Physico-chemical analysis of cationic liposome–DNA complexes (lipoplexes) with respect to *in vitro* and *in vivo* gene delivery efficiency †

Luisa Stewart,[a] Michelle Manvell,[b] Elizabeth Hillery,[b] Christopher J. Etheridge,[a] Robert G. Cooper,[a] Holger Stark,[c] Marin van-Heel,[d] Monika Preuss,[a] Eric W. F. W. Alton[b] and Andrew D. Miller *[a]

[a] *Imperial College Genetic Therapies Centre, Department of Chemistry, Imperial College of Science, Technology & Medicine, South Kensington, London, UK SW7 2AY*
[b] *Department of Gene Therapy, Imperial College School of Medicine at the National Heart and Lung Institute, Manresa Road, London, UK SW3 6LR*
[c] *Institut für Molekularbiologie und Tumorforschung, Emil-Mannkopff-Strasse 2, 35037 Marburg, Germany*
[d] *Department of Biochemistry, Imperial College of Science, Technology and Medicine, South Kensington, London, UK SW7 2AY*

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Cationic liposomes are potential vectors for gene therapy applications. In previous work, our first generation cationic liposome system, formulated from cytofectin 3β-[*N*-(*N′*,*N′*-dimethylaminoethyl)carbamoyl]cholesterol (DC-Chol) and the neutral phospholipid dioleoyl-L-α-phosphatidylethanolamine (DOPE), was shown to transfect the lungs of mice *in vivo*. More recently, we described second generation cationic liposome systems including one formulated from DOPE and the novel pentaamine cytofectin N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP). As a result of formulation changes, CTAP–DOPE cationic liposomes are shown here to be approximately 400-fold more efficient at mediating gene delivery to mouse lung *in vivo* than DC-Chol–DOPE liposomes (2000-fold more effective than plasmid DNA alone). Physico-chemical analyses were performed on CTAP–DOPE, other second generation cationic liposome systems and DC-Chol–DOPE to determine how differences in the structural and physical properties of cytofectins, cationic liposomes and lipoplex mixtures might affect the efficiency of transfection both *in vitro* and *in vivo*. The data suggest that CTAP–DOPE cationic liposomes are effective *in vivo* for two reasons. (1) They are able to efficiently neutralise, condense and encapsulate nucleic acids into lipoplex particles; (2) they present unprotonated amine functional groups ($pK_a < 8$) at neutral pH that could have the capacity for endosome buffering, thereby facilitating nucleic acid escape from endosome compartments into the cytosol following cell entry, like polyethylenimine. Weak, inefficient neutralisation, condensation and encapsulation of nucleic acids and the presence of unprotonated amine functional groups appear to be desirable liposome characteristics for *in vitro* transfection. The inclusion of "natural" propylene and butylene spacings between the amine functional groups of cytofectin head groups appears to promote efficient neutralisation, condensation and encapsulation. The inclusion of some "unnatural" ethylene spacings appears to be a useful way of lowering amine pK_a values.

Introduction‡

The basic concept of gene therapy is that nucleic acid constructs may be identified which should be able to correct basic pathophysiological defect(s) if delivered to the appropriate organs and cells of the body *in vivo*. However, this delivery process is not straightforward. Whilst naked DNA may be administered under certain circumstances, a delivery "vehicle" or vector is usually required for efficient nucleic acid delivery. Several non-viral physical or chemical-based vector systems are known but none are currently efficacious enough for general use in clinical gene therapy.[1] In spite of this, cationic liposome-based nucleic acid delivery systems are showing some promise.[2]

Cationic liposomes are lipid vesicles formed typically from a combination of a cationic amphiphile (cytofectin) and a neutral co-lipid. They mediate nucleic acid delivery by interacting electrostatically with negatively charged nucleic acid sequences forming cationic liposome–nucleic acid complexes (known as lipoplexes) which enter cells by interaction with cell surface proteoglycans,[3] followed by endocytosis,[4,5] or phagocytosis[6] and then release nucleic acids for expression in the cell nucleus.[2] Our first generation cationic liposome system was formed from first generation cytofectin 3β-[*N*-(*N′*,*N′*-dimethylaminoethyl)carbamoyl]cholesterol (DC-Chol) **1** and

† Cationic lipids for gene therapy. Part 4. For Part 3, see ref. 41.
‡ Abbreviations: Chol: cholesteryl; DC-Chol: 3β-[*N*-(*N′*,*N′*-dimethylaminoethyl)carbamoyl]cholesterol; DOPE: dioleoyl-L-α-phosphatidylethanolamine; ACHx: 3-aza-N^1-cholesteryloxycarbonylhexane-1,6-diamine; ACO: 4-aza-N^1-cholesteryloxycarbonyloctane-1,8-diamine; CDAN: N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; CDAD: N^1-cholesteryloxycarbonyl-4,9-diazadodecane-1,12-diamine; CTAP: N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine; CTAH: N^{16}-cholesteryloxycarbonyl-4,8,13-triazahexadecane-1,16-diamine; HEPES: *N*-(2-hydroxyethyl)piperazine-*N′*-(ethane-2-sulfonic acid); CAT: chloramphenicol acetyl transferase; CFTR: cystic fibrosis transmembrane conductance regulator protein; PCS: photon correlation spectroscopy.

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the neutral phospholipid dioleoyl-L-α-phosphatidylethanolamine (DOPE) **2**. This was shown to mediate the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to the lungs of transgenic mice *in vivo*.[7] Our work with DC-Chol–DOPE liposomes was one of the first proof-of-principle studies for non-viral gene therapy. More recently, we described second generation cationic liposome systems that were formulated from polyamine analogues of DC-Chol (second generation cytofectins) and **2**.[8] Of these, one cationic liposome system in particular formulated from the pentaamine N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP) **3** and **2** was found to mediate the delivery of the chloramphenicol acetyl transferase (CAT) reporter gene to the lungs of mice *in vivo* at least 100-fold more efficiently than the first generation DC-Chol–DOPE cationic liposome system, bringing us significantly closer to the necessary efficacy for gene delivery to the human lung.[8] At the time, the CTAP–DOPE cationic liposome system was one of the most efficient non-viral systems available for the delivery of nucleic acids *in vivo* to lung, matched and improved by only one other cholesterol-based cationic liposome system, containing the cationic amphiphile GL67.[9] In general, such cholesterol-based systems as CTAP–DOPE have proved popular owing to versatility and low toxicity.[2,10] Moreover, they have recently shown some exciting clinical potential for cystic fibrosis gene therapy applications as well.[11]

However, in order to progress towards third generation systems appropriate for routine clinical use, we realised the need for some physico-chemical understanding. There have been a number of significant physico-chemical studies reported with cationic liposomes and lipoplex mixtures over the last few years, but in each case structure–activity correlations have proved either difficult to establish or rather limited in scope.[8,9,12–18] For this reason, we decided to carry out our own physico-chemical study to try and identify structure–activity correlations important to nucleic acid delivery mediated by CTAP–DOPE and other related second generation cationic liposome systems. We hoped that results of this physico-chemical study might suggest the next step forward in the process of designing efficient non-viral vectors by revealing useful correlations between transfection efficiency, both *in vitro* and *in vivo* in lung, and the structural–physical properties of cytofectins, cationic liposomes and lipoplex mixtures.

