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RESEARCH ARTICLE

Characterisation of LMD virus-like nanoparticles self-assembled from cationic liposomes, adenovirus core peptide μ (mu) and plasmid DNA

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Liposome:mu:DNA (LMD) is a ternary nucleic acid delivery system built around the μ (mu) peptide associated with the condensed core complex of the adenovirus. LMD is prepared by precondensing plasmid DNA (D) with mu peptide (M) in a 1:0.6 (w/w) ratio and then combining these mu:DNA (MD) complexes with extruded cationic liposomes (L) resulting in a final lipid:mu:DNA ratio of 12:0.6:1 (w/w/w). Correct buffer conditions, reagent concentrations and rates of mixing are all crucial to success. However, once optimal conditions are established, homogeneous LMD particles (120 ± 30 nm) will result that each appear to comprise an MD particle encapsulated within a cationic bilammellar liposome. LMD particles can be formulated reproducibly, they are amenable to long-term storage (>1 month) at −80°C and are stable to aggregation at a plasmid DNA concentration up to 5 mg/ml (15 mM nucleotide concentration). Furthermore, LMD transfections are significantly more time and dose efficient in vitro than cationic liposome-plasmid DNA (LD) transfections. Transfection times as short as 10 min and plasmid DNA doses as low as 0.001 μg/well result in significant gene expression. LMD transfections will also take place in the presence of biological fluids (eg up to 100% serum) giving 15–25% the level of gene expression observed in the absence of serum. Results from confocal microscopy experiments using fluorescent-labelled LMD particles suggest that endocytosis is not a significant barrier to LMD transfection, although the nuclear membrane still is. We also confirm that topical lung transfection in vivo by LMD is at least equal in absolute terms with transfection mediated by GL-67:DOPE:DMPE-PEG$_5$000 (1:2:0.05 m/m/m), an accepted 'gold-standard' non-viral vector system for topical lung transfection, and is in fact at least six-fold more dose efficient. All these features make LMD an important new non-viral vector platform system from which to derive tailor-made non-viral delivery systems by a process of systematic modular upgrading.
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Keywords: *cationic liposomes; cationic lipids; non-viral gene therapy; adenovirus; mu peptide*

Introduction

Non-viral vector systems have many potential advantages compared with viral vector systems. For instance their lower toxicity/immunogenicity and potential for oncogenicity, their size independent delivery of nucleic acids (from oligonucleotides to artificial chromosomes), simpler quality control and easier pharmaceutical and regulatory requirements. However, current non-viral vector systems are not competitive with their viral counterparts and are unlikely to find routine clinical use because they are beset with too many basic problems.[1,2] Previously, we have been trying to develop effective cationic liposome systems beginning from first generation cationic liposomes formulated from cytofectin 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) and the neutral phospholipid dioleoyl L-α-phosphatidylethanolamine (DOPE) (Figure 1).[3] Research culminated in development of a reasonably effective second-generation cationic liposome system formulated from second-generation cytofectin N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (CTAP) and DOPE (Figure 1). This CTAP/DOPE system was found to mediate the delivery of the chloramphenicol acetyl transferase (*CAT*) reporter gene to the lungs of mice up to 400-fold more efficiently than the first generation DC-Chol/DOPE cationic liposome system, bringing us close to the necessary efficacy for gene delivery to the human lung.[4,5]

However, further developments of the CTAP/DOPE

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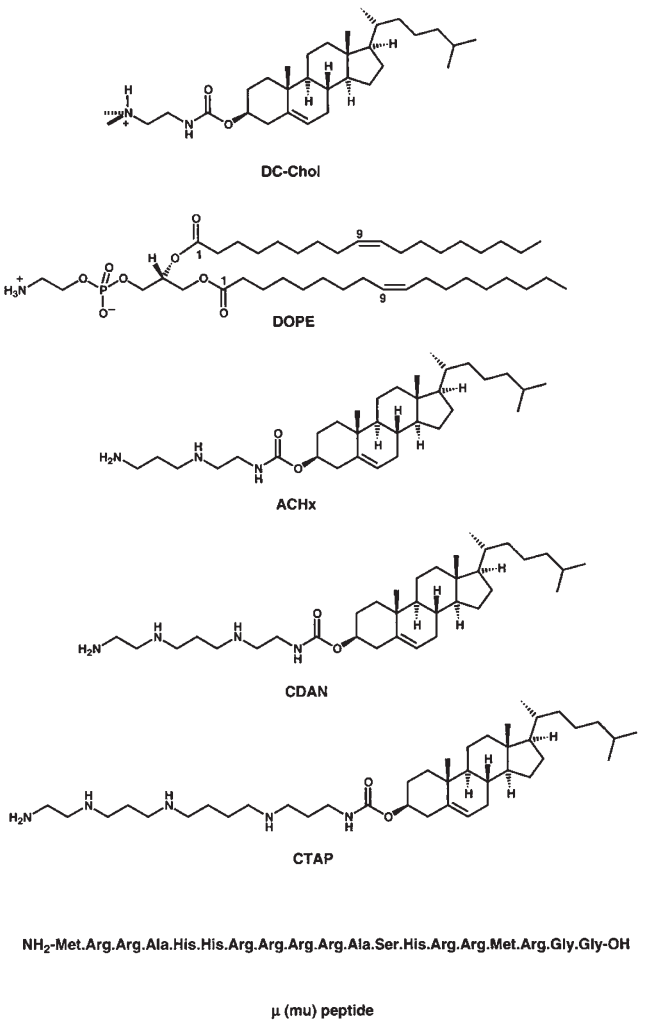

Figure 1 *Summary of all the main chemical structures described in the text.*

system were dogged by serious difficulties in obtaining reproducible transfection outcomes, reproducible formulations, and stable long-term storage. These familiar problems severely compromised our research effort aimed at reaching further systematic improvements in lung gene delivery. As a result, we decided to try and solve these basic problems by developing an alternative ternary non-viral vector system. A number of ternary systems have been described over the past few years, most importantly the lipid:protamine:DNA (LPD) vector systems reported by Huang and coworkers, and others.[6–15] Other ternary systems involving alternatives to protamine have also been described in the past few years including systems based around poly-L-lysine,[16,17] spermidine,[18] lipopolylysine,[19] histone proteins,[20,21] chromatin proteins,[22] human histone derived peptides,[23] oligo-L-lysine,[24–26] L-lysine containing synthetic peptides,[27] and a histidine/lysine (H-K) copolymer.[28] In our case, we chose to use the adenoviral core complex peptide μ (mu).[29]

Mature adenovirus consists of an icosahedral, non-enveloped capsid particle (approximately 90 nm) enclosing a core complex that consists of a linear dsDNA viral genome (~36 kbp) non-covalently associated with two cationic proteins (proteins V [pV] and VII [pVII]), as well as the mu peptide.[29–35] Evidence suggests that pVII and mu peptide are most tightly associated with viral DNA, whilst pV plays a role in assisting the delivery of the adenovirus core complex into the host cell nucleus.[30,32–34] In initial experiments with the mu peptide, we were able to demonstrate recently that mu peptide is able to enhance routine cationic liposome-mediated transfection *in vitro*.[36] However, these initial experiments used a large excess of mu peptide relative to cationic liposome giving rise to large, heterogeneous lipopolyplex particles (approximately 1000 nm in diameter). This paper now documents the process in which these initial experiments with the mu peptide have been turned to effect in developing a new ternary liposome:mu:DNA (LMD) vector system that appears to have the necessary characteristics to be a potent non-viral vector platform system.

Results

LMD formulation and particle characterisation

Formation of mu:DNA (MD) complex mixtures was studied using photon correlation spectroscopy (PCS) and by gel retardation assays as described previously.[5,35,36] The plasmid used for these studies was the β-galactosidase (β-gal) expressing plasmid pCMVβ (7.2 kbp). According to PCS measurements, discrete MD particles could be formed (80–120 nm diameter) in low ionic strength buffers such as HEPES (4 mM, pH 7.0–7.2) provided final plasmid concentration was <0.35 mg/ml, optimally 0.2 mg/ml. Higher salt concentrations increased the particle size. Interestingly, the diameters of the MD complexes varied over a broad range of mu:DNA ratios. Small, stable nanoparticles were formed within the mu:DNA ratio 0.3:1 to 1.2:1 (w/w; range L; 110 ± 30 nm) and over 5:1 (range H; 220 ± 60 nm) (Figure 2). Intermediate ratios resulted in heavy aggregation with the size of complex particles growing over the time of incubation to reach more than 2 μm in size.

