. This figure was adapted with permission from Gershon et al.[47]

charge ratio were also observed in lipid-mixing experiments with the resonance energy transfer (RET) method of Struck et al.[48] In this method, liposomes containing two complementary fluorescent-labeled lipids are combined with unlabeled liposomes. In the event of lipid mixing/fusion, the labeled lipids become diluted into the previously unlabeled liposomes, with the result that fluorescence quenching effects between complementary labeled lipids are reduced and fluorescence intensity is enhanced.

The studies with the RET method showed that whilst lipid mixing/fusion was generally promoted by DNA, in common with other multivalent anions,[49–52] a noticeable change in behavior occurred around the 1:1 charge ratio (Figure 2 a). In interpreting these sharp changes in behavior, Gershon et al. proposed that the cationic liposomes were probably sufficiently neutralizing the negative charge of DNA to induce a cooperative collapse in the DNA structure,[47] in analogy to previous observations.[53, 54] They further proposed that collapsed DNA, whose exposed surface area is then much smaller, could then be efficiently encapsulated by lipid. This proposal was corroborated by Kleinschmidt metal-shadowing electron microscopy experiments in which liposomes appeared as localized DNA-bound spherical clusters, at low liposome-to-DNA ratios, and as much larger (ca. 700 nm) rod-shaped structures above the critical charge ratio (Figure 3). The latter were proposed to arise from the encapsulation of several condensed DNA molecules together.

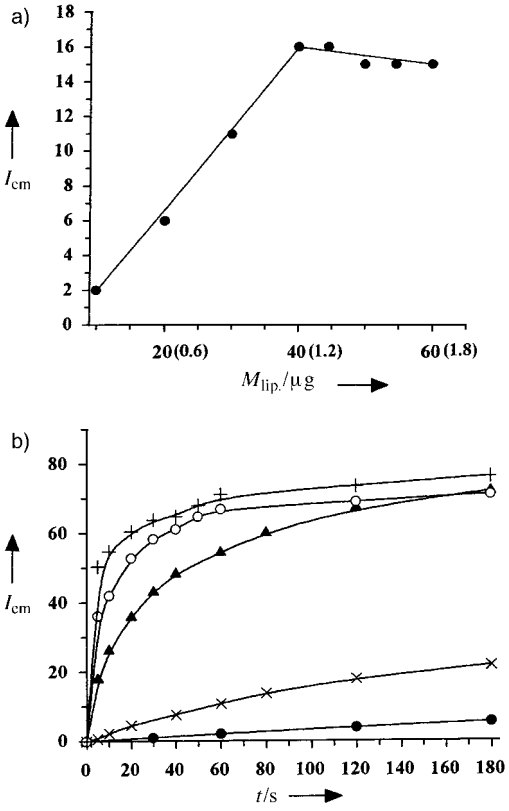


Figure 2. Lipid mixing of labeled and unlabeled liposomes induced by DNA. a) DOPE/DOTMA liposomes (1:1 molar ratio) were combined with fluorescent-labeled DOPE/DOTMA liposomes in the presence of DNA (25 μM, nucleotide concentration), and the increase in fluorescence intensity (I_{em} in arbitrary units) due to lipid mixing was measured with increasing lipid concentration (expressed as mass of liposome $M_{lip.}$ added to DNA). The DNA-induced increase in fluorescence was obtained by subtracting the fluorescence of labeled liposomes alone from that obtained in the presence of DNA. Estimated ratios of positive to negative charges are shown in parentheses This figure was adapted with permission from Gershon et al.[47] b) Liposomes containing 16 μM and 4 μM DOTAP (**3**) were combined with fluorescent-labeled DOTAP liposomes, and the change in fluorescence intensity due to lipid mixing was measured over time t in the presence of different concentrations of DNA (nucleotide concentration). Different DNA concentrations are reflected by the negative-to-positive charge ratio. DNA-to-liposome charge ratios (−/+) in the mixtures after the addition of oligonucleotides were 0 (i.e., no DNA present; ●), 0.03 (×), 0.2 (▲), 0.8 (), and 1.2 (+). This figure was adapted with permission from Jääskeläinen et al.[52]