Results

Cationic liposomes

A selection of six second generation cationic liposome systems were prepared for comparison with the original first generation DC-Chol–DOPE liposome system. These six second generation systems were formulated from DOPE **2** and six different second generation cytofectins. The chosen cytofectins consisted of two triamines 3-aza-N^1-cholesteryloxycarbonylhexane-1,6-diamine (ACHx; CJE52) **4** and 4-aza-N^1-cholesteryloxycarbonyloctane-1,8-diamine (ACO; B130) **5**, two tetraamines N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN; B198) **6** and N^1-cholesteryloxycarbonyl-4,9-diazadodecane-1,12-diamine (CDAD; B185) **7**, and finally two pentaamines N^{16}-cholesteryloxycarbonyl-4,8,13-triazahexadecane-1,16-diamine (CTAH; B222) **8** and CTAP **3** (Fig. 1). Second generation cytofectins **5**, **7** and **8** were selected because the amine functional groups in their head groups are all separated by "natural" propylene and butylene spacings, equivalent to those found in natural polyamines spermidine **9** and spermine **10**. Cytofectins **3**, **4** and **6** were selected for comparison because some of their amine functional groups are separated by shorter "unnatural" ethylene spacings. In our previously published work, we concluded that both the number and spacing of amine functional groups in second generation cytofectins may be important determinants of efficient nucleic acid delivery.[8] Therefore, all six were selected in order to define further the relative importance of both structural features.

All second generation cationic liposome systems were formulated with a constant 6 : 4 mol ratio of cytofectin (3–5 mg, 6 μmol) to **2** (3 mg, 4 μmol) by means of the reverse-phase evaporation vesicle (REV) method as opposed to the dehydration–rehydration used previously.[8,19] The dehydration–rehydration method was sometimes observed to result in heterogeneous cationic liposome mixtures with some large (>3000 nm) aggregate particles. This aggregation problem was completely averted by using a combination of the REV method and the mol ratio of cytofectin to **2** given above. REV cationic liposome suspensions (5 ml) (1.2 mM cytofectin concentration, [cyt]) were found to contain liposome vesicles of between 100–200 nm in diameter with the exception of the DC-Chol–DOPE system (average 400–500 nm in diameter). After preparation, all cationic liposome suspensions were stored at 4–8 °C without any apparent change in size or aggregation-state and generally used to prepare lipoplex mixtures within one week.

In vitro and *in vivo* gene delivery

Lipoplex mixtures were prepared for *in vitro* gene delivery experiments by combining aliquots of pCIβgal plasmid (final nucleotide concentration, [nt], 0.1 mM; dose 1 μg) with cationic liposome suspensions in OptiMEM solution. The pCIβgal plasmid is a close relative of the well-known pCMVβ plasmid that expresses the marker gene for the enzyme β-galactosidase (βgal) and the level of βgal enzyme activity in cells post-transfection is a direct measure of the efficiency of gene delivery. The optimised transfection results [in African Green Monkey kidney cells (COS-7)] are summarised in Fig. 2 [lipid : DNA (w/w) ratios are expressed as the mol ratio of cytofectin to nucleotide concentration ([cyt]/[nt]) so as to facilitate comparison with the physico-chemical data]. Only one second generation cationic liposome system, CDAN–DOPE, was found to mediate gene delivery into COS-7 cells more efficiently (3-fold) *in vitro* than DC-Chol–DOPE, within experimental error. These *in vitro* results are broadly in line with our previous *in vitro* results. Differences between these and the previous data probably reflect the fact that a different cell line (CFT1 cells) and different liposome formulation procedures were used previously.[8,19]

Lipoplex mixtures for *in vivo* gene delivery were prepared by adding aliquots of cationic liposome suspensions ([cyt] 1.2 mM) directly to fixed aliquots (50 μl) of pCF1-CAT plasmid solution (initial [nt] 4.8 mM; dose 80 μg). This pCF1-CAT plasmid expresses the marker gene for the enzyme chloramphenicol acetyl transferase (CAT). The level of CAT enzyme activity post-transfection is well known to be one of the most sensitive measures of direct gene delivery efficiency *in vivo*. Furthermore, the pCF1-CAT plasmid is similar in size to the pCIβgal and pCMVβ plasmids mentioned above, thereby ruling out any effect of plasmid size on transfection efficiency.[13] Optimised transfection results [in female Balb/c mice] using the CTAP–DOPE system are shown alongside results obtained with DC-Chol–DOPE and CDAN–DOPE (Fig. 3). *In vivo* results obtained with the other the second generation systems (not shown) were the same within experimental error or less than CDAN–DOPE, broadly in line with *in vivo* data trends reported previously.[8] There is one important difference, in this study CTAP–DOPE was found to be some 400-fold more effective on average than DC-Chol–DOPE (equivalent to approx. 2000-fold the efficacy of DNA alone), 4-fold higher than observed previously.[8] This may reflect the fact that CTAP–DOPE liposomes were formulated by the more reliable REV method in this study (see above) rather than by the dehydration–rehydration method used previously. The CTAP–DOPE system was the only second generation cationic lipo-