An analysis of MD complex mixtures by cryo-electron microscopy confirmed the formation of small MD particles (approximately 110 nm in size) in range L (Figure

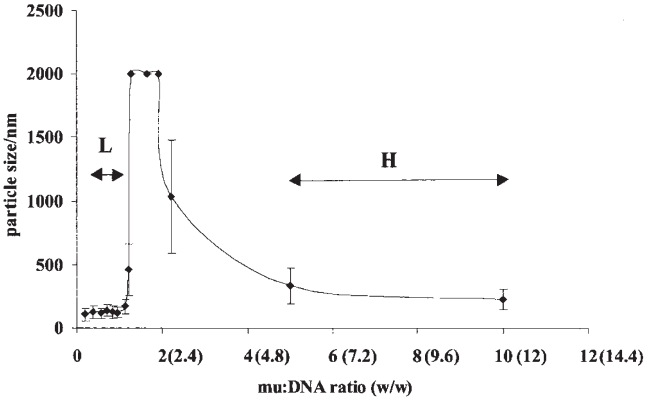

Figure 2 *Results of analysis of mu:DNA (MD) complex mixtures by photon correlation spectroscopy (PCS) at different mu:DNA w/w ratios of mu and pCMVβ plasmid. MD complexes were prepared as described in the Experimental section and then diluted with 4 mM HEPES buffer, pH 7.2, to a final DNA concentration of 24 μg/ml before PCS analysis. Note that stable particles can only be achieved for mu:DNA (w/w) ratios between 0.2-1.5 and above 5. N/P ratios have been added in brackets for the sake of completeness.*

2 and 3). Where observations were made, particles appeared to be dense and spherical, but otherwise lacking ordered nano-metric structure (Figure 3a). Poly L-lysine hydrobromide (pLL, average MW 3970 Da) and protamine sulfate (grade X from salmon) also form similar particles with plasmid DNA. However, in both cases, pLL and protamine sulfate particles proved unstable to aggregation with time even at low polypeptide:DNA w/w ratios (>0.6). By contrast, MD particles were stable to aggregation at mu:DNA w/w ratios up to 1.2. A tendency to aggregate into larger particles with time (as judged by PCS analysis) is highly undesirable if properly defined, size stable non-viral vector formulations are to be produced. In this respect, the aggregation properties of MD particles are notably better than those of particles formed between either pLL or protamine sulfate and plasmid DNA.

LMD particles were prepared from MD particles in the following way. Results from this study and our previous biophysical investigation of the interactions between mu and plasmid DNA (pNGVL1, 7.5 kbp),[35] suggested that the optimal mu:DNA ratio was 0.6:1.0 (w/w). At this ratio, saturation binding of approximately 1300 mu peptides per pNGVL1 plasmid was observed giving rise to small discrete, stable particles.[35] In spite of achieving saturation binding, particles retained an overall negative charge (N/P = 0.7 at mu:DNA 0.6:1.0 w/w) (Figure 2), and were therefore expected to interact freely with extruded cationic liposomes. Initially, extruded DC-Chol/DOPE liposomes (6:4, m/m; 109 ± 15 nm diameter) (total lipid concentration determined by colorimetric Stewart assay) were added directly to optimal MD complex mixtures but this approach proved unreliable, aggregation being a persistent problem. However, by carrying out slow addition of optimal MD complex mixtures to suspensions of extruded cationic liposomes under continuous vortex mixing conditions, LMD nanoparticles were formed reliably and highly reproducibly. According to PCS measurements, LMD particles of 120 ± 30 nm were formulated routinely from extruded DC-Chol/DOPE cationic liposomes with a final lipid:mu:DNA ratio of 12:0.6:1 w/w/w (lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w) (final DNA concentration 0.14 mg/ml). An analysis of LMD complex mixtures by cryo-electron microscopy was able to confirm the formation of LMD particles with a dense spherical core (Figure 3b and c). Each particle appears to consist of an MD particle encapsulated within a cationic bilammellar liposome. Therefore, an LMD particle could be described as a double-walled virus-like nanoparticle (VNP). The existence of the dense spherical core was further confirmed by PCS measurements that revealed a significant increase in scattering intensity from LMD mixtures in comparison to scattering intensities associated with either MD complex mixtures or cationic liposome suspensions at identical matching concentrations (results not shown).

LMD mixtures were prepared for transfection studies using two different plasmids, namely the pCMVβ (7.2 kbp) and the CAT expressing plasmid pCF1-CAT (4.7 kbp), employing a mu:DNA ratio of 0.6:1.0 (w/w) in both cases. Accordingly, since average nucleotide molecular weight (329 Da) is the same for both plasmids, then the mole ratio of mu:nucleotide was also the same in both cases. The average diameter of pCF1-CAT containing LMD particles was 80–90% of the average diameter of pCMVβ containing LMD particles, consistent with the encapsulation of a smaller plasmid. In both cases, the exact number of plasmids encapsulated per particle cannot be established with certainty, although Zhang *et al*[37] have reported that stabilised plasmid-lipid particle (SPLP) systems (diameter 100 ± 40 nm) formed with the pCMVLuc plasmid (5.6 kbp) do contain a single plasmid

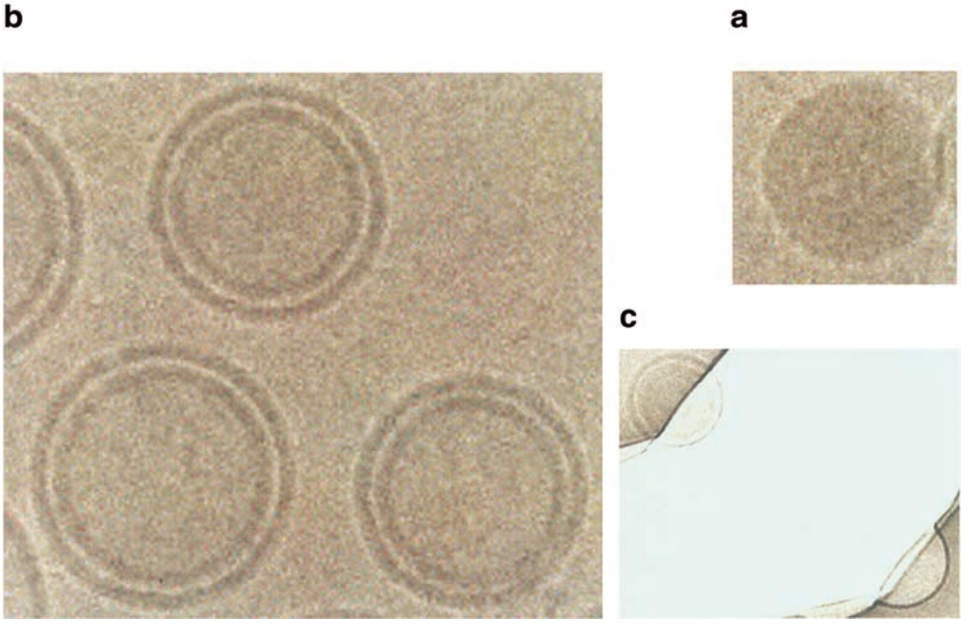

Figure 3 *Cryo-electron microscopy images of: (a) A putative mu:DNA (MD) (mu:DNA ratio 0.6:1 w/w) particle (1 cm = 40 nm); (b) Liposome:mu:DNA (LMD) particles (lipid:mu:DNA ratio 12:0.6:1, w/w/w) prepared with DC-Chol/DOPE cationic liposomes and pCMVβ plasmid particle (1 cm = 40 nm); (c) An LMD particle (lipid:mu:DNA ratio 12:0.6:1, w/w/w) that has fractured between the inner and outer leaflets of the first of the two bilayers that commonly surround the higher density MD core particle (1 cm = 95 nm). Images of LMD mixtures were completely dominated by the structures shown together with a small population of free cationic liposomes and occasional MD particles.*

per particle, suggesting that LMD particles may similarly contain a single plasmid per particle.

Further characterisation of LMD particle suspensions formulated from extruded DC-Chol/DOPE cationic liposomes revealed that these particles were stable when extruded through >0.1 μm pore-size membranes. There was no noticeable loss in particle size integrity according to PCS analysis, and little mechanical loss of LMD particles resulting from extrusion, as judged by total lipid concentration assays (colorimetric Stewart assay) (data not shown). The integrity of LMD particles, as determined by PCS measurements, was likewise preserved over repetitive cycles of freezing and thawing over 41 days in the presence of 10% sucrose (w/v). Moreover, particles showed evidence of long-term cryopreservation (months) when stored at –80°C in the presence of sucrose 10% (w/v). After extended periods of storage, LMD particle suspensions exhibited no substantial loss in transfection efficiency of COS7 cells, nor any substantial increase in particle size as determined by PCS measurements (data not shown). Finally, controlled evaporation of buffer *in vacuo* at 30°C of LMD prepared at 0.14 mg/ml was found to result in very large increases in plasmid DNA concentration (up to 5 mg/ml) without any sign of particle aggregation or reduced transfection efficiency, as judged by PCS analysis and COS7 cell LMD transfection experiments (data not shown).

In vitro transfections

Initial experiments were performed with the neuronally derived ND7 cell line that had proved previously amenable to cationic liposome-mediated transfection.[38] We also used this same cell line previously to evaluate the transfection efficiency of lipopolyplex mixtures formulated with excess mu peptide.[36] LMD transfections were compared directly with the results of transfections involving the optimal cationic liposome-plasmid DNA (LD) system formulated from extruded DC-Chol/DOPE (6:4, m/m) cationic liposomes. In contrast to the LD, LMD transfections were found to mediate significant levels of gene expression in a transfection time of only 10 min. At this time-point, LMD treatment resulted in 16 times more CAT enzyme activity than LD (Figure 4a). This difference was narrowed to six times for transfection times of 60 min, while absolute levels of gene expression were increased in both cases as well. In the case of a similar comparative transfection study performed with a second COS7 cell line, LMD transfection kinetics appeared slower, but LMD was found to mediate levels of gene expression at least one order of magnitude higher than LD when transfection times of 30 and even 60 min were used (Figure 4b). Results obtained with a third cell line (Panc-1) also provided evidence of rapid LMD transfection kinetics compared with optimal DC-Chol/DOPE LD transfection, but this time in the presence of 10% serum (Figure 4c). In this case, LMD treatment resulted in 35 times more β-gal enzyme activity than LD with a 30-min transfection time, 30 times after 1 h and seven times after 2 h. LMD transfection after 2 h was five times more effective even than transfection mediated by the commercial agent TransFast, done for comparison under optimal conditions according to the manufacturer's instructions (Figure 4c). Successful LMD transfections of HBE or COS7 cells were possible even in the presence of up to 100% serum, resulting in routine transfection levels 15–25% of those levels obtained in the absence of serum (data not shown).