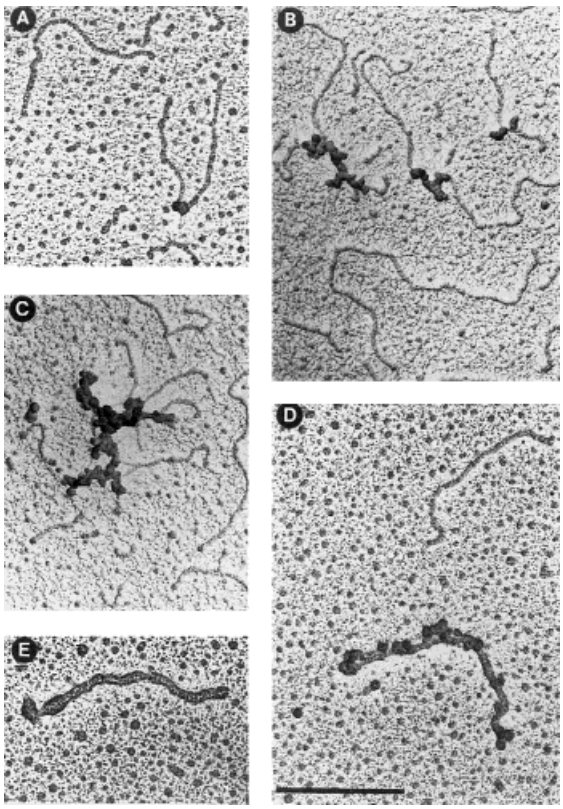


Figure 3. Kleinschmidt metal-shadowing electron microscopy of liposome/DNA complexes. The complexes were prepared with gradually increasing amounts of DOPE/DOTMA liposomes and a constant amount of DNA (3.5 μg mL^{-1}). The liposome-to-DNA (+/− charge) ratios were 0.2 (A), 0.4 (B), 0.6 (C), 1.0 (D), and 1.5 (E). The scale bar represents 0.5 μm. This figure was reproduced with permission from Gershon et al.[47]

These observations and the two main proposals have since been supported by the results of other research groups. In particular, Gustafsson et al. have obtained cryo-transmission electron microscope images of DNA complexed with DOTAP and DOPE/DDAB liposomes which revealed the presence of "free" or loosely bound DNA, when the positive/negative charge ratio was less than 1, and large (> 100 nm) lipid/DNA structures at liposome-to-DNA molar ratios in excess of the neutral charge ratio.[55] They proposed that these large structures were characteristic of multilamellar lipid structures within which DNA was totally encapsulated.

Encapsulation of DNA has also been supported by observations that cationic liposomes are able to protect DNA from degradation by nucleases, shearing by aerosoliza-

tion, and ultrasonic damage during sonication.[56, 57] The exact nature of this encapsulation has been very recently revealed through some spectacular X-ray diffraction data obtained by Rädler and co-workers in studying DOPE/DOTAP liposomes (1:1) complexed with λ-phage DNA.[58] They observed that DNA was encapsulated in highly ordered multilammelar structures and sandwiched between cationic bilayers. The DNA itself is arranged in highly ordered one-dimensional monolayer arrays forming a regular lattice (Figure 4). The

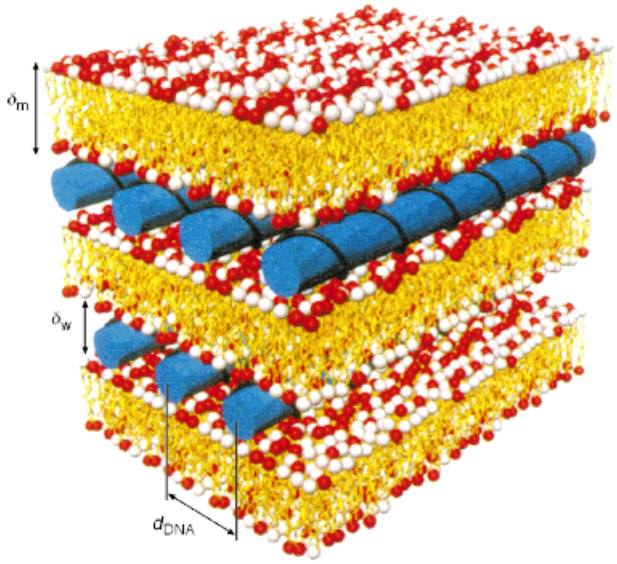


Figure 4. Schematic diagram of the proposed local arrangement of cationic liposome/DNA complexes. The DNA molecules are represented as blue rods. The head groups of anionic/zwitterionic lipids are shown as white spheres, and those of cytofectins as red spheres. Cytofectins are present in a higher concentration near the DNA. δ_m refers to the thickness of the bilayer, δ_w to the separation between the bilayers, and d_{DNA} to the interaxial spacing of the DNA. The magnitude of d_{DNA} increases from about 25 to 60 Å as the ratio of cationic liposome to DNA (positive/negative charge ratio) increased from less than 1 to more than 1. This figure was reproduced with permission from Rädler et al.[58]

formation of these ordered arrays was suggested to arise from initial condensation of DNA on the surface of the cationic liposomes. This results in screening of the electrostatic repulsion between lipid bilayers, thereby allowing multilayer formation driven by the release of DNA-associated counterions.

However, this is probably not the whole story. Behr orginally showed how DNA may coat or wrap around the surface of cationic liposomes.[59] This observation has since been supported by Eastman et al., whose new data suggest that DNA binding to the surface of cationic liposomes may also conveniently mediate the formation of the large cationic liposome/DNA complexes described above.[60] Therefore, it seems most probable and conceivable that the interaction between cationic liposomes and DNA involves a combination of both total encapsulation and surface association.