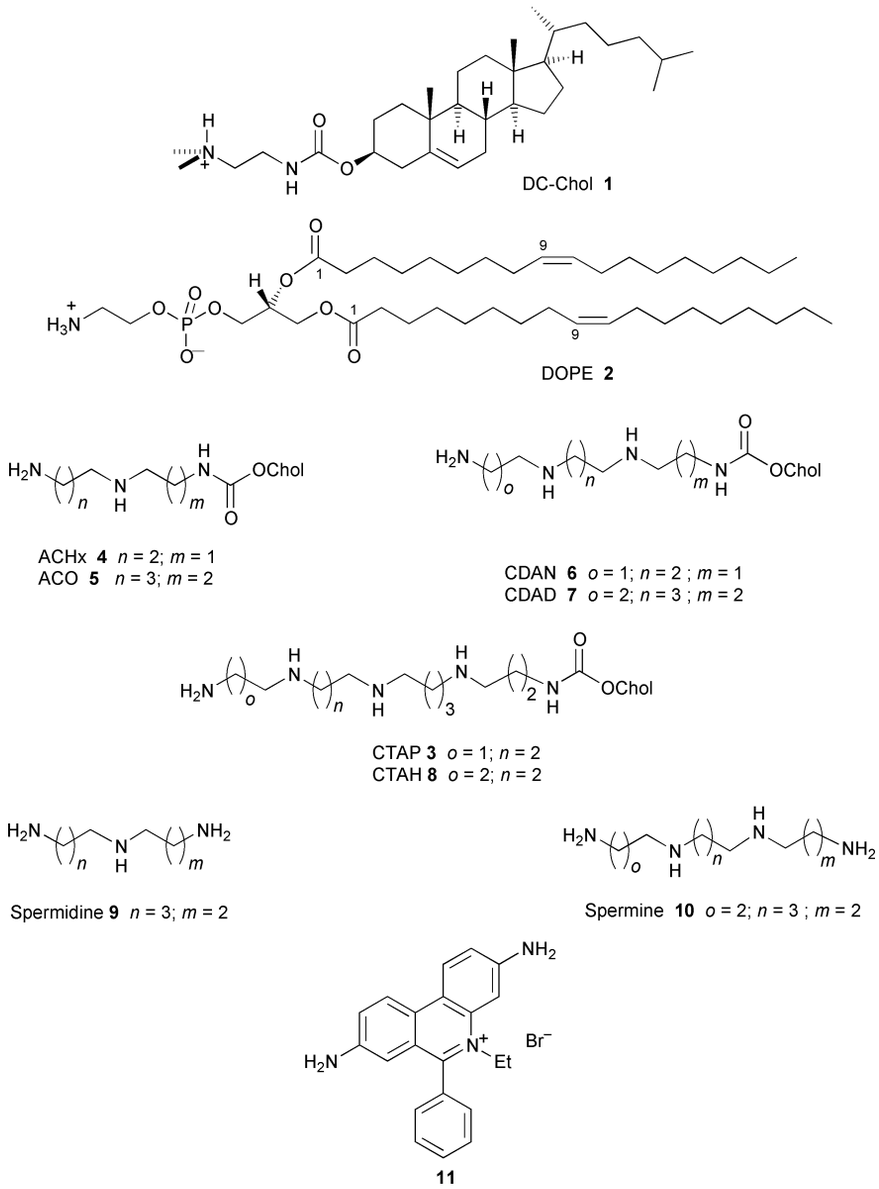

Fig. 1 Summary of main chemical structures.

some system to show any truly significant improvement over DC-Chol–DOPE *in vivo*.

Light scattering analysis of lipoplex formation

The first physico-chemical studies performed were light scattering experiments. Light scattering is sensitive to changes in the state of nano-particles such as cationic liposomes. Therefore, we expected that changes in the dynamic light scattering behaviour of cationic liposome suspensions, following the addition of a plasmid (pCMVβ), would allow the equilibration of the resulting lipoplex mixtures to be followed as a function of time. This indeed appeared to be the case. When a fixed concentration of a given cationic liposome system (final [cyt] 0.06 mM) was mixed with pCMVβ plasmid, the intensity of light scattered was found to increase with time and reached a plateau in 5 min or less, irrespective of whether the [cyt]/[nt] ratio was 0.6, 1.1 or 3.3. Representative results are shown for the DC-Chol–DOPE system (Fig. 4). These equilibration times correlate well with times determined by resonance energy transfer.[20] Since lipoplex mixtures used in all studies were routinely left to equilibrate for at least 15 min prior to administration, there is no doubt that all had reached structural equilibrium before use. The difference in concentration between lipoplex mixtures used *in vivo* and those used *in vitro* would not appear to influence equilibration rates according to available data published elsewhere.[9]

Electron microscopy

Electron microscopy studies were performed to determine the physical state of an equilibrated lipoplex mixture prepared at the [cyt]/[nt] ratio optimal for efficient *in vitro* and *in vivo* gene delivery (see Figs. 2 and 3). A CDAN–DOPE lipoplex mixture (final [cyt] 0.1 mM) prepared with the pCMVβ plasmid at an optimal [cyt]/[nt] ratio of 0.6 was observed by cryo-electron microscopy (Fig. 5). At this optimal [cyt]/[nt] ratio the lipoplex mixture was actually found to have an overall negative charge (see below). In this environment, discrete lipoplex particles (60 to 250 nm in diameter) were observed consisting of some fused structures, reminiscent of those reported elsewhere,[4,21–24] and some single spherical entities also bearing close resemblance to particles reported previously.[4,21,23,25] Free DNA was visible (not shown) but strands or "spaghetti"-like structures as well as other large and/or heterogeneous structures that have been reported previously[4,24,26,27] were not observed. In short, these lipoplex particles that we were observing at an optimal [cyt]/[nt] ratio for *in vitro* and *in vivo* transfection were surprisingly homogeneous. On many of the particles, regular striations were visible with a periodicity of 4.2 ± 2 nm. This distance compares

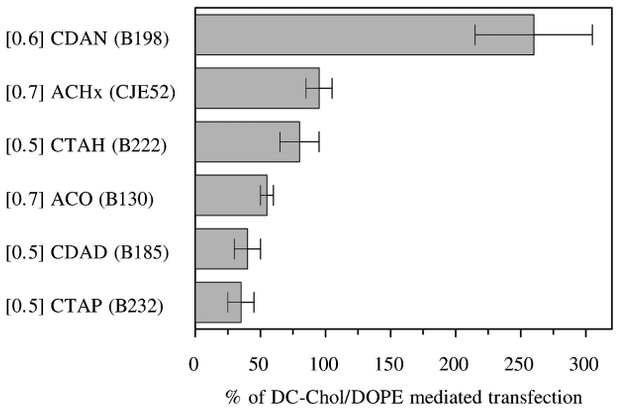

Fig. 2 Efficiency of cationic liposome-mediated gene delivery *in vitro*. Results are expressed as a percentage of β-galactosidase enzyme activity following delivery of pCIβgal plasmid mediated by DC-Chol–DOPE cationic liposomes. The number in square brackets refers to the mol ratio of cytofectin to nucleotide ([cyt]/[nt]) in the optimal lipoplex mixtures used in each case to transfect the COS-7 cells. See Fig. 1 for structures of cytofectins. The Mann–Whitney U Test was used to compare group means and the null hypothesis rejected at $\rho < 0.05$. Error bars indicate SEM; $n = 6$ for each optimized lipoplex mixture studied. Optimal DC-Chol–DOPE cationic liposome-mediated transfection was obtained at a [cyt]/[nt] ratio of approximately 1.7. All optimal [cyt]/[nt] ratios for transfection correspond to 2 : 1 lipid–DNA (w/w).