LMD transfections were found generally to be more dose efficient then DC-Chol/DOPE LD transfections in addition to being more transfection time efficient. Usually, *in vitro* LD transfections are performed with approximately 1 μg of plasmid/well.[5,38] Therefore, a comparison was made between LMD and LD transfection of ND7 cells involving 1, 0.1, 0.01 and 0.001 μg of plasmid DNA/well. LMD was able to mediate detectable gene delivery and expression even at a dose as low as 0.001 μg, whereas LD transfection yielded only modest levels of expression at a dose of 1 μg and negligible expression at lower doses (Figure 4d). A similar comparison was also made between LMD and LD transfection of COS7 cells. In this case, an interesting divergence was observed with LD transfection being more efficient than LMD at a dose of 5 μg of plasmid DNA/well. However, this situation was found to reverse at lower doses (0.5, 0.05 μg) (Figure 4e), in line with the ND7 results. Finally, a comparison was made between the cell coverage (ie percentage of cells transfected) mediated by both LMD and DC-Chol/DOPE LD transfections of three separate cell lines. LMD transfection resulted in uniformly high cell coverage (approximately 30% cells positive for transfection with LMD) in ND7 cells in comparison with LD transfection (approximately 2% cells) (Figure 5a). Similar results were observed with COS7 and HBE cells (results not shown).

Having established the efficacy of the LMD system based upon extruded first generation DC-Chol/DOPE (6:4, m/m) cationic liposomes, alternative LMD systems were formulated with extruded second generation cationic liposomes in place of extruded DC-Chol/DOPE liposomes. Second generation liposomes used were ACHx/DOPE, CDAN/DOPE and CTAP/DOPE (all 6:4, m/m), each of which had been shown previously to possess some advantages over first generation DC-Chol/DOPE liposomes.[4,5,39] LMD(ACHx), LMD(CDAN) and LMD(CTAP) (lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w) were prepared and particle sizes found to be comparable with original LMD formulated with DC-Chol/DOPE cationic liposomes. In the event, only LMD(CDAN) formulated from CDAN/DOPE cationic liposomes proved more efficient at *in vitro* transfection of ND7 cells than original LMD (Figure 5a). Otherwise, the rank order of transfection efficiency was LMD(CDAN) > LMD > LMD(ACHx) >> LMD(CTAP). Cationic liposome-mediated transfection was found to follow an equivalent rank order of transfection efficiency (CDAN/DOPE > DC-Chol/DOPE > CTAP/DOPE) (Figure 5a). Variation of the mu:DNA ratio was observed to have some effect upon LMD(CDAN) transfection efficiency. The most effective mu:DNA ratio remained 0.6:1 (w/w), although the 0.3:1 (w/w) variant was not significantly less effective (Figure 5b).

By contrast, variations in the total amount and composition of cationic liposomes used to prepare LMD(CDAN) did appear to have more wide ranging consequences for transfection efficiency. Different LMD(CDAN) systems were prepared by combining CDAN/DOPE cationic liposomes, formulated using mole ratios of either 6:4 (m/m), 1:1 (m/m) or 1:2 (m/m) respectively, with optimal MD complex mixtures (mu:DNA 0.6:1 w/w) employing a variety of different ratios from 6.2–21.8 μmol of total

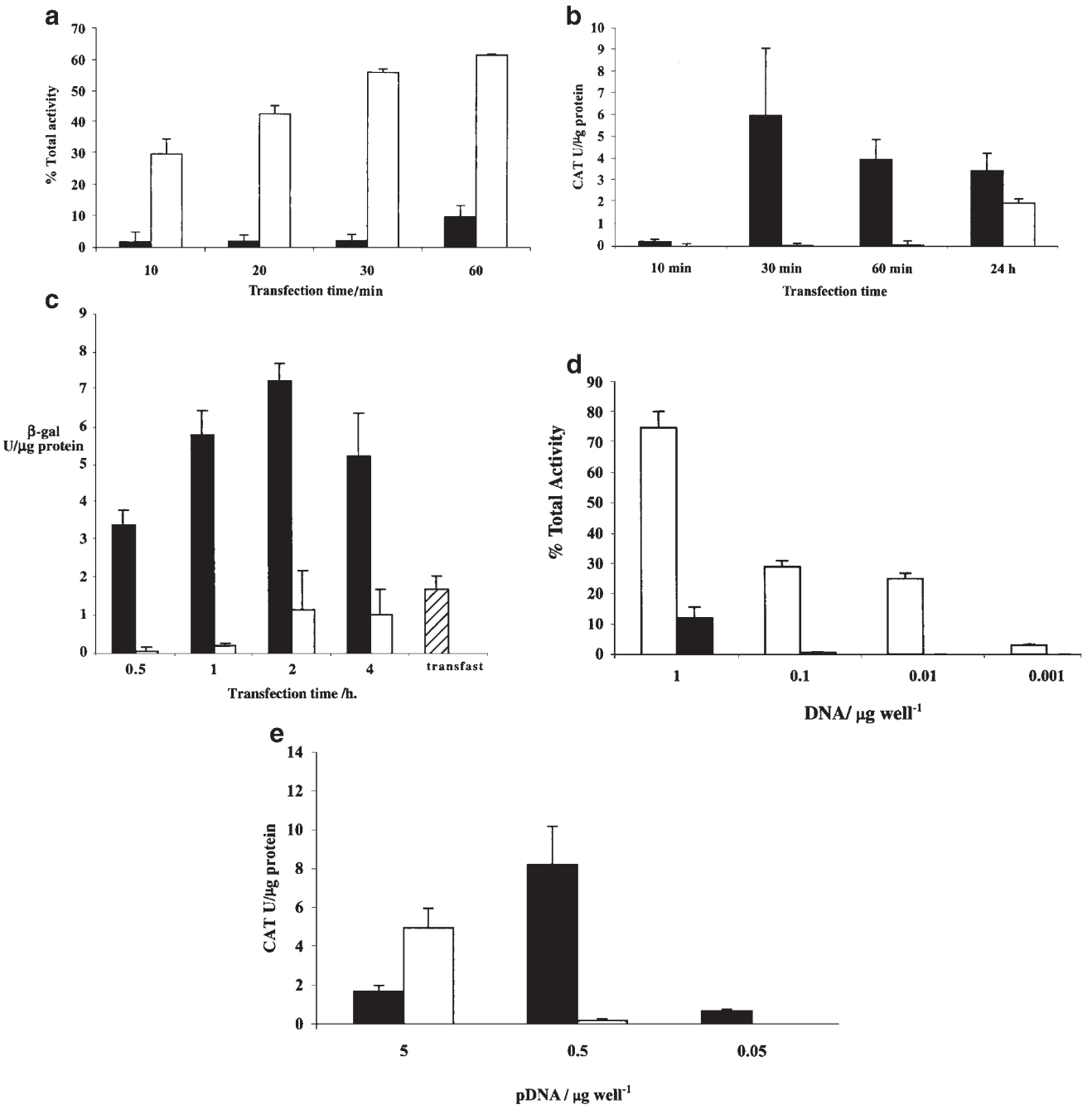

Figure 4 *Data to illustrate the time and dose efficiency of LMD transfection in vitro compared with DC-Chol/DOPE cationic liposome mediated transfection. (a) A comparison between cationic liposome–plasmid DNA (LD) (black) and LMD (white) transfection of ND7 cells as a function of transfection times. The pCF1-CAT plasmid DNA dose was 1 μg/well in all cases. Transfection efficiency was judged to be proportional to the chloramphenicol acetyl transferase (CAT) enzyme activity measured in cells after transfection, as described in the experimental section. (b) LMD (black) and LD (white) transfections of COS7 cells as a function of transfection times. The pCF1-CAT plasmid DNA dose was also 1 μg/well in all cases. Transfection efficiency was judged to be proportional to the CAT enzyme activity measured in cells post transfection. (c) Data to illustrate the time efficiency of LMD transfection of Panc-1 cells. LMD (black) and LD (white) transfections are shown as a function of transfection times and the outcomes compared with optimal transfection mediated by TransFast, used according to the maufacturer's instructions. The pCMVβplasmid DNA dose was 1 μg/well. Transfection efficiency was judged to be proportional to the β-galactosidase (β-gal) enzyme activity measured in cells after transfection. (d) LMD (white) and LD (black) transfections of ND7 cells as a function of plasmid DNA dose/well. The transfection time was 2 h in all cases. Transfection efficiency was judged to be proportional to the CAT enzyme activity measured in cells after transfection. (e) LMD (black) and LD (white) transfections of COS7 cells as a function of pCF1-CAT plasmid DNA dose/well. Transfection times were 2 h in all cases. Transfection efficiency was judged to be proportional to the CAT enzyme activity measured in cells after transfection.*