The variation of the size of the cationic liposome/DNA complex by changing the molar ratio of cationic liposome to DNA has been investigated by a number of groups. For instance, quasielastic light scattering measurements of DOTAP or DOPE/DDAB liposomes in association with DNA have shown that at low liposome-to-DNA ratios, only small particles (ca. 40 nm) form.[52] The average size then appeared to increase dramatically (ca. 3000 nm) as a positive/negative charge ratio of 1 is approached, but then gradually declined again as the liposome-to-DNA molar ratio was increased further (Figure 5). The same phenomenon has been observed

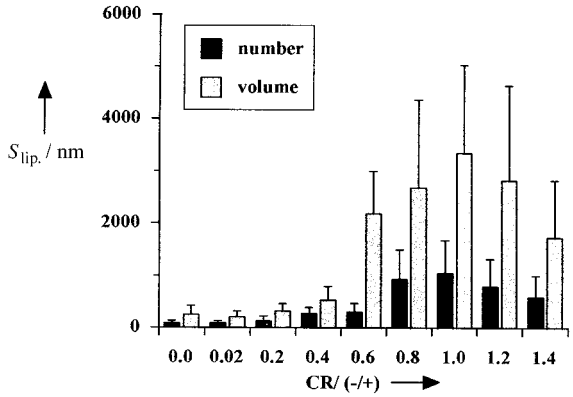


Figure 5. Quasi-elastic light scattering measurements of the particle size of liposome/DNA complexes. The complexes were prepared by incubating increasing amounts of a 15-mer S-oligonucleotide with cationic liposomes containing 10 μM DOTAP (**3**); the average particle size was then measured. The increase in DNA concentration is reflected by the increasing ratio of DNA to liposome [CR/(−/+)]. Size distributions $S_{lip.}$ were determined as the mean ± standard deviation of the diameter on the basis of vesicle number and volume. This figure was adapted with permission from Jääskeläinen et al.[52]

by Rädler and co-workers with a combination of dynamic light scattering measurements and differential interference microscope imaging.[58] Whilst large (> 100 nm) multilammelar particles with encapsulated DNA are almost certainly formed readily around and above the neutral charge ratio, these particles are probably in dynamic equilibrium with many other smaller complex species and even free DNA. Zabner et al. have provided evidence for this in studies with DOPE/DMRIE liposomes complexed to DNA.[61] Their electronphotomicrographs (Figure 6) showed not only typical large, dense lipid/DNA structures (> 100 nm) with looped DNA extensions, but also smaller clusters and free DNA. They also observed what appeared to be DNA strands coated with a thin layer of lipid, analogous to some "spaghettilike" structures which had been previously described in freeze-fracture electron microscope images of DNA complexed to DOPE/DC-Chol liposomes.[62]

The kinetics of liposome/nucleic acid complex formation have been analyzed at two levels. Using ethidium bromide as a fluorescent probe for exposed DNA base pairs, Gershon et al. observed that DNA encapsulation by DOPE/DOTMA liposomes was almost instantaneous when liposome and DNA were combined at concentrations where the positive/negative charge ratio significantly exceeded unity (> 1.2).[47] However, between a charge ratio of 1.0 to 1.1, more complex behavior was observed, and equilibration times of at least 60 min were required (Figure 1b). The observed oscillatory behavior is certainly indicative of a highly dynamic system, although it is

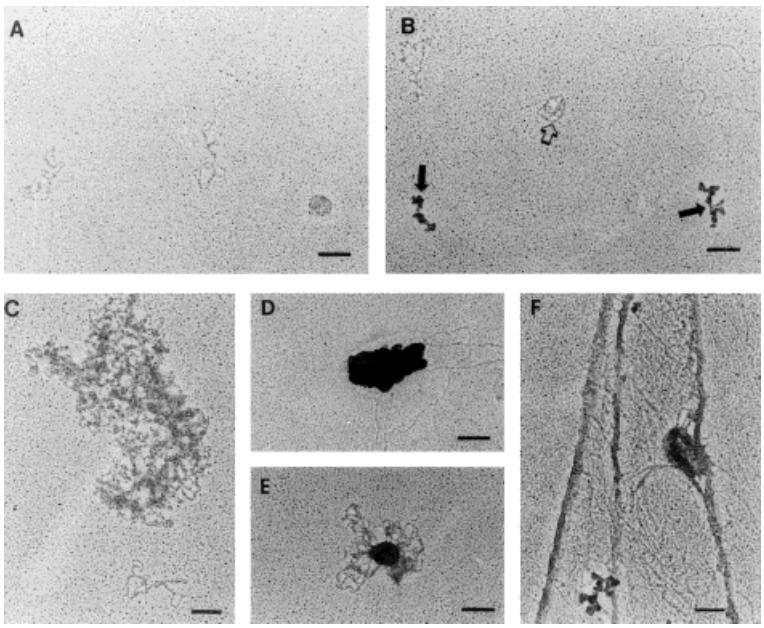


Figure 6. Transmission electron photomicrographs of liposome/DNA complexes. The complexes were prepared at a liposome-to-DNA ratio of 5:1 with DOPE/DMRIE liposomes (1:1 molar ratio). A) DNA without lipid, B) – F) examples of various types of complex. In (B) the open arrow shows uncomplexed plasmid, and the solid arrow plasmid complexed to lipid. The bar corresponds to 100 nm. This figure was reproduced with permission from Zabner et al.[61]

somewhat difficult to account for. At a ratio of 1.2, equilibration was achieved in 10 min (Figure 1b). A more detailed kinetic analysis using resonance energy transfer (RET) lipid-mixing experiments[48] revealed that DNA was able to induce aggregation and subsequent fusion of DOTAP and DOPE/DDAB liposomes at rates consistent with equilibration after a few minutes (<5 min, Figure 2b); however, no results were reported when the positive/negative charge ratio is between 1.0 and 1.1, as above.[52]