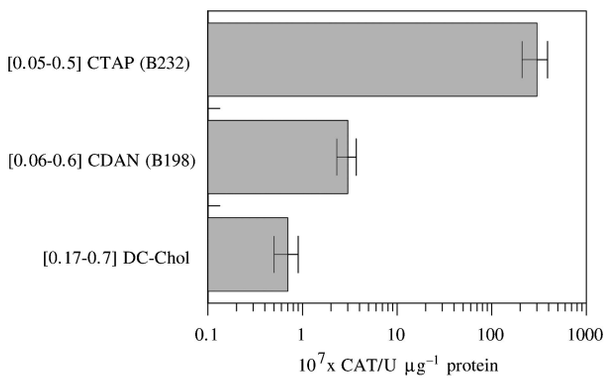

Fig. 3 Efficiency of cationic liposome-mediated gene delivery *in vivo*. Results are given as amount of chloramphenicol transferase (CAT) enzyme activity measured per μg of total lung protein, 2 days following cationic liposome-mediated delivery of CAT gene expressing plasmid (pCF1-CAT). In each case, the numbers in square brackets refer to the [cyt]/[nt] mol ratios of the optimal lipoplex mixtures used. See Fig. 1 for structures of cytofectins. The Mann–Whitney U Test was used to compare group means and the null hypothesis rejected at $\rho < 0.05$. Error bars indicate SEM; $n = 6$ for each optimized lipoplex mixture studied. Optimal CTAP–DOPE and CDAN–DOPE [cyt]/[nt] ratios of [0.05–0.5] and [0.06–0.6] respectively both correspond to a 0.2–2 : 1 lipid–DNA (w/w). Optimal DC-Chol–DOPE [cyt]/[nt] ratio of [0.17–0.7] corresponds to 0.5–2 : 1 lipid–DNA (w/w).

well with periodicities of 6.5 and 3.5 nm measured by others in small-angle X-ray scattering (SAXS) and cryo-electron microscopic analysis of alternative lipoplex mixtures.[23,25,28] Therefore, a similar form of lipid–DNA close packing is probably being observed in which pCMVβ plasmid molecules are condensed and encapsulated in regular periodic arrays within a multilammellar lipid assembly. The observed homogeneity of the lipoplex mixture may well suggest that the observed particles are the actual structures responsible for transfection activity.

Cationic liposome–nucleic acid interaction assays

Ethidium§ bromide exclusion, gel retardation and photon correlation spectroscopy assays were performed to determine

§ IUPAC name for ethidium is 3,8-diamino-5-ethyl-6-phenylphenanthridinium.

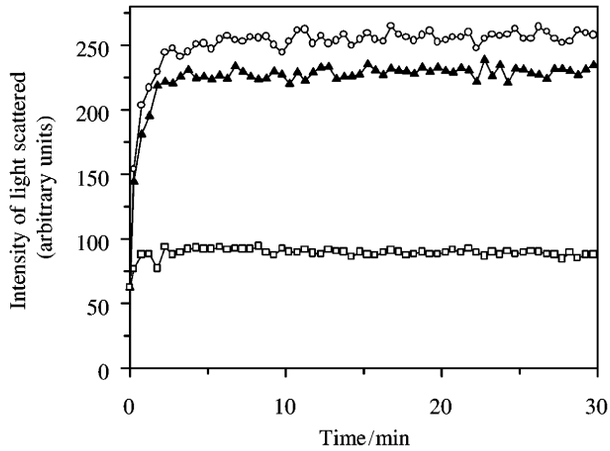

Fig. 4 Light scattering analysis of lipoplex formation. Change in light scattering intensity was monitored as a function of time following the combination of DC-Chol–DOPE cationic liposomes with pCMVβ plasmid, giving lipoplex mixtures with [cyt]/[nt] mol ratios of 0.6 (○), 1.1 (▲) and 3.3 (□). The final lipid concentration was 0.1 mM in each mixture.

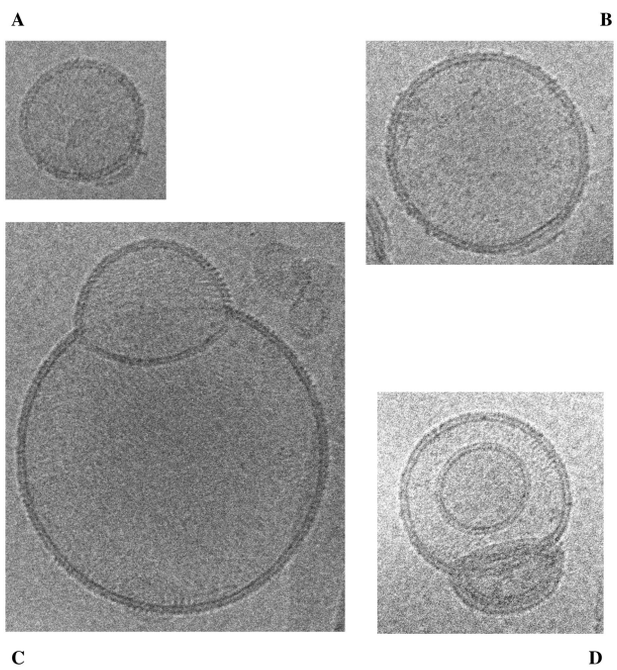

Fig. 5 Cryo-electron microscopy images of lipoplex particles. Representative selection of electron micrographs of lipoplex particles formed after the combination of CDAN–DOPE cationic liposomes and pCMVβ plasmid in the [cyt]/[nt] mol ratio of 0.6. Final lipid concentration was 0.17 mM. Magnification is 180 000 × (1 cm = 56 nm).

how well each cationic liposome system was able to charge–neutralise, condense and encapsulate nucleic acids within lipoplex particles. In our previous work,[8] it was indicated that efficient gene delivery *in vivo* may require cationic liposome systems able to bind DNA more tightly than is appropriate for *in vitro* use. All three assays were employed to test this proposal.

Exclusion assays were performed as follows. Fixed aliquots of the pCMVβ plasmid (final [nt] 0.03 mM) were incubated with different amounts of cationic liposomes then ethidium bromide **11** (final [**11**] 4.6 μM) (Fig. 1) was added and the fluorescence emission intensity recorded at 590 nm (excitation at 260 nm), and plotted as a function of [cyt]/[nt] (Fig. 6). Assays of this type were pioneered by Gershon *et al.* who showed that the formation of lipoplex particles excludes the intercalation of nucleic acid base pairs by **11**, thereby causing a corresponding reduction in fluorescence emission intensity.[26] Emission intensity reaches a basal minimum when nucleic acid encapsulation

Table 1 Summary of cytofectin and cationic liposome data[a]

Cationic liposome system	Cytofectin head group[b] pK_a values	Nominal cytofectin head group charge at pH 7.8	$([cyt]/[nt])_R$
DC-Chol **1**–DOPE **2**	8.4	1	>1.8
ACHx **4**–DOPE **2**	10.4, 7.7	1.5	1.6
CDAN **6**–DOPE **2**	10.7, 7.9, 7.5	1.5	1.6
ACO **5**–DOPE **2**	10.6, 9.8	2	1.0
CDAD **7**–DOPE **2**	10.7, 9.8, 9.5	3	0.8
CTAP **3**–DOPE **2**	10.7, 9.8, 9.1, 7.5	3	0.8
CTAH **8**–DOPE **2**	10.7, 9.8, 9.5, 9.5	4	0.4

[a] The table includes cytofectin pK_a values and nominal charges at pH 7.8 (estimated by standard procedures),[30] and the mol ratios of cationic liposome to nucleotide, $([cyt]/[nt])_R$, at which pCMVβ plasmid electrophoretic mobility in agarose gels became retarded by complexation with cationic liposomes (see text for details). [b] See Fig. 1 for structures of cytofectins.