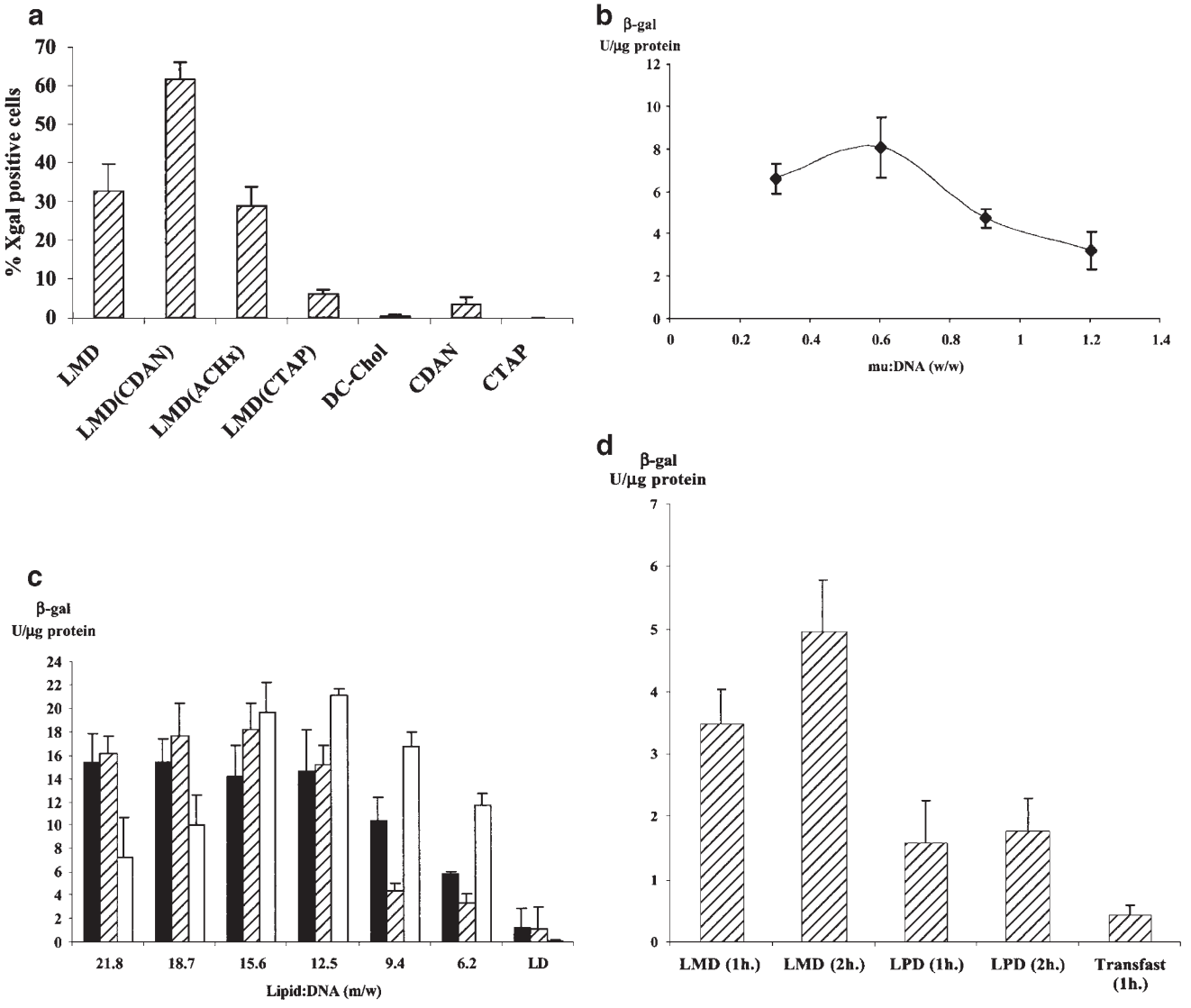

Figure 5 *A comparison between LMD systems formulated with second generation cationic liposomes. (a) ND7 cells were transfected with first and second generation LMD or LD systems prepared from either DC-Chol/DOPE or second-generation liposomes (ACHx/DOPE, CDAN/DOPE or CTAP/DOPE) as indicated. All LMD systems were formulated with a standard lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg (m/w/w), LD systems with a standard lipid:DNA ratio of 3:1 (w/w). In all cases, transfection times were 2 h and the pCMVβ plasmid DNA dose was 1 μg/well. Transfection efficiency was judged to be proportional to the number of transfected (blue) cells as observed by inverted microscope; (b) comparison between the transfection efficiencies of Panc-1 cells by LMD(CDAN) systems formulated with different overall ratios of mu:DNA (w/w). All LMD systems were formulated with a standard lipid:DNA ratio of 20 μmol:1.0 mg (m/w), pCMVβ doses of 1 μg/well, transfection times 30 min. Transfection efficiency was judged to be proportional to the β-galactosidase (β-gal) enzyme activity measured in cells post transfection; (c) comparison between the transfection efficiencies of Panc-1 cells by LMD(CDAN) systems formulated with a fixed mu:DNA w/w ratio of 0.6:1, but with varying amounts of CDAN/DOPE cationic liposomes prepared with CDAN:DOPE m/m ratios of either 6:4 (black), 1:1 (shaded) or 1:2 (white). Results are compared with LD transfections using CDAN/DOPE cationic liposomes formulated with the same alternative m/m ratios. In all cases, plasmid pCMVβ dose was 1 μg/well, transfection time 2 h. Transfection efficiency was judged to be proportional to the β-gal enzyme activity measured in cells after transfection; (d) comparison between the transfection efficiencies of confluent Swiss 3T3 cells by LMD(CDAN) and LPD(CDAN) systems formulated with a fixed peptide:DNA w/w ratio of 0.6:1 and CDAN/DOPE (6:4 m/m) cationic liposomes. In all cases, pCMVβ dose was 1 μg/well, transfection times were as indicated. Transfection efficiency was judged to be proportional to the β-gal enzyme activity measured in cells after transfection.*

lipid/mg of plasmid DNA. Transfection efficiencies were then compared (Figure 5c). LMD(CDAN) systems prepared with 6:4 (m/m) or 1:1 (m/m) CDAN/DOPE cationic liposomes reached a plateau in transfection efficiency at high lipid composition (≥12.5 μmol lipid/mg DNA). Reduced transfection efficiency below this lipid composition threshold appeared to correlate with particle instability. According to PCS analysis, LMD(CDAN) systems formulated with ≥12.5 μmol lipid/mg DNA produced small, stable particles (<200 nm). However, below 12.5 μmol, particle size was in excess of 200 nm and aggregation was even visible at 6.2 μmol. In a similar way, LMD(CDAN) systems prepared with 1:2 (m/m) CDAN/DOPE cationic liposomes exhibited a peak in transfection efficiency at 12.5 μmol lipid/mg DNA marginally higher than the transfection plateau observed with other LMD(CDAN) systems. This could represent a useful alternative formulation to the

standard formulation (lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w) described above. Once again, decline in transfection performance appeared to correlate with particle size instability. Finally, a comparison was made between LMD(CDAN) (lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w formulated with CDAN/DOPE 6:4 m/m) and LPD(CDAN) formulated in a similar way (lipid:protamine:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w). We found that LMD(CDAN) was twice as effective as LPD(CDAN) after a 1 h transfection time and nearly three times as effective after a 2 h transfection time (Figure 5d). These results suggest an influence of the peptides on the transfection levels with mu being superior to protamine for this particular system, in line with the results of our previous biophysical analyses comparing the interactions of mu and protamine with plasmid DNA.[35]

Confocal microscopy

LMD transfection of 56 FHTE8o$^-$ cells was investigated *in vitro* by confocal microscopy using DC-Chol/DOPE-based LMD particles formulated with Cy5-labelled plasmid DNA.[40] After 15 min incubation with dividing cells, labelled DNA was detected in the cytoplasm and after 2 h, significant amounts were visible in the nucleus of the vast majority of cells investigated (Figure 6a and b). By contrast, DNA entry into the nucleus was compromised in experiments performed using growth arrested cells. Little DNA was actually visible in these cells except for the appearance of blue-fluorescent spots attached to the nuclear envelope, suggestive of plasmid DNA attached to nuclear pore complexes but unable to traverse the pore and gain entry into the nucleus (Figure 6c).

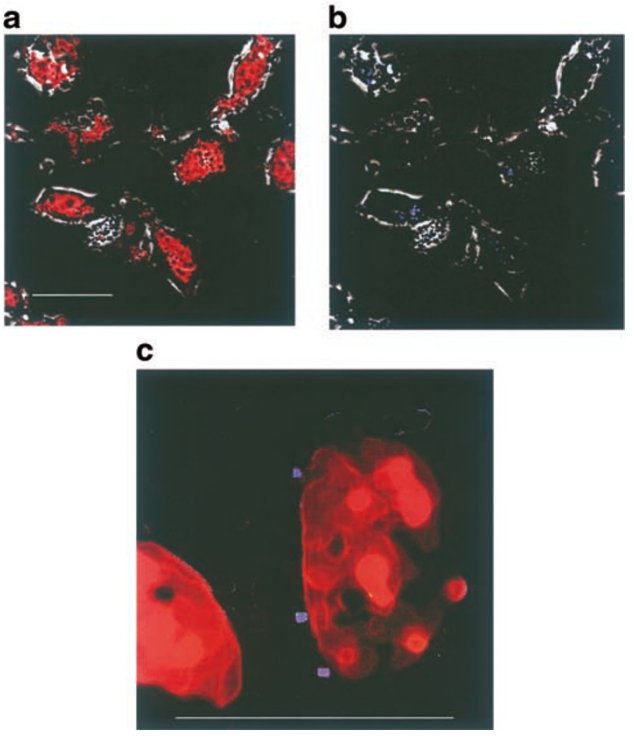

Figure 6 *Confocal microscopy images of 56 FHTE8o$^-$ cells[61] after transfection with LMD prepared from cyanine dye Cy5-labelled plasmid DNA.[40] The transfection time was 2 h before fixing and analysis according to previous protocols.[62] (a) Locations of cell nuclei labelled with propidium iodide. (b) Localisation of Cy5-labelled plasmid DNA (blue) within the nuclei. Both images are a montage of fluorescence image and a phase-contrast picture of the cells. (c) Localisation of Cy5-labelled plasmid in growth arrested 56 FHTE8o$^-$ cells by aphidicholin.[51] Note that the dark blue spots on the outer cell membrane represent plasmid DNA prevented from entry into the cell. The bar represents a length of 1 μm.*

Ex vivo transfections

Previously, we reported upon the development of an organotypic *ex vivo* rat brain model, set up to evaluate DC-Chol/DOPE LD transfection of neuronal tissue.[41] Such brain slices are usually considered excellent model systems to study the problems of *in vivo* brain transfection owing to their well preserved cellular connectivities and cyto-architecture. Therefore, LMD transfection efficiency was compared directly with the efficiency of optimised DC-Chol/DOPE LD transfection studied previously.[41] LMD transfection at a plasmid DNA dose of 5.0 μg/slice (2 ml culture) was 19 times more efficient than LD transfection at the same dose (in terms of numbers of cell coverage). LMD transfection was even found to be four times more efficient than L[LT-1]D transfection, the L[LT-1]D system being prepared using the commercially available, low toxicity polyamine reagent LT-1 in place of mu peptide. Moreover, even low dose LMD transfection (plasmid DNA 129 ng/slice) appeared to be more efficient than normal dose LD transfection (plasmid DNA 5.0 μg/slice) (Figure 7). These results are encouraging and suggest that LMD systems could be important new transfection agents for neuronal tissue *in vivo*.