It is interesting to note how the results described above do begin to interlock to form a coherent picture of how cationic liposomes and DNA interact, inspite of the fact that these studies were performed by different research groups with a wide range of DNA lengths, lipids, liposome formulations, buffers, etc. Clearly the stylized depiction of Felgner and Ringold[46] is untenable. Cationic liposome/DNA complexes appear to be highly heterogeneous and encompass every manner of structure—from naked DNA to ordered multilammellar arrays of encapsulated DNA—which are almost certainly at equilibrium 30 – 60 min after the initial mixing of the cationic liposome and DNA. Given the fact that cationic liposome mediated nucleic acid delivery frequently appears to be most efficient when the molar ratio of cationic liposome to DNA is such that the positive/negative charge ratio is around or above 1, it is tempting to suggest that the large multilammellar particles containing ordered, encapsulated DNA are the principle structures responsible for nucleic acid delivery. However, since cationic liposome/DNA complexes are also highly dynamic and in equilibrium with smaller structures, this may not necessarily be true.

4.2. Entry of Complexes into Cells

The entry of complexes into cells is the first step of cationic liposome mediated nucleic acid delivery. Formerly, it was thought that membrane fusion between liposome and cell membrane was the primary means of cell entry[11, 16] on the premise that cationic and anionic liposomes readily fuse.[51, 63] However, cationic liposome/DNA complexes fuse much less readily with negatively charged membranes, indicating that cell entry by membrane fusion may not apply in this case.[17] Instead, evidence now suggests that slow endocytosis of intact complexes is the primary method,[64–66] and it is mediated by proteoglycan interactions.[67, 68] This process was first suggested by Behr et al.[22, 69] and has since been beautifully observed by Zabner et al., who used electron microscopy to follow the cell entry (into COS and HeLa cells) of gold-labeled DNA complexed to DOPE/DMRIE liposomes (positive/negative charge ratio = 1.2).[61] After initial association to the cell surface, complexes enter by endocytosis and remain localized within a vesicle or endosome (Figure 7). With confocal microscopy, the subsequent intracellular fate of the endosome-trapped complex was then monitored. Upwards of

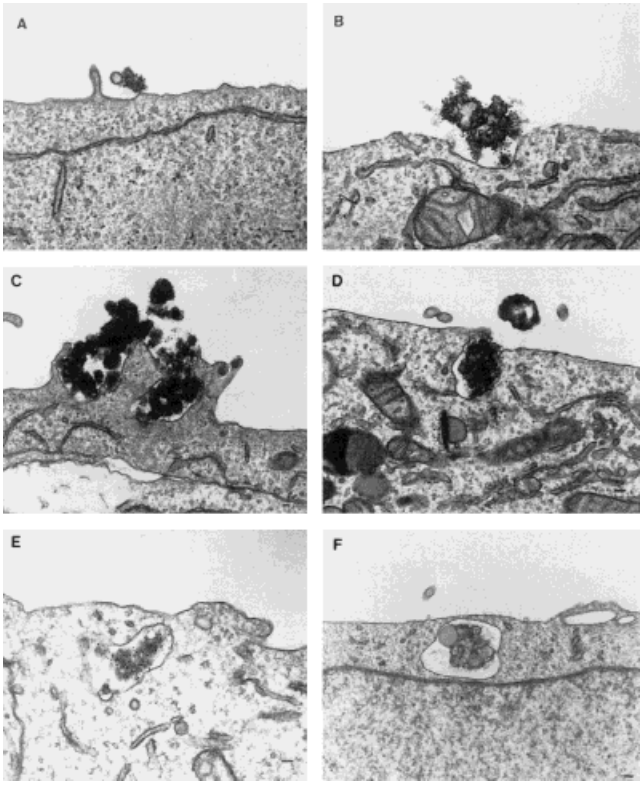


Figure 7. Transmission electron photomicrographs of COS cells transfected with gold-labeled DNA complexed with liposomes. Cells were exposed to complexes of DOPE/DMRIE with gold-labeled DNA, which were prepared with a liposome-to-DNA ratio of 5:1; cells were removed for electron microscopy at the indicated times: A) 5 min, B) 30 min, C) 1 h, D) 6 h, E) 24 h; F) cells transfected with unlabeled DNA. The bar corresponds to 100 nm, gold particles were 10 nm. This figure was reproduced with permission from Zabner et al.[61]

24 h after internalization, complexes were found to accumulate in the perinuclear region. This was interpreted to be the result of endosome migration toward the cell nucleus followed by endosome fusion, thereby allowing the enclosed complexes to coalesce and form macromolecular lipid/DNA structures. These macromolecular lipid/DNA structures are highly ordered and comprise an array of regularly packed tubules in which DNA is surrounded by bilayers or tubular monolayers of lipid. There was no evidence of lysozome fusion with endosomes, although this has been reported elsewhere.[66]

Zabner et al. were also able to examine the kinetics and efficiency of cell entry by liposome/DNA complexes by using fluorescence-activated cell sorting (FACS) to follow the uptake into COS cells of fluorescent-labeled DNA complexed with DOPE/DMRIE liposomes.[61] Only 5% of cells had internalized complexes after 30 min, but the process appeared to be 70% complete after 6 h, which is consistent with other data on cellular uptake.[12]