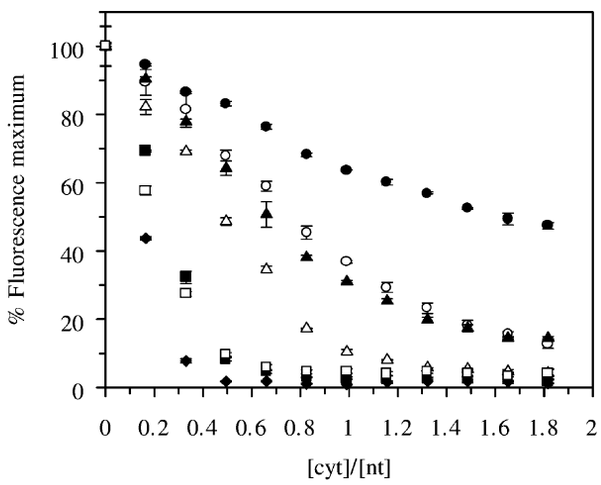

Fig. 6 Ethidium bromide exclusion assays. Changes in pCMVβ plasmid-induced ethidium bromide fluorescence as a function of increasing concentrations of cationic liposomes. Results are expressed in terms of [cyt]/[nt] mol ratios. A fixed concentration of pCMVβ plasmid (final [nt] 0.03 mM) was separately combined with various amounts of DC-Chol–DOPE (●), ACHx–DOPE (○), CDAN–DOPE (▲), ACO–DOPE (△), CDAD–DOPE (■), CTAP–DOPE (□) or CTAH–DOPE (◆) cationic liposomes (final [cyt] range 0 to 0.06 mM) giving a complete set of lipoplex mixtures with different [cyt]/[nt] mol ratios. After 15 min equilibration, ethidium bromide **11** was added to each mixture (final [**11**] 4.6 μM giving a saturating final [nt]/[**11**] mol ratio of 6.5) and after a further 2 min, the fluorescence emission intensities at 590 nm were measured for each set of lipoplex mixtures. The excitation wavelength was 260 nm.

is complete leaving no base pairs free for intercalation.[29] A saturating 6 : 1 ([nt]/[**11**]) mol ratio was used throughout our experiments to avoid complications from limiting amounts of **11**, as described previously.[21] In order to interpret these results more easily, the nominal charge of each cytofectin at pH 7.8 was determined from amine functional group pK_a values estimated according to standard procedures (see Table 1).[30] These estimates have been corroborated by an alternative experimental evaluation of cholesterol polyamine pK_a values reported by Geall *et al.*[12] There appears to be a direct correlation between the estimated nominal charge of each cytofectin head group (see Table 1) and the ability of each corresponding cationic liposome system to interact with, condense and encapsulate nucleic acids. The higher the nominal charge, the more readily fluorescence emission was observed to decline with increasing [cyt]/[nt], consistent with more effective condensation and encapsulation (Fig. 6).

A similar correlation was observed with agarose gel retardation assays. For each cationic liposome system in turn, aliquots of the pCMVβ plasmid (final [nt] 0.06 mM) were separately incubated for 15 min with different amounts of cationic liposome (final [cyt] range 0 to 0.12 mM) and the resulting lipoplex mixtures analyzed side by side on a single agarose gel. Nucleic acids migrate towards the anode at a rate proportional to their molecular weight in response to an electrical potential applied across an agarose gel. This migration is known to be hindered by the formation of lipoplex particles, owing to complex formation and negative charge neutralization, and is totally hindered or retarded when complete nucleic acid encapsulation has occurred.[21,26,31] The term $([cyt]/[nt])_R$ represents the [cyt]/[nt] ratio at which complete complex formation and charge neutralisation have occurred. The lower the ratio, the more readily does a given cationic liposome system neutralise and encapsulate nucleic acids within lipoplex particles. Once again, there was also observed to be a direct correlation between the estimated nominal charge of each cytofectin head group and the ability of each corresponding cationic liposome system to complex and neutralise nucleic acids. The higher the nominal charge, the lower was the observed value of $([cyt]/[nt])_R$, consistent with more effective complexation and neutralisation (see Table 1).

The apparent correlation between the estimated nominal charge of each cytofectin head group and the ability of each corresponding cationic liposome to neutralise nucleic acid charge was also observed by photon correlation spectroscopy (PCS) assays. PCS experiments were performed in the following way. For each cationic liposome system, a series of lipoplex mixtures were prepared with a fixed concentration of cationic liposome (final [cyt] 0.06 mM) and various concentrations of pCMVβ plasmid (final [nt] range 0.18 to 0.009 mM; [cyt]/[nt] ratio range 0.3 to 6.6). Mean lipoplex particle diameter was then plotted as a function of the [cyt]/[nt] ratio (Fig. 7). PCS uses light scattering to determine a mean particle diameter assuming essentially monodisperse, spherical particles. Although neither assumption is necessarily true for lipoplex mixtures,[4,26] PCS will still give a reasonable measure of gross changes in particle behaviour in solution. There is a general tendency for cationic liposome systems to form small particles with nucleic acids at low [cyt]/[nt] ratios owing to electrostatic repulsion of components with an excess of negative charge, and also at high ratios due to repulsion of components with an excess of positive charge. Large particles form as a consequence of colloidal instability when the positive and negative charges in the lipoplex mixture are neutralised.[4,20,28,32] Therefore, PCS gives an alternative measure of the [cyt]/[nt] ratio at which nucleic acid charge neutralisation has taken place. As for gel retardation assay data, there was observed to be a direct correlation between the estimated nominal charge of each cytofectin head group and the ability of each corresponding cationic liposome system to neutralise nucleic acid charge. The higher the nominal charge, the lower the [cyt]/[nt] ratio at which neutralisation (judged by the formation of large particles) was achieved (see Table 1, Fig. 7).

It is noteworthy from the PCS data that optimal [cyt]/[nt] ratios for *in vitro* and *in vivo* transfection (Figs. 2 and 3) mostly