In vivo transfections

Topical lung transfection *in vivo* by LMD was compared directly with transfection by the LD[GL-67] system prepared from the cationic liposome system GL-67:DOPE:DMPE-PEG$_{5000}$ (1:2:0.05 m/m/m).[42] This cationic liposome system containing cytofectin GL-67, a branched cholesterol polyamine compound, is a currently accepted 'gold-standard' non-viral vector system for topical lung transfection and was recently used with some success in topical lung cystic fibrosis clinical trials.[43] Therefore, topical lung transfection by LD[GL-67] represents an important positive control for *in vivo* transfec-

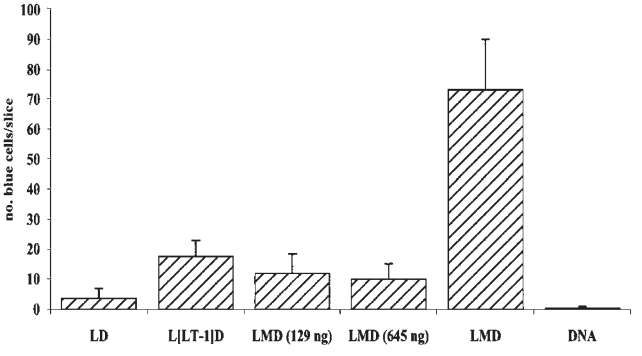

Figure 7 *Ex vivo LMD transfection of rat brain organotypic explant slices. LMD transfection was compared with L[LT-1]D, LD and naked DNA transfections. In all cases, slices were given pCMVβ doses of 5 μg/slice, unless otherwise indicated, transfection time was 2 h. After transfection, slices were maintained for a further 48 h before X-gal staining and slice analysis according to previously described protocols.[41] Transfection efficiency was judged to be proportional to the number of transfected (blue) cells as observed by inverted microscope.*

tion by non-viral vector systems. LMD and LD[GL-67] systems were prepared with pCF1-CAT plasmid and transfection experiments were performed introducing two different plasmid DNA doses (14 and 80 μg/mouse) into the lungs of Balb/c mice. LMD transfection (plasmid DNA dose 14 μg/mouse) was found to be equivalent to LD[GL-67] transfection at the higher dose (80 μg/mouse) (Figure 8), showing that LMD transfection was at least six-fold more dose efficient than LD[GL-67] transfection *in vivo*. These results are also encouraging. DC-Chol/DOPE cationic liposome-mediated topical lung transfection typically results in levels of transgene expression two orders of magnitude lower than the levels reported here following LMD and LD[GL-67] transfection of mouse lung.[5,42] Naked DNA transfection is typically reported to result in transgene expression three orders of magnitude lower.[4,5,42] Hence, topical lung transfection *in vivo* by LMD shows considerable improvements over optimal transfection *in vivo* by LD systems prepared from DC-Chol/DOPE cationic liposomes.

Discussion

The problems of cationic liposome-mediated gene delivery have been well documented.[1,2,44,45] LD particles formed from cationic liposomes and plasmid DNA are difficult to formulate reproducibly. They are susceptible to aggregation, are difficult to store long-term and do not mediate reproducible transfections *in vivo*, *ex vivo* and even *in vitro*. Moreover, LD particles are not cell-type specific, they appear to be slow to enter cells (hours), are prone to endosome entrapment, and appear to be only weak facilitors of nucleic acid entry into the cell nucleus. DNA entry within the nuclear envelope appears impossible without the intervention of M-phase in the cell cycle, when the nuclear membrane is partially dismantled to allow mitosis and cell division to take place. If this were not enough, LD particles are very highly unstable in biological fluids (eg high salt and serum). In spite of these problems, cationic liposome systems have been used with partial success in some clinical trials, not the least for topical lung delivery of the *CFTR* gene in cystic fibrosis clinical trials.[43] Nevertheless, problems such as these will need to be solved convincingly if non-viral vector systems including LD are to be brought to a state of meaningful clinical readiness competitive with viral vector systems. However, systematic attempts to achieve this cannot be fruitful unless non-viral systems are derived in which all of the most fundamental problems of reproducible and scalable formulation, stability to aggregation, long-term storage and reproducible transfection outcomes are properly addressed at the outset. Provided that such properly defined, stable systems can then be easily upgraded in clearly defined ways, these may justifiably be called non-viral platform systems. LMD was developed as a potential non-viral platform system.

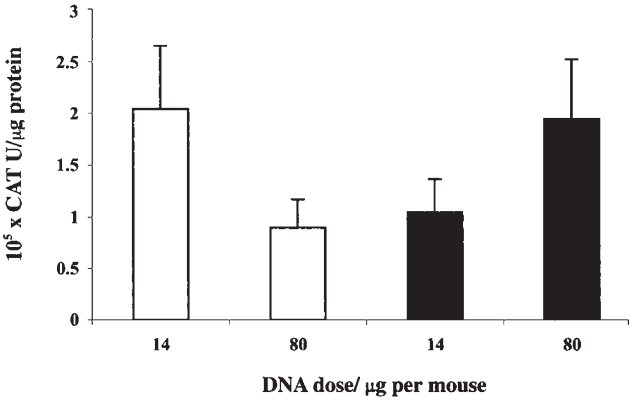

Figure 8 *In vivo transfection of Balb/c mice with LD[GL-67] system prepared from GL-67:DOPE:DMPE-PEG$_{5000}$ (1:2:0.05 m/m/m) or LMD. Transfection experiments were performed using Balb/c mice according to previously described protocols.[5] LMD (white) and LD[GL-67] (black) transfections were compared at pCF1-CAT doses of 14 μg/mouse and 80 μg/mouse as indicated. Transfection efficiency was judged to be proportional to the CAT enzyme activity measured in lung homogenates 48 h after transfection.*

The key selection of mu peptide as the basis for the LMD ternary system was made for several reasons. First, we had prior experience with mu as an enhancer of routine cationic liposome-mediated transfection *in vitro*.[36] Second, we suspected that this peptide could be a useful 'template' to control the process of plasmid DNA neutralisation and condensation into discrete particles owing to its central role in the adenovirus core complex.[29,31] Third, mu peptide has three histidine residues (pKa 6.0), that might have the capacity to promote endosome buffering and osmotic shock of endosome compartments which has been described as a prerequisite for the escape of plasmid DNA from endosome compartments into the cytosol.[5,46–48] Our results clearly show that mu peptide is an excellent plasmid DNA neutralisation and condensation 'template'. The variation of MD particle size as a function of mu:DNA ratio (w/w) (Figure 2) is reminiscent of LD particle size variation as a function of liposome:DNA ratio.[5] At low mu:DNA ratios, small stable particles probably form because of the electrostatic repulsion of components with an excess of negative charge, whilst at high ratios small stable particles probably exist owing to the presence of excess positive-charge. 'Colloidal instability' close to charge neutrality is probably the reason for large particle formation in the MD mixture. By contrast, MD particles formed at the optimal mu:DNA ratio (0.6:1 w/w) for LMD formation are small (80–120 nm) and negative in overall charge, providing an ideal template for LMD particle (120 ± 30 nm) formation upon combination with extruded cationic liposomes (approximately 100 nm). The visual appearance, dimensional size and homogeneity of LMD particles observed by cryoelecton microscopy (Figure 3), are all in complete agreement with the results of PCS analysis justifying the description of LMD particles as double-walled virus-like nanoparticle (VNP) structures. Huang and co-workers have reported similar such structures following the formulation of LPD systems.[8–10,13,49,50]

In comparison with our experiences with LD formulation, we have found LMD formulation to be absolutely consistent provided that relative component ratios, concentrations, and buffer conditions described in the Results are properly adhered to. Therefore once optimised, formulation of LMD systems appears simple, reproducible, scalable and flexible, irrespective of the extruded cationic liposome system (DC-Chol/DOPE, ACHx/DOPE, CDAN/DOPE and CTAP/DOPE) or plasmid type (pCMVβ or pCF1-CAT) used. Moreover, LMD particles once formulated appear to be amenable to long-term storage at −80°C in the presence of sucrose 10%

(w/v), and stable up to plasmid DNA concentrations of 5 mg/ml (nucleotide concentration 15 mM; assuming an average nucleotide molecular weight of 329 Da). Such DNA concentrations are appropriate for facile use *in vivo*. In comparison, LD systems are typically unstable towards aggregation above 4 mM nucleotide concentration, even in low ionic strength buffer.[4,5,11] Furthermore, LMD transfections are significantly more reliable, time and dose-efficient *in vitro* than LD transfections, providing more widespread cell coverage (Figures 4 and 5). LMD transfection times as short as 10 min and plasmid DNA doses as low as 0.001 μg/well result in significant gene expression, whereas LD transfections typically require transfection times of several hours (typically 24 h) and plasmid DNA doses of approximately 1 μg/well. LMD transfections even take place in the presence of biological fluids (eg up to 100% serum), giving 15–25% the level of gene expression observed in the absence of serum, suggesting that LMD possesses an additional element of stability. LD transfection will typically not take place under such conditions at all. All these characteristics fulfil many of the requirements for LMD to be considered a non-viral vector platform.