Whilst the recent evidence for endocytosis and endosome localization is strong, cell entry may still involve some elements of membrane fusion. For instance, Felgner and coworkers have observed that cationic liposome/DNA complexes containing fluorescent labeled DOPE (**2**) appear to stain the cell membrane.[16] However, it is unlikely that fusion processes dominate. Very recently, evidence has been advanced to suggest that phagocytosis may also play a role in complex entry alongside endocytosis.[70]

4.3. Trafficking of Nucleic Acids to the Nucleus

Cell entry of liposome/nucleic acid complexes appears to be a suprisingly efficient if slow process.[61, 64] However, not all the nucleic acid which enters the cell will be functional. This point has been illustrated by Zabner et al. They followed cationic liposome mediated gene delivery into COS or HeLa cells and observed that gene expression occured in less than 50% of the cells, even though endocytosis was determined to have an efficiency of at least 80%.[61] In other cell lines, the divergence between gene expression levels and endocytosis efficiency was much worse.[61] Unfortunately, the largest proportion of delivered nucleic acid is usually unable to escape from the endosomal compartments into the cell cytoplasm after entry. As a result, cationic liposome mediated delivery actually appears to be a very wasteful and inefficient way to deliver nucleic acids into cells, depriving them of most of the potential therapeutic benefit of the nucleic acids—whether this be from gene expression, antisense oligonucleotide inhibition, and so on.

Zabner et al. have clearly shown how inefficient the escape of nucleic acids from the endosome is by attempting to use electron and confocal microscopy to detect DNA in the cytoplasm of COS cells after cell entry mediated by DOPE/DMRIE liposome.[61] They were unable to detect any significant quantities, although enough DNA was apparently escaping for significant cytoplasmic gene transcription to be driven by a recombinant vaccinia virus expressing T7 polymerase. Nevertheless, the vast majority of delivered DNA accumulated in perinuclear endosomes in the form of the macromolecular lipid/DNA structures described in the previous section. Similar observations have also been made by Gao and Huang.[71]

When nucleic acids do succeed in escaping from endosomes, the most recent evidence indicates that they do so at an early stage after endocytosis has taken place, that is, when the cationic liposome/nucleic acid complexes are in an "early" endosome compartment.[65, 66] The mechanism of early endosome escape is not well understood, but when DOPE (**2**) forms part of the cationic liposome, escape may be aided by the tendency of **2** to promote significant polymorphic changes in the lipid phase under physiologically relevant conditions.[72–74] In particular, **2** readily promotes the formation of an inverted hexagonal phase (at room temperature and neutral pH) from the lamellar liquid crystal phase characteristic of most biological membranes.[72] This hexagonal phase is frequently observed when membranes fuse.[3] Therefore, **2** could provide the means for endosome disruption by promoting membrane fusion. When **2** is not part of the cationic liposome [e.g., liposomes of DOTAP (**3**) or DOGS (**9**)], local cationic lipid detergent effects may be the cause of endosome escape.[18] Very recently, Xu and Szoka proposed a model for early endosome breakout in which cationic liposome/DNA complexes destabilize the endosome membrane to allow the DNA to escape.[75] Electrostatic interaction between cationic liposome and endosome membrane induces the flip-flop of anionic lipids from the monolayer of the endosome membrane that faces the cytoplasm; these lipids laterally diffuse into the complex where they form charge neutral ion pairs with cytofectins. Ionic interaction between DNA and cytofectins is thereby disrupted allowing DNA to diffuse freely into the cytoplasm (Figure 8).

Once in the cytoplasm, nucleic acids may face a further difficulty in entering the nucleus to perform their therapeutic task. This is not always necessary, for instance, if mRNA is delivered then it does not need to enter the nucleus to function. However, where nuclear entry is required, this can be as serious a problem facing the net efficiency of cationic liposome mediated nucleic acid delivery as escape from the endosome. The difficulty of nucleic acid trafficking to the nucleus has been clearly demonstrated by Capecchi. Using a mouse cell line, he showed that nuclear-injected DNA led to protein expression in over 50% of the cells, whilst cytoplasm-injected DNA led to expression in less than 0.01% of the cells.[76]

Zabner et al. obtained similar results in comparable experiments with a Xenopus oocyte system.[61] The size of the nucleic acids appears to be the most important factor in determining whether they will reach the nucleus or not. Small oligonucleotides (between approximately 20 and 30 base pairs in length) readily accumulate in the nucleus whether delivered by cationic liposomes[65, 77] or cytoplasmic injection.[78] However, larger DNA is probably excluded from the nucleus because the pore sizes of the nuclear membrane have a diffusion cutoff of about 40 kDa.[79] That is not to say that larger DNA may not enter the nucleus at all, otherwise the expression of delivered genes would be impossible. However, the pore-size limitations of the nuclear membrane do require that assistance is given for larger DNA to enter the nucleus.