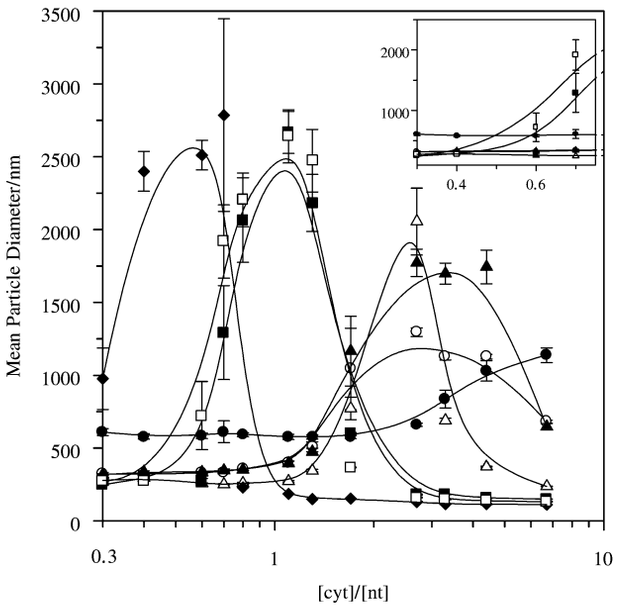

Fig. 7 Photon correlation spectroscopic analyses of lipoplex mixtures. Changes in lipoplex particle size as a function of increasing concentrations of pCMVβ plasmid. Results are expressed in terms of [cyt]/[nt] mol ratios. Fixed concentrations (final [cyt] 0.06 mM) of either DC-Chol–DOPE (●), ACHx–DOPE (○), CDAN–DOPE (▲), ACO–DOPE (△), CDAD–DOPE (■), CTAP–DOPE (□) or CTAH–DOPE (◆) cationic liposomes were separately combined with various amounts of pCMVβ plasmid (final [nt] range 0.009 to 0.18 mM) giving a set of lipoplex mixtures with different [cyt]/[nt] mol ratios for each different cationic liposome system. The average diameters of lipoplex particles in each set of lipoplex mixtures were determined by separate spectroscopic runs comprising of five consecutive scans, each of 200 s in duration.

coincide with lipoplex mixtures that have an overall negative charge and contain small lipoplex particles (200–300 nm in diameter). This observation makes an interesting contrast with some reports,[33] but is well corroborated by others too.[9,34] The CTAH–DOPE system is the exception, the optimal [cyt]/[nt] ratio for *in vitro* transfection (0.5) corresponds to neutral lipoplex mixtures with very large aggregates (possibly aggregates of smaller particles). However, this system was not observed to mediate effective transfection *in vivo* either here or previously.[8]

Discussion

Ethidium bromide exclusion, gel retardation and PCS assays all support a direct correlation between the estimated nominal charge at pH 7.8 of each cytofectin head group and the ability of each corresponding cationic liposome system to neutralise, condense and encapsulate nucleic acids into lipoplex particles at the same pH. The higher the charge, the more effective the neutralisation, condensation and encapsulation. This nominal charge appears to be a function of the number of amine functional groups per cytofectin head group and their relative distances of separation, where second generation cytofectins are concerned (Table 1). When amine functional groups are separated by three or four methylene groups (propylene or butylene spacings respectively), pK_a values are apparently >9, thereby ensuring essentially complete functional group protonation at pH 7.8. However, if the separation is only two methylene groups (ethylene spacing), then pK_a values are apparently perturbed to <8, ensuring that only partial protonation would take place at pH 7.8. The consequence of this combined effect appears to be, for instance, that cationic liposomes formulated with "unnatural" tetraamine CDAN **6** were less effective at neutralising, condensing and encapsulating nucleic acids into lipoplex particles than liposomes formulated with the "natural" triamine ACO **5**. Similarly, cationic liposomes formulated with "unnatural" pentaamine CTAP **3** were equivalent to liposomes formulated with the "natural" tetraamine CDAD **7** and less effective than liposomes formulated with the "natural" pentaamine CTAH **8**.

In effect, the inclusion of ethylene spacings appears to significantly weaken and perturb cationic liposome–nucleic acid interactions. Geall *et al.* have reached a similar conclusion in their recently published work on the interactions between cholesterol polyamines and nucleic acids.[12] Published work on polyamine–DNA interactions also supports this conclusion by showing that natural propylene and butylene spacings are required for a tight binding interaction between polyamines and double helical DNA.[35] However, Geall *et al.* went on to conclude that "high affinity binding" of nucleic acids by their naked polyamines resulted in higher *in vitro* transfection of CHO cells.[12] Our *in vitro* data obtained using cationic liposome systems, rather than naked polyamines, show the opposite effect (Fig. 2). In this study, the CDAN–DOPE cationic liposome system was not only our most effective transfection system *in vitro* but the only second generation system to surpass DC-Chol–DOPE. A direct comparison between our CDAN–DOPE and DC-Chol–DOPE systems (not to mention the ACHx–DOPE system) reveals that none were able to neutralise, condense and encapsulate nucleic acids into lipoplex particles particularly efficiently according to exclusion, gel retardation and PCS assay data (Figs. 6 and 7, Table 1). In comparison, the other second generation cationic liposome systems studied were able to do so more efficiently but were obviously less effective transfection systems than DC-Chol–DOPE. In other words, the efficient *in vitro* transfection of COS-7 cells appears to require cationic liposome systems that exhibit weak, inefficient neutralisation, condensation and encapsulation of nucleic acids.

The primary objective of the work described here was to try and identify structure–activity correlations important to nucleic acid delivery mediated by CTAP–DOPE and other second generation cationic liposomes, thereby suggesting the next step forward in the design process for efficient non-viral vectors with future lung applications. Therefore, alongside *in vitro* transfections *in vivo* lung transfection experiments were also performed (Fig. 3). In this case, only the CTAP–DOPE cationic liposome system showed any truly significant improvement over and above the first generation DC-Chol–DOPE system, in line with previous data.[8] By contrast, the CTAP–DOPE liposome system was poor at mediating *in vitro* transfection (Fig. 2). In comparison to CDAN–DOPE or DC-Chol–DOPE systems, CTAP–DOPE was able to neutralise, condense and encapsulate nucleic acids into lipoplex particles with generally high efficiency according to exclusion, gel retardation and PCS assay data (Figs. 6 and 7, Table 1). Certainly, such a characteristic might be expected to be useful *in vivo* given the greater complexity of the extracellular milieu *in vivo* as compared to *in vitro*. However, were that all required, then CTAH–DOPE, CDAD–DOPE and even ACO–DOPE systems would have performed as well *in vivo* and they did not either in this study or previously.[8] Therefore, other factors are involved. In our opinion, the presence of amine functional groups with low pK_a values (<8) in the cytofectin head groups may make an important contribution. Whilst unprotonated amine functional groups may not be able to contribute to the neutralisation, condensation and encapsulation of nucleic acids into lipoplex particles (as shown above), they could assist in the release of nucleic acids following entry of lipoplex particles into the cell. Amine functional groups with low pK_a values (<8) in polyethylenimine (PEI) have already been proposed to have the capacity to buffer intracellular endosome compartments following endocytosis, thereby promoting osmotic shock of endosome compartment membranes and escape of nucleic acids into the cytosol.[36] Therefore, our proposal is not unreasonable.

Taking such amine functional groups into account, it is interesting to note that the head group of CDAN **6** presents two

secondary amines and one primary amine with estimated pK_a values of 10.7, 7.9 and 7.5 respectively (Table 1). This is the only cytofectin studied here with two such amine functional groups with low pK_a values (<8). The head group of DC-Chol **1** presents only one tertiary amine of pK_a 8.4. Therefore, we would suggest that the CDAN–DOPE system outperforms the DC-Chol–DOPE system *in vitro* by possessing a weak capacity to neutralise, condense and encapsulate nucleic acids (like DC-Chol–DOPE) but augmented by a PEI-like low pK_a-enhanced capacity to release nucleic acids from endosome compartments following endocytosis. The potential availability of unprotonated amine functional groups for this purpose is substantiated experimentally by the fact that CDAN–DOPE neutralises, condenses and encapsulates nucleic acids significantly less effectively than ACO–DOPE liposomes (Figs. 6 and 7, Table 1) even though CDAN **6** presents one more amine functional group than ACO **5**.