Formulation of LMD systems with second generation cationic liposome systems such as CDAN/DOPE in place of DC-Chol/DOPE seems to be beneficial (Figures 1 and 5). We recently reported that CDAN/DOPE-mediated *in vitro* transfection of COS7 cells was three-fold more efficient than DC-Chol/DOPE-mediated transfection. CDAN presents two amine functional groups (pKa <8) at neutral pH that may be partially unprotonated and therefore could have the capacity for endosome buffering, thereby facilitating nucleic acid escape.[5] We would suggest that similar effects may also serve to explain why LMD(CDAN) transfection *in vitro* was also three-fold times more effective than first generation DC-Chol/DOPE LMD transfection. Further optimisations were attempted by introducing variations in the LMD lipid:mu:DNA ratio. It is interesting to note that levels of *in vitro* transfection of Panc-1 cells were relatively insensitive to many changes in the preferred lipid:mu:DNA ratio 20 μmol:0.6 mg:1.0 mg m/w/w. Huang and co-workers have observed similar levels of ratio tolerance in working with various LPD systems.[8–10,13,49,50] In our hands, LMD(CDAN) transfections compared favourably with transfections using an equivalent system formulated with protamine sulfate (Figure 5d). We equally observed LMD(CDAN) transfections to be similarly more effective than transfections with equivalent systems prepared from poly L-lysine hydrobromide (pLL), or poly L-arginine hydrochloride (pLA) (known as LKD(CDAN), and LRD(CDAN) respectively) (results not shown). These results emphasise the usefulness of mu peptide in comparison with other commercially available DNA condensing peptides/polypeptides, including protamine. Mechanistic investigations of the transfection process are now under way to determine if the mu peptide sequence may be adapted to enhance transfection efficiency further.

Confocal microscopy experiments were carried out using LMD particles containing fluorescent-labelled plasmid DNA as a means to obtain some initial mechanistic information. The kinetics of LMD transfection appear to be rapid. Cell entry is rapid as suggested by the significant levels of transgene expression after incubation of cells with LMD for transfection times as short as 10 min. Furthermore according to confocal microscopy analysis, intracellular trafficking of plasmid DNA is rapid (minutes) suggesting that endocytosis and late endosome accumulation are not significant barriers and/or impediments to the LMD transfection process (Figure 6), in comparison with what is thought to be the case with LD transfection.[1,2] However, more detailed data are required for a more complete mechanistic picture. In cells growth-arrested using aphidicolin which inhibits the polymerases of eukaryotic cells thus stopping the cell cycle in the S phase,[51] mu peptide did not appear to facilitate plasmid DNA entry into the nucleus (Figure 6c). The nuclear membrane is one of the major barriers of non-viral transfection due to the small inner diameter (50–70 nm) of the nuclear pore complex.[21,22,52–56] Only condensed plasmid DNA can be capable of fulfilling the volumetric requirements to pass through the nuclear pore. Our current data are not conclusive as to whether plasmid DNA is still in the condensed form when entering the nucleus or whether the mu peptides become detached after LMD particles enter the cell leaving plasmid DNA in a free and only partially condensed state. However, the failure of mu peptides to promote plasmid DNA entry into the nucleus is consistent with a failure to continue to condense plasmid DNA adequately following cell entry. All transfection experiments described here were carried out on dividing cells where the integrity of the nuclear cell membrane is perturbed due to the ongoing cell cycles.[57] Therefore, when plasmid DNA entry into the nucleus has been observed, the most likely mechanism is through breakdown of the membrane structure rather than by an active transport process involving the nuclear pore complex.[56]

Ex vivo and *in vivo* LMD transfection data suggest that LMD has sufficient elements of stability in biological tissue and fluids to be a viable and effective transfection agent *in vivo* after further modification (Figures 7 and 8). Certainly there is little doubt that topical lung transfection *in vivo* by LMD compares favourably with transfection mediated by the current 'gold standard' GL-67:DOPE:DMPE-PEG_{5000} (1:2:0.05 m/m/m) cationic liposome system and, if anything, is more dose efficient. Nevertheless, substantive efforts will now have to be introduced to develop a triggerable LMD system; that is, a system truly stable and non-reactive in extracellular fluids but unstable once recognised and internalised by target cells in the target organ of choice. Nevertheless, although a fully triggerable system is not yet developed, taking all the characteristics of LMD together, we would suggest that the first generation LMD non-viral vector system described here does fulfil all the main criteria to be considered a non-viral vector platform system.

Materials and methods

General

Poly L-lysine hydrobromide (pLL) (3970 Da), poly L-arginine hydrochloride (pLA, average MWt 11 800 Da), protamine sulfate (grade X from salmon), dioleoyl phosphatidyl L-α-ethanolamine (DOPE), ammonium thiocyanate and ferric chloride hexahydrate were all purchased from the Sigma-Aldrich (Poole, UK). Cationic liposome system GL-67:DOPE:DMPE-PEG_{5000} (1:2:0.05 m/m/m) was provided by Genzyme (Framingham, MA, USA), TransFast

was bought from Promega (Southampton, UK), and LT-1 from PanVera (Madison, WI, USA). A solution of 0.1 M ammonium ferrothiocyanate was prepared by the addition of ammonium thiocyanate (final concentration 30.4 g/l) and ferric chloride hexahydrate (final concentration 27 g/l) to deionised water. This solution was stored at room temperature before use. Cytofectins 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol); 3-aza-N^1-cholesteryloxycarbonylhexane-1,6-diamine (CJE52, ACHx), N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (B198, CDAN) and N^{15}-cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine (B232, CTAP) were prepared as described previously[4] and stored under argon at −20°C before use. Agarose (biotechnology grade) was obtained from Anachem (Luton, UK). The extruder was obtained from Lipex Biomembranes (Vancouver, Canada), whilst the poretics polycarbonate filters (0.4, 0.2 and 0.1 μm) were purchased from Millipore (Watford, UK). Endotoxin-free plasmids (pCMVβ and pCF1-CAT) were obtained from Bayou BioLabs (LA, USA) and concentrations determined as described previously.[5] The lipid concentrations of cationic liposome suspensions or complexes of lipid, mu peptide and plasmid DNA were determined using an ALC PK12R or a Hitachi RCM150G centrifuge together with an Ultrospec 4000 spectrophotometer (Amersham-Pharmacia Biotech, Amersham, UK). Sizes of cationic liposome suspension or complexes of liposome, mu peptide and plasmid DNA were determined with 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. Sterile filtration was achieved using a Sartorius Minisart (0.45 μm) membrane.

Mu peptide preparation

Mu-peptide was synthesised by standard Merrifield solid phase peptide synthesis[58] procedures on Wang resin (Novabiochem, Nottingham, UK) and standard (9-fluorenyl)methoxycarbonyl (Fmoc)-protected L-amino acids (Novabiochem). In order to prevent potential aggregation of the growing protected-polypeptide chains during solid phase synthesis and consequent loss of recovered peptide yields, the pseudo-proline method was applied to overcome these drawbacks. In this case the Ala-Ser sequence was coupled in the form of a temporary oxazolidine dipeptide building block.[59] After synthesis, peptide purification was carried out on a Hitachi semi-preparative HPLC system at a flow rate of 30 ml/min, using a LiChrospher C_{18} (300 Å, 5 μm) column. Analytical HPLC was run on a Hitachi system using a Purospher RP-18 endcapped column (5 μm) at a flow rate of 1 ml/min, gradient 0–100% acetonitrile (20 min). After elution, fractions containing the desired peptide were combined and lyophilized to give the peptide as a white powder. The identity of the peptide was confirmed by mass spectrometry (MALDI) ($C_{95}H_{170}N_{52}O_{21}S_2$: $[M + H]^+$ calculated 2440.7, found 2440.6) and purity by analytical HPLC (>95%). Desalting of mu was achieved on a Sephadex G-25 M column (Amersham Pharmacia Biotech) after purification by semi-preparative HPLC.

Cationic liposomes

DC-Chol (15 mg, 30 μmol) and DOPE (15 mg, 20 μmol) were combined in dichloromethane. The solution was transferred to a round-bottomed flask (typically 100 ml) and organic solvent removed under reduced pressure (rotary evaporator) giving a thin-lipid film that was dried for 2–3 h *in vacuo*. Following this, HEPES buffer (4 mM, pH 7.2, 3 ml) was added to the round-bottomed flask so as to hydrate the thin-lipid film. After brief sonication (2–3 min) under argon, the resulting cationic liposome suspension (lipid concentration 5 mg/ml) was extruded by means of an extruder device. Initially, three times through two stacked polycarbonate filters (0.2 μm) and then 10 times through two stacked polycarbonate filters (0.1 μm) to form small cationic liposomes (average diameter 109 ± 15 nm according to PCS analysis). Lipid concentrations (approximately 4.5 mg/ml, 7.5 μmol/ml) were determined by the Stewart assay. Cationic liposomes formulated with cytofectins other than DC-Chol were prepared in the same way with identical molar quantities.