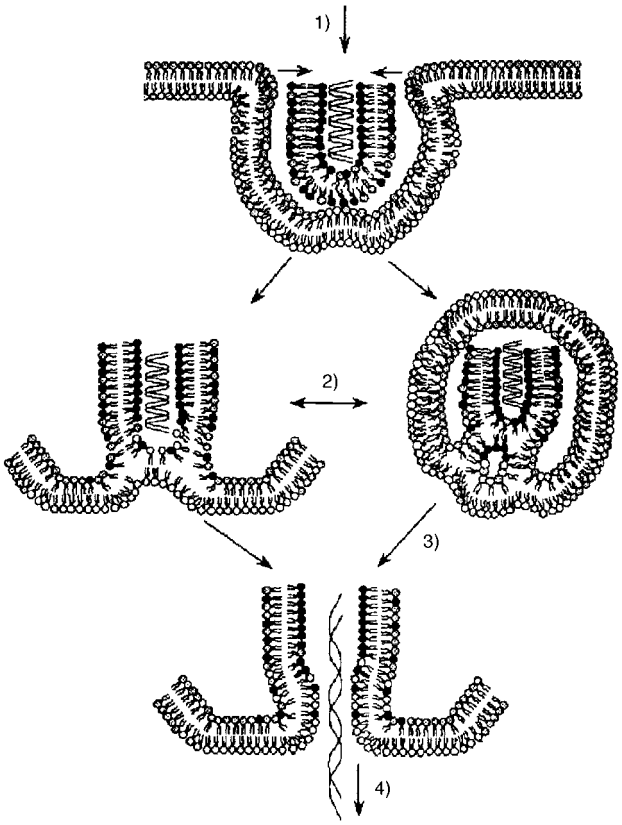


Figure 8. Proposed mechanism for the entry of cationic liposome/DNA complexes into cells and subsequent release of DNA from endosomes. Step 1: electrostatic interaction of cationic liposome/DNA complex with cell membrane followed by endocytosis. Step 2: membrane destabilization in the early endosome results in anionic/zwitterionic lipid flip-flop. Step 3: anionic lipids diffuse into the complex and form a charge neutral ion pair with cytofectins. Step 4: DNA dissociates from the complex and diffuses into the cytoplasm. The head groups of anionic/zwitterionic lipids are shown as open or shaded circles, those of cytofectins are shown as black circles. This figure was reproduced with permission from Xu and Szoka.[75]

Quite clearly, viruses are very efficient at this process. It remains to be seen whether cationic liposomes can be adapted to give all sizes of nucleic acid oligomers similar levels of assistance to enter the nucleus.

5. General Applicability of Cationic Liposome Formulations

Owing to the relative simplicity of use and reasonable efficiency, cationic liposome mediated gene delivery has been used widely to introduce foreign genes into a variety of primary and cultured cells in vitro. Perhaps unsurprisingly, commercially available cationic liposome formulations have tended to be used extensively, in part because they have proven, widespread efficacy. However, other formulations have also been used. Some recent examples include the use of Lipofectin (Table 1) to transfect rat oligodendrocytes[80] and rabbit endothelial cells;[81] LipofectAMINE to transfect murine myoblasts,[82] human hepatoma,[83] prostatic epithelial,[84] and ovarian cells;[85] DOTAP (**3**) to transfect murine cell lines[86] and human cervical carcinoma (CaSki) cells;[87] and finally DDAB liposomes to transfect CaSki cells[87] and human breast adenocarcinoma cells.[88] Liposomes containing DOPE (**2**) and DC-Chol (**14**) have also achieved fairly widespread use in vitro, for example in the transfection of human ovarian,[85] epidermoid carcinoma,[89, 90] epithelial,[91] and neuronal cells.[44]

There appear to be few structural principles to define which series of cytofectin and corresponding liposome formulations will be efficacious as in vitro vectors for nucleic acid delivery and which not. Often, where structural restrictions have been noted with one series of cationic amphiphiles, other series appear to flout those restrictions. For instance, quaternized derivatives of cholesterol-derived cationic amphiphiles are much less efficient at in vitro delivery than their tertiary amine counterparts.[43] However, DOTMA (**1**) and its analogues are all quaternary amines. As a result, structure–activity comparisions, within a given series of cytofectins and liposomes, have often been the only way to optimize nucleic acid delivery rationally. For example, both Felgner et al.[18] and Balasubramaniam et al.[20] carried out extensive, carefully controlled studies to assess the relative merits of liposomes containing DOTMA analogues. In a similar way, Lee et al.,[32] Cooper et al.,[33] and Walker et al.[31] recently optimized the transfection abilities of a series of polyamine cholesterol derived cytofectins. Other more limited comparisions have been made between homologues of other cytofectins.[23, 43]

In searching for a general link between cytofectin structure and efficiency of gene delivery, Akao et al. have suggested that the primary requirement for successful transfection is that the phase-transition temperature T_c of the cationic liposome formulation should be less than 37 °C.[23] However, a recent analysis of liposomes containing DOTMA analogues does not support this suggestion.[20] Instead, cell toxicity may well be the most important criterion. In this respect, DOPE/DC-Chol liposomes are very useful in that they have low toxicity.[92] Nevertheless, this is probably not the only factor. Recently, it has been suggested that gene delivery efficiency may be determined in part by the zeta potential of the cationic liposome, which is itself a partial function of cytofectin structure.[93] By comparing the efficiency of gene delivery for a series of cationic liposomes—formulated with cholesterol-derived cytofectins such as DC-Chol (**14**), **15**, and **16**—there appeared to be a direct proportional relationship between delivery efficiency and the zeta potential, as measured by laser Doppler spectroscopy.