In a similar way, the head group of CTAP **3** presents three secondary amines and one primary amine with estimated pK_a values of 10.7, 9.8, 9.1 and 7.5 respectively (Table 1). By contrast, the head groups of ACO **5**, CDAD **7** and CTAH **8** do not present amine functional groups with low pK_a values (<8). Therefore, we suggest that CTAP–DOPE outperforms others *in vivo* by possessing a strong capacity to neutralise, condense and encapsulate nucleic acids (like ACO–DOPE, CDAD–DOPE and CTAH–DOPE), but augmented by the same low pK_a-enhanced capacity to release nucleic acids from endosome compartments after cell entry as described above. The potential availability of unprotonated amine functional groups for this purpose is also substantiated experimentally by the fact that CTAP–DOPE and CDAD–DOPE cationic liposomes neutralise, condense and encapsulate nucleic acids to the same extent (Figs. 6 and 7, Table 1) although CTAP **3** presents one more secondary amine than CDAD **7**.

Conclusion

Previous attempts to form correlations between structural–physical properties have made a number of interesting but limited correlations. Akao *et al.*[14] have suggested that successful gene delivery will occur when the phase-transition temperature, T_c, of the cationic liposome formulation is less than 37 °C. However, this has not been supported by more recent studies,[15] although lower phase transition temperatures appear to be helpful in some cases.[16] Other authors have suggested a proportional relationship between the zeta potential of cholesterol-based cationic liposomes and their gene delivery efficiency.[17] In our case, the zeta potentials of our cationic liposome systems were too similar within experimental error to confirm any such conclusions. Still others have systematically catalogued the deleterious effects of electron withdrawing groups in cytofectin head groups on transfection efficiency.[18] In this study, we have shown how the differing ability of cationic liposome systems to efficiently neutralise, condense and encapsulate nucleic acids into lipoplex particles as well as to present unprotonated amine functional groups (pK_a <8) (presumably for endosome buffering and osmotic shock of endosome compartments) affects transfection efficiency *in vitro* and in lung *in vivo*. Both the number of amine functional groups per cytofectin head group and their relative distances of separation have crucial roles to play in governing these properties. The inclusion of "natural" propylene and butylene spacings between amine functional groups appears to promote efficient neutralisation, condensation and encapsulation. The inclusion of some "unnatural" ethylene spacings appears to be a useful way of lowering amine pK_a values. On this basis, CTAP–DOPE cationic liposomes appear to be effective *in vivo* because they are able to efficiently neutralise, condense and encapsulate nucleic acids into lipoplex particles, and present unprotonated amine functional groups (pK_a < 8) at neutral pH. By contrast, weak, inefficient neutralisation, condensation and encapsulation of nucleic acids and the presence of unprotonated amine functional groups appear to be desirable liposome characteristics for *in vitro* transfection.

Our observations have been made using DC-Chol–DOPE, CTAP–DOPE and other related second generation cationic liposome systems. Therefore, given the close family relationship of these systems, it is necessary to be cautious about the general applicability of our results. Nevertheless, to the best of our knowledge, this is the first study to identify structure–activity correlations that appear to relate to both *in vitro* and *in vivo* cationic liposome-mediated transfection. We might speculate that a polyamine polymer designed around the particular spacings of CTAP **3** could represent an interesting alternative non-viral vector system to CTAP–DOPE cationic liposomes. With respect to the future of our cationic liposome-based systems for lung applications *in vivo*, the requirement for strong, efficient neutralisation, condensation and encapsulation of nucleic acids with an additional facility for the buffering of endosome compartments suggests that stable nucleic acid encapsulation systems like lipid–protamine–DNA particles,[37] HVJ-cationic liposomes,[38] or indeed stabilized plasmid–lipid particles (SPLP),[39] would be appropriate systems from which to draw inspiration.

Experimental

General

All lipids, media, general supplements and general chemicals were obtained from the Sigma Chemical Company (Poole, Dorset, UK) unless otherwise stated. Cytofectins used in this study were prepared as described previously.[8] Fluorescence spectroscopy and light scattering experiments were performed using a Shimadzu RF 5001 PC spectrofluorophotometer fitted with a thermostated cuvette holder. Zeta potential measurements were made using a Malvern Instruments Zetamaster 3000. Photon correlation spectroscopy was carried out using a Beckman Coulter N4 MD sub-micron particle analyzer. Electron microscopy was performed with a Gatan cryo-holder mounted in a Philips CM200 FEG electron microscope.

Formulation of cationic liposomes

Cytofectin (6 μmol) was combined with dioleoyl-L-α-phosphatidylethanolamine (DOPE) (3 mg, 4 μmol; provided as a 10 mg ml^{-1} solution in chloroform) in redistilled dichloromethane (5 ml). Following this, 20 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-(ethane-2-sulfonic acid) (HEPES), pH 7.8 (5 ml) was added and the mixture sonicated (bath sonicator) for 3 min. The organic phase was then removed under reduced pressure (rotary evaporator) at ambient temperature, after which the aqueous liposome suspension (approx. 1.2 mg ml^{-1}, 2 mM total lipid concentration; 1.2 mM cytofectin concentration, [cyt]) was sonicated for a further 3 min. Liposomes were stored at 4–8 °C before use.

Preparation of plasmid DNA

Escherichia coli strain DH1 was transformed with plasmid pCMVβ (7 kbp) (Clontech) and the recombinant bacteria grown to saturation in 2LB medium (500 ml). Plasmid DNA (0.5–1 mg) was extracted using a Plasmid Mega Kit (Qiagen, Crawley, Surrey, UK). Only plasmid showing an A_{280}/A_{260} ratio of greater than 1.8 was used in subsequent studies. Plasmid was stored at 4 °C in either de-ionised, sterile water or TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) at a concentration of approx. 1 mg ml^{-1} (nucleotide concentration, [nt], 3 mM). The concentration of this DNA was determined using an $A_{260}^{0.005\%}$ of 1,[29] and nucleotide concentrations, [nt], were determined using an average nucleotide molecular weight of 329 Da. The plasmids pCIβgal (for *in vitro* gene delivery

experiments) and pCF1-CAT (for *in vivo* gene delivery experiments) were prepared in a similar way.