Stewart assay

Cationic liposome suspensions (100 μl) were diluted into chloroform (2 ml) (final lipid concentration in the range 0.3–2.5 mg/ml) followed by the addition of 0.1 M ammonium ferrothiocyanate (2 ml). After vortex mixing for 30 s, and centrifugation (5 min, 2000 *g* at 4°C), the lower chloroform layer was collected and optical density measured at A_{485}. The lipid concentration was calculated using a standard curve obtained with a dilution series of pre-extrusion cationic liposome suspensions of known concentration. All measurements taken at A_{485} were measured in triplicate.[60]

Lipoplex preparation

Cationic liposome-plasmid DNA (LD) complexes (lipoplexes) were prepared for transfection of ND7 or Panc-1 cells with an optimised lipid:DNA ratio of 3:1 (w/w) unless otherwise stated, as described previously.[38] Transfection of COS7 cells was performed with an optimised lipid:DNA ratio of 2:1 (w/w) as described previously.[5] Unless otherwise indicated, DC-Chol/DOPE cationic liposomes (6:4, m/m) were used throughout for comparison with LMD.

Preparation of LMD complexes

Initially, mu:DNA (MD) particles were prepared by mixing as follows. Plasmid DNA stock solutions (typically 1.2 mg/ml) were added to a vortex-mixed solution of mu peptide (1 mg/ml) in HEPES (4 mM, pH 7.2). The final mu:DNA ratio was 0.6:1 (w/w), unless otherwise stated, and final plasmid DNA concentration was 0.27 mg/ml. MD containing solutions were further diluted with HEPES (4 mM, pH 7.2) and then added slowly under vortexing to suspensions of extruded DC-Chol/DOPE cationic liposomes (4.5 mg/ml, 7.5 μmol/ml) resulting in the formation of small LMD particles with narrow size distribution (120 ± 30 nm), as measured by photon correlation spectroscopy (PCS). Final lipid:mu:DNA ratio 12:0.6:1 w/w/w (20 μmol:0.6 mg:1 mg; m/w/w). A solution of sucrose (100%, w/v) in 4 mM HEPES buffer, pH 7.2, was then added to obtain LMD particle suspensions at the desired DNA concentration (final DNA concentration typically 0.14 mg/ml and final sucrose concentration 10% w/v). The suspension was divided into small aliquots and these were stored at –80°C until use. Before transfection, an aliquot was thawed on ice, placed in the

bottom of a bijou container (Bibby Sterilin, Staffordshire, UK) and diluted in an appropriate medium (usually 400 μl) for the cells being transfected. Diluted LMD suspensions were then allowed to stand for 15 min at 20°C before being applied to cells. Some transfections were performed in the presence of serum. For these transfections, LMD suspensions were initially diluted with serum-free medium and then further diluted with serum medium before transfection. The LMD(ACHx), LMD(CDAN) and LMD(CTAP) variants described in the text were prepared in the same way as basic LMD with the same final lipid:mu:DNA ratio 20 μmol:0.6 mg:1 mg (m/w/w) unless otherwise stated.

MD and LMD particle characterisation

MD complexes were prepared at a plasmid DNA concentration of 0.27 mg/ml in HEPES (4 mM, pH 7.2) and diluted to 24 μg/ml with the same buffer for size analysis by PCS. Measurements were recorded at 20°C, with a viscosity of 0.0890 cP, a reflex index of 1.33, angle of 90° and at a wavelength of 632.8 nm. Unimodal analysis was used throughout to calculate the mean particle size and standard deviation (s.d.). The size distribution program (SDP) accessing the CONTIN algorithm was utilised, where appropriate, to differentiate multi-modal peaks and detect particle populations of different sizes. Cryo-electron microscopy was used to image preparations of MD and LMD particles. LMD or MD mixtures (5 μl) were applied to holey carbon grids that had been freshly washed with refluxing acetone. After blotting the specimen to a thin film, grids were quench-frozen using a Reichert KF80 freezing device, by plunging into liquid ethane (approximately −186°C). Transfer to the microscope was achieved using a GATAN 626 cryo-transfer system and holder operating at approximately −170°C. Images of LMD or LMD particles embedded in a thin film of amorphous ice were recorded as defocus pairs at 200 kV, ×20 000–50 000 magnification under low-dose conditions on Kodak SO163 film developed to an OD of approximately 0.7 for 12 min in D19 developer.

In vitro transfection experiments

Transfection of ND7 cells: ND7 cells are derived from the fusion of a neuroblastoma (N18Tg2) with neonatal rat sensory neurons and are a well-characterised cell line.[38] These cells were seeded in normal growth medium (NGM) (containing 10% serum) at a density of approximately 4×10^4 cells per well, in a 24-well culture plate. After 24 h, cells were washed by brief exposure to normal growth medium (NGM) (serum free) and then treated with solutions containing LMD or LD particles (formulated with plasmid DNA pCF1-CAT) prediluted with NGM (serum free), for the transfection periods indicated. The final plasmid DNA concentration was 3.2 μg/ml/well in all cases (1 μg dose/well), or else as indicated. Cells were then washed again and incubated for a further 48 h before harvesting. Levels of transfection in each well were determined by chloramphenicol acetyl transferase (CAT) enzyme assay using a fixed initial quantity of ^{14}C-chloramphenicol (^{14}C-CAM) as substrate (Promega, Southampton, UK) per well. Acetylated ^{14}C-labelled chloramphenicol (acetyl-^{14}C-CAM) product was recovered by ethyl acetate extraction and lyophilised, then samples were redissolved in ethyl acetate, spotted on to a Whatman TLC plate and developed in chloroform:methanol (95:5, v/v). Plates were dried, after which acetyl-^{14}C-CAM radioactivity recovered from each sample was quantified relative to initial ^{14}C-CAM radioactivity using a Beckman Phosphoimager. CAT enzyme activities in each sample were then expressed in terms of the percentage (% total activity) of ^{14}C-CAM converted into acetyl-^{14}C-CAM. Transfections were also performed with solutions containing LMD or LD particles formulated with plasmid pCMVβ. In these cases, ND7 cells were treated with solutions containing LMD or LD particles prediluted with NGM (serum free), for a transfection period of 2 h. After this, cells were then washed again and incubated for a further 48 h before processing for histochemical staining with X-gal according to previously published procedures.[36,38,41] The numbers of cells stained blue were counted under a Nikon Diaphot inverted microscope.

Transfection of COS7 cells: COS7 (African Green Monkey kidney cells) were grown and transfected as described previously.[5] Transfection periods and serum levels during transfection were varied as described in the text.

Transfection of Panc-1 cells: Panc-1 cells (human pancreatic cancer cell line) were seeded at an approximate density of 5×10^4 per well in a 24-well culture plate in RPMI supplemented with 10% fetal calf serum (FCS) and grown for 24 h at 37°C in the presence of 5% CO_2. Cells were washed by brief exposure to RPMI and then treated with solutions containing LMD or LD particles (formulated with plasmid pCMVβ) prediluted with RPMI containing 10% FCS, for the transfection periods indicated. The final DNA concentration was 5.0 μg/ml/well in all cases (1 μg dose/well). Cells were then washed again and incubated for a further 48 h in RPMI supplemented with 10% FCS before harvesting and the assay of β-galactosidase (β-gal) enzyme activity using a standard assay kit (Promega). Where appropriate, transfections with TransFast (Promega) were performed according to the manufacturer's instructions in serum-free medium (optimum conditions) with a 1 h transfection time period.

Transfection of confluent Swiss 3T3 cells: Swiss 3T3 cells (fibroblast cell line) were seeded at an approximate density of 2×10^4 per well in a 24-well culture plate in DMEM supplemented with 10% FCS and grown for 48 h to complete confluence in the presence of 5% CO_2 at 37°C. Cells were washed by brief exposure to DMEM and then treated with solutions containing LMD or LD complexes (formulated with plasmid pCMVβ) prediluted with DMEM containing 10% FCS, for the transfection periods indicated. In all cases final plasmid DNA concentration was 5.0 μg/ml/well (1 μg dose/well). Cells were then washed again and incubated for a further 48 h in DMEM supplemented with 10% FCS before harvesting. The level of β-galactosidase (β-gal) enzyme activity was determined with the standard assay kit (Promega).

