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Physical stability and in-vitro gene expression efficiency of nebulised lipid–peptide–DNA complexes

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Abstract

The lower respiratory tract provides a number of disease targets for gene therapy. Nebulisation is the most practical system for the aerosolisation of non-viral gene delivery systems. The aerosolisation process represents a significant challenge to the maintenance of the physical stability and biological activity of the gene vector. In this study we investigate the role of a condensing polycationic peptide on the stability and efficiency of nebulised lipid–DNA complexes. Complexes prepared from the cationic lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and plasmid DNA (pDNA) at mass (w/w) ratios of 12:1, 6:1 and 3:1, and complexes prepared from DOTAP, the polycationic peptide, protamine, and pDNA (LPD) at 3:2:1 w/w ratio were nebulised using a Pari LC Plus jet nebuliser. Samples from the nebuliser reservoir (pre- and post-nebulisation) and from the aerosol mist were collected and investigated for changes, including: particle diameter, retention of in-vitro transfection activity and the relative concentration and nature of the complexed pDNA remaining after the nebulisation procedure. The process of jet nebulisation adversely affected the physical stability of lipid:pDNA complexes with only those formulated at 12:1 w/w DOTAP:pDNA able to maintain their pre-nebulisation particle size distribution (145 ± 3 nm pre-nebulisation vs. 142 ± 2 nm aerosol mist) and preserve significant pDNA integrity in the reservoir (35% of pre-nebulisation pDNA band intensity). The LPD complexes were smaller (102 ± 1 nm pre-nebulisation vs. 113 ± 2 nm aerosol mist) with considerably greater retention of pDNA integrity in the reservoir (90% of pre-nebulisation pDNA band intensity). In contrast the concentration of pDNA in the aerosol mist for both the 12:1 w/w DOTAP:pDNA and LPD complexes were significantly reduced (10 and 12% of pre-nebulised values, respectively). Despite reduced pDNA concentration the transfection (% cells transfected) mediated by aerosol mist for the nebulised complexes was comparatively efficient (LPD aerosol mist 26 vs. 40% for pre-nebulised complex; the respective values for 12: 1 w/w DOTAP:pDNA were 12 vs. 28%). The physical stability and biological activity of nebulised lipid:pDNA complexes can be improved by inclusion of a condensing polycationic peptide such as protamine. The incorporation of the peptide precludes the use of potentially toxic excesses of lipid and charge and may act as a platform for the covalent attachment of peptide signals mediating sub-cellular targetting.

Keywords: Gene therapy; Cationic lipid; Plasmid DNA; Polycationic peptide; Protamine; Nebulisation; Aerosolisation; Lung

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1. Introduction

The lower respiratory tract provides a number of disease targets for the gene therapy of both acquired and inherited genetic defects, including cancer (Smith et al., 1997), potentially asthma (Demoly et al., 1997), α1-anti-trypsin deficiency (Canonico et al., 1994) and most notably cystic fibrosis (Alton et al., 1998; Sheng and Scheule, 1998). Further, a number of acute pulmonary indications such as protection from endotoxin lung injury (Conary et al., 1994) and modulation of transplant rejection (Pierson et al., 1996) may, in the future, benefit from the delivery of therapeutic genes. Aerosol administration of gene vectors is necessary to achieve widespread lower respiratory tract deposition and afford localised and potentially direct access to the appropriate epithelial and sub-mucosal target cells. Currently, nebulisation is the most acceptable and practical system for aerosolisation of gene vectors (Eastman et al., 1998; Geddes and Alton, 1998), although metered dose (Brown and Chowdhury, 1997), aerosol unit-dose (Sorgi et al., 1998) and dry-powder (Holzner et al., 1997) inhalation strategies are also receiving attention. However, the physical stability of nucleic acid throughout storage and during the aerosolisation process itself will remain an important and determining issue.