In vitro gene delivery experiments

In vitro cell studies were performed with COS-7 (African Green Monkey kidney) cells cultured in Dulbecco's minimum essential medium (DMEM) with penicillin and streptomycin (each 1% w/v) and supplemented with 10% fetal bovine serum (FBS). Cells were seeded in 24-well plates at an approximate density of 2.5×10^4 and grown to approximately 70% confluence in DMEM media at 37 °C under an atmosphere of carbon dioxide (5%, v/v). The cells were then washed in phosphate-buffered saline (PBS) before OptiMEM (with added GLUTAMAX) was administered to each well (370 μl per well). Lipoplexes were then prepared in the following standard manner for each transfection in each well. For each transfection, OptiMEM was added to two separate Falcon tubes (approx. 15 μl per tube) and an appropriate aliquot of pCIβgal plasmid in de-ionized water (containing 1 μg of plasmid DNA) diluted into one Falcon tube, whilst an appropriate aliquot of cationic liposome suspension (containing 0.5, 2.0, 5.0 or 10.0 μg of cationic liposome) was diluted into the other Falcon tube. The two were then combined (cationic liposome suspension added to plasmid DNA solution) at 25 °C and incubated for 15 min to form an equilibrated lipoplex mixture (total combined volume 30 μl; plasmid concentration 0.033 mg ml^{-1}, [nt] 0.1 mM). Subsequently, this lipoplex mixture was diluted into the OptiMEM solution of a given well. After 24 h at 37 °C, this OptiMEM solution containing lipoplex was replaced with supplemented DMEM (500 μl) and the cells incubated for a further 48 h at 37 °C. Finally, cells were lyzed by the addition of an aliquot (350 μl) of 250 mM Tris-HCl, pH 8.0, containing Triton X-100 (0.1%, v/v). After lysis, cells were frozen and then thawed to measure the levels of β-galactosidase expression in each well as a result of transfection.

The level of β-galactosidase expression in each well was determined by a 96-well plate photometric assay.[40] Cell lysate (50 μl) from each given well was combined with an aliquot (50 μl) of PBS, containing bovine serum albumin (BSA) (0.5%, w/v), and an aliquot (150 μl) of 60 mM phosphate buffer, pH 8.0, containing 1 mM $MgSO_4$, 10 mM KCl, 50 mM β-mercaptoethanol, and chlorophenol red galactopyranoside (CPRG) (1 mg ml^{-1}). Values of A_{578} were recorded (microtitre plate reader) at 10 min, 1 h, 3 h, and occasionally after 24 h. Levels of β-galactosidase expression (in pg $well^{-1}$) were deduced from a standard curve correlating A_{578} values (after subtraction of background values) with β-galactosidase between 20 and 20000 pg. Protein levels were quantified by standard Bradford assay (Biorad).

In vivo gene delivery experiments

For each transfection experiment, an appropriate aliquot of undiluted cationic liposome suspension (approx. 1.2 mg ml^{-1}) (containing either 16, 40, 160 or 400 μg of cationic liposome) was added directly to a fixed aliquot (approx. 50 μl) of pCF1-CAT plasmid in sterile, de-ionized water (containing 80 μg of plasmid DNA) to form a lipoplex mixture which was left to equilibrate for 15 min at 25 °C (total combined volumes ranged from approx. 63–383 μl; DNA concentration 1.3–0.2 mg ml^{-1}, [nt] 4.0–0.6 mM). Balb/c mice were anaesthetised using metofane[9] and then supported in a vertical position with the lower jaw held shut while lipoplex mixtures were applied to the nose and sniffed down into the lung. After 2 days, the mice were sacrificed and the lungs excised, homogenised in 250 mM Tris-HCl, pH 8, and the levels of CAT determined. Homogenised tissue was put through 4 freeze–thaw cycles, followed by 10 min of heating at 60 °C, to inactivate any endogenous deacetylase activity. Finally, CAT levels were measured in the homogenate by the LSC CAT assay (Promega).

Light scattering analysis of cationic liposome–DNA complex formation

Solution aliquots (50 μl) of pCMVβ plasmid DNA in de-ionized water and equal volumes of cationic liposome suspensions (0.2 mM total lipid concentration; total [cyt] 0.12 mM) in 20 mM HEPES buffer, pH 7.8, were separately pre-equilibrated at 20 °C for 5–10 min. After addition of cationic liposome suspensions to DNA solution aliquots, changes in light scattering intensity at 640 nm were followed as a function of time at 20 °C. In each mixture studied, the final lipid concentration was 0.1 mM (final [cyt] 0.06 mM) and final [nt] were either 0.1, 0.055 or 0.018 mM.

Electron microscopy of lipoplex mixtures

Lipoplex mixtures (5 μl) were applied to a freshly glowing discharged electron microscopy grid covered with a carbon film. After blotting off excess solution, a thin layer of lipoplex suspension was left on the grid spanning the holes on the carbon film. Grids were then rapidly frozen by plunging them into liquid ethane leading to the formation of lipoplex particles embedded in a thin film of amorphous ice. Images of these lipoplex particles in ice holes were obtained using a 160 kV accelaration voltage at the temperature of liquid nitrogen, with a magnification of 66000 and a defocus of −1.5 micron.

Ethidium bromide exclusion assays

Solution aliquots (55 μl) of pCMVβ plasmid DNA in de-ionized water (total [nt] 0.071 mM) were combined with equal volumes of cationic liposome suspensions in 20 mM HEPES buffer, pH 7.8. After 15 min equilibration at 20 °C, ethidium bromide **11** (20 μl of 0.03 mM solution in 20 mM HEPES buffer, pH 7.8) was added. In each mixture, final [nt] was 0.03 mM, final [**11**] was 4.6 μM, and the final lipid concentration varied through a concentration range from 0 to 0.1 mM (final [cyt] range 0 to 0.06 mM). After a further 2 min equilibration time, the fluorescence emission intensity at 590 nm (excitation wavelength 260 nm) of each mixture was monitored and reported as a function of the final cytofectin concentration.

Gel retardation assays

Solution aliquots (4 μl) of pCMVβ plasmid DNA in de-ionized water (total [nt] 0.12 mM) were combined with equal volumes of cationic liposome suspensions in 20 mM HEPES buffer, pH 7.8, and the mixtures left to equilibrate for 15 min at 20 °C. In each mixture, final [nt] was 0.06 mM and the final lipid concentration varied through a concentration range from 0 to 0.2 mM (final [cyt] range 0 to 0.12 mM). Mixtures were then supplemented with gel loading buffer (2 μl) and then run out on agarose gels according to standard procedures.[29] All gels were run out at 110 V for 40 min. DNA bands were visualized using ethidium bromide staining.[29]

Photon correlation spectroscopy

Solution aliquots (100 μl) of pCMVβ plasmid DNA in de-ionized water were combined with equal volumes of cationic liposome suspensions (0.2 mM total lipid concentration; total [cyt] 0.12 mM) in 20 mM HEPES buffer, pH 7.8, and the mixtures left to equilibrate for 15 min at 20 °C. Mixtures were then analyzed by photon correlation spectroscopy using light scattered at 90° to the incident light to determine the mean complex diameters. For each mixture, a mean complex diameter was determined as an average from five consecutive scans, each of 200 s in duration. In each mixture studied, the final lipid concentration was 0.1 mM (final [cyt] 0.06 mM) and final [nt] varied over a range from 0.009 to 0.18 mM.

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