The very simplicity of cationic liposome mediated nucleic acid delivery in vitro has resulted in a rapid increase in the application of cationic liposomes to gene delivery in vivo as well.[3, 94] The same liposomes whose in vitro efficacy has been described above have also proved to have some in vivo efficacy as well. However, in vitro efficacy frequently appears to be a poor guide to the absolute efficacy of a given cationic liposome in vivo.[32, 33] A number of recently reported examples of uses in vivo are presented in the following.

Liposomes containing DOPE (**2**) and DDAB (**12**) have been found to transfect murine lung, spleen, and kidney cells with cytokine genes after intraperitoneal injection of liposome/DNA complexes.[95] In addition, cholesterol/DDAB liposomes produced high-level systemic transfection with a

chloramphenicol acetyltransferase (CAT) marker gene in mice following intravenous injection.[96] Lipofectin has been shown to transfect porcine arterial wall cells in vivo with foreign histocompatibility genes.[97] Better results were obtained with DOPE/GAP-DLRIE cationic liposomes.[98] Murine tumors have also been transfected in vivo with a tumor necrosis factor α gene following direct subcutaneous injection of the tumor with Lipofectin/DNA complexes.[99] Correspondingly, DOPE/DMRIE liposomes have been used to transfect cerebral murine tumors in vivo with the thymidine kinase gene by means of a continuous injection method.[100] A remarkable recent discovery was that liposomes containing DOGS (**9**) were able to transfect the embryos of pregnant mice with a CAT marker gene following intravenous injection of liposome/DNA complexes. The transgene was expressed in both the fetuses and progeny.[101] There are as yet no further reports to corroborate this finding, and so this fascinating discovery should be viewed with some care until that has been done.

Some of the most successful in vivo applications of cationic liposome mediated gene delivery can be found in recent work concerned with the transfection of airway passages. Following a demonstration that DOPE/DOTMA liposomes could deliver a CAT marker gene to murine lung by aerosol administration of the complex,[102] Alton et al.[103] and Hyde et al.[104] reported that DOPE/DC-Chol and Lipofectin liposomes could be used in a similar way to deliver the cystic fibrosis transmembrane conductance regulator (CFTR) gene to the lungs of cystic fibrosis transgenic mice; this resulted in correction of lung defects associated with cystic fibrosis. This demonstration of physical benefit has led to the application of DOPE/DC-Chol liposomes in clinical trials directed at the human gene therapy of cystic fibrosis.[105] Since then Cooper et al.[33] and Lee et al.[32] have reported that DOPE/CTAP and DOPE/lipid 67 cationic liposomes respectively are at least 100-fold more efficient than DOPE/DC-Chol liposomes at delivering a CAT marker gene to murine lung. As a result, lipid 67 (**18**) is now the subject of new cystic fibrosis gene therapy trials. In the future, many more cationic liposome formulations are likely to be tested for their ability to act as gene delivery agents for therapeutic genes in human gene therapy trials.

6. Summary and Outlook

The use of cationic liposomes for the transfer of nucleic acids into primary or cultured cells in vitro is now a well-established technique. There is also a rapidly increasing use of cationic liposomes for the gene delivery in vivo of both marker and potentially therapeutic genes. What are the prospects for cationic liposome mediated gene therapy? I believe the prospects are good. Cationic liposomes have already been involved in human gene therapy clinical trials.[92, 105] Whilst the results indicated that the cationic liposomes used in these trials were not efficient enough to bring about significant clinical benefit, there is every reason to believe that two of the most recently reported cationic liposome formulations, involving lipid 67 (**18**)[32] and CTAP (**19**),[33] are efficient enough for them to be clinically useful in gene therapy approaches to lung disorders.

However, ever improved cationic liposome formulations will be required with ever more improved cytofectins if cationic liposomes are to see widespread use in the human gene therapy of the future. Most notably, cationic liposome/nucleic acid complexes are not very cell-type selective, which may not be a problem for the gene therapy of some lung disorders, but will almost certainly be one for other diseases. Even if the complexes were selective, in general they still bind nucleic acids with relatively low efficiency, so that the amount of liposome required to deliver therapeutically useful levels is too large to be clinically useful. Moreover, the entry of cationic liposome/nucleic acid complexes into cells is really much too slow. Finally, once internalized, too much of the nucleic acids remain trapped in endosome compartments unable either to function in the cytoplasm or traffick to the nucleus to perform a therapeutic function. It is conceivable and possible that some or all of these problems may be solved by the design of new cytofectins. However, to overcome this substantive list of problems, cationic liposomes will probably have to be equiped not only with lipid components, but also with batteries of appropriate ligands designed to deal with cell recognition, accelerating cell entry, endosomolysis, nuclear targeting, etc. In effect, chemists will need to construct artificial viruses.