Confocal microscopy analyses

56 FHTE8o$^-$ cells provided by Dr Dieter Gruenert[61] were cultured in 50% DMEM 50% Ham F12 supplemented with 10% FCS. For transfection experiments, cells were seeded on 12-well culture plates and for confocal

microscopy analysis on Labteck slides and grown until semi-confluent. Cell growth arrest was effected with aphidicolin (Sigma-Aldrich) in the manner described previously.[51]

Ex vivo and in vivo transfection experiments

Ex vivo transfection of organotypic explant cultures of the rat brain ventral mesencephalon were performed essentially as described previously.[36,41] Histochemical staining with X-gal was also performed according to previously published procedures.[36,38,41] The numbers of cells stained blue/slice were counted under a Nikon Diaphot inverted microscope as above. *In vivo* transfections were performed with Balb/c mice as described previously.[5]

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References

1 Miller AD. Cationic liposomes for gene therapy. *Angew Chem Int Ed* 1998; **37**: 1768–1785.
2 Miller AD. Nonviral delivery systems for gene therapy. In: Lemoine NR (ed.). *Understanding Gene Therapy*. BIOS Scientific Publishers: Oxford, 1999, pp 43–69.
3 Alton EWFW *et al*. Noninvasive liposome-mediated gene delivery can correct the ion-transport defect in cystic-fibrosis mutant mice. *Nature Genet* 1993; **5**: 135–142.
4 Cooper RG *et al*. Polyamine analogues of 3-β-[N-(N′N′-dimethylaminoethane)carbomoyl]cholesterol (DC-Chol) as agents for gene delivery. *Chem Eur J* 1998; **4**: 137–151.
5 Stewart L *et al*. Cationic lipids for gene therapy. Part 4 Physicochemical analysis of cationic liposome-DNA complexes (lipoplexes) with respect to *in vitro* and *in vivo* gene delivery efficiency. *J Chem Soc-Perkin Trans 2* 2001; 624–632.
6 Li S, Huang L. Protamine sulfate provides enhanced and reproducible intravenous gene transfer by cationic liposome/DNA complex. *J Lipos Res* 1997; **7**: 207–219.
7 Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Therapy* 1997; **4**: 891–900.
8 Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. *Gene Therapy* 1997; **4**: 961–968.
9 Li S, Rizzo MA, Bhattacharya S, Huang L. Characterization of cationic lipid-protamine-DNA (LPD) complexes for intravenous gene delivery. *Gene Therapy* 1998; **5**: 930–937.
10 Li S *et al*. Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Therapy* 1999; **6**: 585–594.
11 Li B *et al*. Lyophilization of cationic lipid-protamine-DNA (LPD) complexes. *J Pharm Sci* 2000; **89**: 355–364.
12 Whitmore M, Li S, Huang L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Therapy* 1999; **6**: 1867–1875.
13 Chesnoy S, Huang L. Structure and function of lipid-DNA complexes for gene delivery. *Annu Rev Biophys Biomolec Struct* 2000; **29**: 27–47.
14 Dokka S *et al*. High-efficiency gene transfection of macrophages by lipoplexes. *Int J Pharm* 2000; **206**: 97–104.
15 Birchall JC, Kellaway IW, Gumbleton M. Physical stability and *in vitro* gene expression efficiency of nebulised lipid-peptide-DNA complexes. *Int J Pharm* 2000; **197**: 221–231.
16 Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. *Biochemistry* 1996; **35**: 1027–1036.
17 Vitiello L *et al*. Condensation of plasmid DNA with polylysine improves liposome-mediated gene transfer into established and primary muscle cells. *Gene Therapy* 1996; **3**: 396–404.
18 Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient *in vivo* gene delivery. *FEBS Lett* 1997; **400**: 233–237.
19 Zhou XH, Huang L. DNA transfection mediated by cationic liposomes containing lipopolylysine. Characterization and mechanism of action. *Biochim Biophys Acta-Biomembr* 1994; **1189**: 195–203.
20 Fritz JD, Herweijer H, Zhang GF, Wolff JA. Gene transfer into mammalian cells using histone-condensed plasmid DNA. *Hum Gene Ther* 1996; **7**: 1395–1404.
21 Hagstrom JE *et al*. Complexes of non-cationic liposomes and histone H1 mediate efficient transfection of DNA without encapsulation. *Biochim Biophys Acta-Biomembr* 1996; **1284**: 47–55.
22 Namiki Y, Takahashi T, Ohno T. Gene transduction for disseminated intraperitoneal tumor using cationic liposomes containing non-histone chromatin proteins: cationic liposomal gene therapy of carcinomatosa. *Gene Therapy* 1998; **5**: 240–246.
23 Schwartz B *et al*. Synthetic DNA-compacting peptides derived from human sequence enhance cationic lipid-mediated gene transfer *in vitro* and *in vivo*. *Gene Therapy* 1999; **6**: 282–292.
24 Jenkins RG *et al*. An integrin-targeted non-viral vector for pulmonary gene therapy. *Gene Therapy* 2000; **7**: 393–400.
25 Cooper RG *et al*. Peptide mini-vectors for gene delivery. *Angew Chem Int Ed* 1999; **38**: 1949–1952.
26 Colin M *et al*. Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells. *Gene Therapy* 1998; **5**: 1488–1498.
27 Vaysse L, Arveiler B. Transfection using synthetic peptides: comparison of three DNA-compacting peptides and effect of centrifugation. *Biochim Biophys Acta-Gen Subj* 2000; **1474**: 244–250.
28 Chen QR, Zhang L, Stass SA, Mixson AJ. Co-polymer of histidine and lysine markedly enhances transfection efficiency of liposomes. *Gene Therapy* 2000; **7**: 1698–1705.
29 Anderson CW, Young ME, Flint SJ. Characterization of the adenovirus-2 virion protein Mu. *Virology* 1989; **172**: 506–512.
30 Shenk T. In: Fields BN *et al* (eds). *Fields Virology*. Lippincott-Raven Publishers, Philadelphia, PA, 1996, pp 2111–2148.
31 Hosokawa K, Sung MT. Isolation and characterisation of an extremely basic protein from adenovirus type 5. *J Virol* 1976; **17**: 924–934.
32 Chatterjee PK, Vayda ME, Flint SJ. Interactions among the 3 adenovirus core proteins. *J Virol* 1985; **55**: 379–386.
33 Matthews DA, Russell WC. Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J Gen Virol* 1998; **79**: 1671–1675.
34 Matthews DA, Russell WC. Adenovirus core protein V interacts with p32. A protein which is associated with both the mitochondria and the nucleus. *J Gen Virol* 1998; **79**: 1677–1685.
35 Keller M, Tagawa T, Preuss M, Miller AD. Biophysical characterization of the DNA binding and condensing properties of adenoviral core peptide μ (mu). *Biochemistry* 2002; **41**: 652–659.
36 Murray KD *et al*. Enhanced cationic liposome-mediated transfection using the DNA-binding peptide μ (mu) from the adenovirus core. *Gene Therapy* 2001; **8**: 453–460.
37 Zhang YP *et al*. Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties. *Gene Therapy* 1999; **6**: 1438–1447.
38 McQuillin A *et al*. Optimization of liposome mediated transfection of a neuronal cell line. *Neuroreport* 1997; **8**: 1481–1484.
39 Fellowes R *et al*. Amelioration of established collagen induced

arthritis by systemic IL-10 gene delivery. *Gene Therapy* 2000; **7**: 967–977.
40 Wheeler VC, Coutelle C. Nondegradative *in vitro* labeling of plasmid DNA. *Anal Biochem* 1995; **225**: 374–376.
41 Murray KD *et al*. Cationic liposome-mediated DNA transfection in organotypic explant cultures of the ventral mesencephalon. *Gene Therapy* 1999; **6**: 190–197.
42 Lee ER *et al*. Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum Gene Ther* 1996; **7**: 1701–1717.
43 Alton EWFW *et al*. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet* 1999; **353**: 947–954.
44 Romano G, Claudio PP, Kaiser HE, Giordano A. Recent advances, prospects and problems in designing new strategies for oligonucleotide and gene delivery in therapy. *In Vivo* 1998; **12**: 59–67.
45 Behr JP. Overcoming barriers to nonviral gene delivery. *Blood Cells Mol Dis* 2000; **26**: 58–60.
46 Simoes S *et al*. Mechanisms of gene transfer mediated by lipoplexes associated with targeting ligands or pH-sensitive peptides. *Gene Therapy* 1999; **6**: 1798–1807.
47 MacLachlan I, Cullis P, Graham RW. Progress towards a synthetic virus for systemic gene therapy. *Curr Opin Mol Ther* 1999; **1**: 252–259.
48 Wattiaux R *et al*. Endosomes lysosomes: their implication in gene transfer. *Adv Drug Del Rev* 2000; **41**: 201–208.
49 Li B, Feng S. Effects of component cholesterol on the structure of lipid monolayer and bilayer membranes. *Biophys J* 1999; **76**: 62.
50 Li S *et al*. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am J Physiol-Lung Cell Mol Physiol* 1999; **276**: L796–L804.
51 Spadari S, Sala F, Pedralinoy G. Aphidicolin: a specific inhibitor of nuclear-DNA replication in eukaryotes. *Trends Biochem Sci* 1982; **7**: 29–32.
52 Aronsohn AI, Hughes JA. Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy. *J Drug Target* 1998; **5**: 163–169.
53 Subramanian A, Ranganathan P, Diamond SL. Nuclear targeting peptide scaffolds for lipofection of non-dividing mammalian cells. *Nat Biotechnol* 1999; **17**: 873–877.
54 Zanta MA, Belguise-Valladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci USA* 1999; **96**: 91–96.
55 Udvardi A *et al*. Uptake of exogenous DNA via the skin. *J Mol Med* 1999; **77**: 744–750.
56 Neves C *et al*. Intracellular fate and nuclear targeting of plasmid DNA. *Cell Biol Toxicol* 1999; **15**: 193–202.
57 Tseng WC, Haselton FR, Giorgio TD. Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. *Biochim Biophys Acta-Gene Struct Expression* 1999; **1445**: 53–64.
58 Merrifield RB. Solid-phase synthesis. *Science* 1986; **232**: 341–347.
59 Keller M, Miller AD. Access to the inaccessible sequence of Cpn 60.1 (195-217) by temporary oxazolidine protection of selected amide bonds. *Bioorg Med Chem Lett* 2001; **11**: 857–859.
60 Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Anal Biochem* 1980; **104**: 10–14.
61 Gruenert DC *et al*. Characterization of human tracheal epithelial cells transformed by an origin-defective Simian virus-40. *Proc Natl Acad Sci USA* 1988; **85**: 5951–5955.
62 Colin M *et al*. Cell delivery intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells. *Gene Ther* 2000; **7**: 139–152.