Some progress has already been made to suggest that cationic liposomes could be developed and improved in the way just described. For instance, a Transfectam/DNA complex has been used to target hepatoma cells using a triantennary galactose ligand attached to the liposome.[106] Recently, mannosylated poly(L-lysine) has been found to promote receptor-mediated gene transfer into macrophages expressing a cell-surface mannose receptor.[107, 108] Other glycosylated poly(L-lysines) also promote receptor-mediated gene transfer into lung epithelial cells[109] and monocyte-derived macrophages.[110] Therefore, the covalent attachment of a variety of monosaccharides, and even oligosaccharides, to cationic liposomes could represent a fruitful area for the development of cell-targeting cationic liposomes. Proteins are also credible cell-targeting ligands, including lectins[111] and/or antibodies.[112] The latter approach has recently been demonstrated with an antithrombomodulin antibody coupled to poly(L-lysine), which was used in combination with DOPE/DC-Chol liposomes to bring about successful targeted transfection of mouse lung endothelial cells.[113] Clean and selective ways of coupling proteins to cationic liposomes will almost certainly have to be developed in the future. The potential use of peptide ligands has been recently demonstrated by Hart et al., who constructed a peptide with an N-terminal integrin-binding amino acid sequence motif [arginine-glycine-aspartic acid (RGD)] and a C-terminal hexadeca(L-lysine) which was able to promote gene delivery by an integrin receptor mediated uptake mechanism.[114] When combined with cationic liposome/DNA complexes, a variant of this peptide enhanced cationic liposome mediated DNA delivery.[127] Barry et al. have also described the selection of cell-binding peptides, derived from random peptide-presenting phage

libraries, which could easily be attached to cationic liposomes in the future.[115]

One useful by-product of this receptor-mediated approach to cell targeting is that the entry of cationic liposome/nucleic acid complexes into cells is generally accelerated. However, the other problems of low nucleic acid binding efficiency, inadequate endosome escape, and inefficient trafficking of nucleic acids to the nucleus still remain. The first problem should easily be solved through the inclusion of polycations with cationic liposomes. For example, polycationic histone proteins have recently been used to enhance lipofectin-mediated gene delivery.[116] In other cases, polycations such as lipopoly(L-lysine) (LPLL),[117] and poly(L-lysine) have proved efficaceous.[118] Polyamine dendrimers may also prove useful in this regard.[118, 119] The remaining problems can almost certainly be overcome, and potential solutions appear to be emerging. For example, Lyons et al. reported some time ago the existence of a pentapeptide nuclear localization signal in adenovirus protein E1a,[120] whilst Gottschalk et al. recently reported an amphipathic peptide capable of disrupting endosomal membranes.[121] Either or both peptides could prove to be useful tools to enhance cationic liposome mediated nucleic acid transfer inside cells.

In addition to all the problems and potential solutions outlined above, there will undoubtedly be additional problems to face depending upon the desired site of action of cationic liposome/nucleic acid complexes in vivo. For instance, administration of complexes to the lung by aerosol, mentioned in connection with cystic fibrosis gene therapy above, involves a completely different set of problems to systemic administration. In the latter case, the need to prolong systemic circulation time and avoid interactions with either plasma components[122] or the Complement system[123] may require that cationic liposomes adopt "stealth" technology,[124, 125] where moieties such as poly(ethylene glycol)[126] or ganglioside G_{M1} oligosaccharide[112] are covalently linked to cationic liposomes.

There are clearly many different possiblities to improve cationic liposome mediated nucleic acid delivery, and a good deal of research into the use of general modifications to confer improvement will be be necessary. However,this potential variety lends hope that cationic liposomes will fulfill their promise as key vector technology for human gene therapy, irrespective of disease type or site of action. Therefore, the design, synthesis, and testing of ever more efficient cytofectins and cationic liposomes offers superb opportunities both now and in the future for the chemists interested in working at the interface between chemistry and medicine.

Appendix: List of Abbreviations

BGTC	bis(guanidinium)-TREN-cholesterol (**21**)
CAT	chloramphenicol acetyl transferase
CEβA	cholesterol ester of β-alanine (**39**)
CTAP	N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (**19**)
DC-Chol	3β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]cholesterol (**14**)
DDAB	dimethyldioctadecylammonium bromide (**12**)
di C 14 amidine	*N*-*tert*-butyl-*N*′-tetradecyl-3-tetradecyl aminopropionamidine (**11**)
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (**4**)
DOGS	dioctadecylamidoglycylspermine · 4 trifluoroacetic acid (**9**)
DOPE	dioleoyl-L-α-phosphatidylethanolamine (**2**)
DORI	1,2-dioleoyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (**7**)
DORIE	1,2-dioleyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (**6**)
DOSPA	2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (**5**)
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)-propane (**3**)
DOTIM	1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (**26**)
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (**1**)
DPPES	dipalmitoylphosphatidylethanolamidospermine · 4 trifluoroacetic acid (**22**)
GAP-DLRIE	(±)-*N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis(dodecyloxy)-1-propanammonium bromide (**8**)
L-PE	lysinyl phosphatidylethanolamine (**23a**)
Lys-Pam_2-Gro*P*Etn	lysinyl-dipalmitoyl-(L)-α-phosphatidylethanolamine · 2 trifluoroacetic acid (**23**)
RET	resonance energy transfer
14 Dea 2	tetradecanoyl-*N*-(trimethylammonioacetyl)diethanolamine chloride (**10**)
$2C_{12}$-L-Glu-ph-C_2-N^+	*O*,*O*′-didodecyl-*N*-[*p*-(2-trimethylammonioethyloxy)benzoyl]-(L)-glutamate (**24**)
$2C_{14}$-L-Glu-C_2-N^+	O,O′-ditetradecyl-*N*-(2-trimethylammonioethanoyl)-(L)-glutamate (**25**)

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