Complexes formed between cationic liposomes and plasmid DNA (pDNA) have been the predominant non-viral vectors utilised in studies of inhalational gene delivery to the respiratory tract (Sheng and Scheule, 1998). Such complexes self-assemble through electrostatic interactions and, although considerable physical heterogeneity may be displayed even within a single formulation (Birchall et al., 1999), they provide for the relative ease in production of large quantities of condensed pDNA particles. With the aim of improving the efficiency of lipid-mediated gene transfer and reducing some of the dose-dependent pulmonary inflammation associated with cationic lipid administration (Scheule et al., 1997), many new cationic lipids have been synthesised and evaluated (Lee et al., 1996). Nevertheless, cationic lipid based gene transfer remains relatively inefficient compared to that mediated by viral vectors such as adenovirus, and there is increasing realisation that the design of efficient non-viral vectors is likely to benefit from the incorporation of certain virus-like characteristics, e.g. nucleic-acid condensing core surrounded by a lipid coat and with the vector possessing appropriate cellular and sub-cellular targeting signals.

The work from the laboratory of Huang (Gao and Huang, 1996; Li and Huang, 1997; Sorgi et al., 1997) has shown that pre-condensation of pDNA with polycationic peptides, such as poly-(L)-lysine and protamine, potentiates cationic liposome-mediated gene delivery. The high proportion of cationic amino acids in these peptides enables them to condense and stabilise pDNA into a highly compact structure. Indeed, these triplex systems form small condensed pDNA particles that are less prone to the aggregation observed with some lipid–pDNA complexes (Gao and Huang, 1996). Although molecules such as poly(L-lysine) and protamine will probably serve only as laboratory models, the inclusion of a linear condensing peptide in a complex clearly provides a suitable scaffold for the covalent attachment of peptide targeting 'signals', either through contiguous extension of the primary peptide sequence itself, or through coupling via side-chain modifications. Such 'signals' may include ligands that mediate sub-cellular targeting, i.e. endosome disruption (Plank et al., 1994; Puyal et al., 1994) or nuclear localisation (Kaneda et al., 1989; Conary et al., 1996) in a manner analogous to viruses (Pouton et al., 1998; Rolland, 1998). With incorporation of multifunctional units within the non-viral vector there will be even more requirement to ensure not only that the aerosolisation process affords retention of pDNA integrity, but also the appropriate orientation of targeting and condensing units.

In this in vitro study we show that lipid:peptide:DNA (LPD) vectors, comprising the polycationic peptide protamine, give rise to multilamellar lipid-coated particulates that are smaller and more homogenous compared to lipid:pDNA complexes alone, and whose structure remains unaltered by jet nebulisation. Further, compared to lipid: pDNA complexes prepared even at very

high mass (w/w) ratios, i.e. 12:1, the LPD complexes afford significantly greater protection to pDNA against the shearing forces encountered in a jet nebuliser. This protection afforded by LPD complexes is achieved without the use of excess quantities of cationic lipid and indeed with particles that provide for a lower theoretical and experimental positive charge.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) was purchased from Avanti Polar Lipids (AL, USA). Protamine sulphate Grade X from salmon sperm was obtained from Sigma Chemical (Poole, UK). The 4.7 kb pEGFP-N1 plasmid construct containing the green fluorescent protein (GFP) reporter gene expressed under the control of the immediate early promoter of human cytomegalovirus was amplified and purified from a commercial construct (Clontech, Palo Alto, USA). The plasmid was propagated using a transformed DH5α strain of *Eschericia coli* colonised onto an ampicillin selective LB agar plate and cultured overnight at 37°C. The plasmid DNA was harvested and purified using a Qiagen Plasmid Mega Kit (Qiagen, Crawley, UK). The following compounds were used as received: agarose LE (Promega, Southampton, UK), ethidium bromide solution (Pharmacia Biotech, St Albans, UK). All other reagents were of analytical grade and purchased from Fisher Scientific UK (Loughborough, UK). Cell culture plastics were obtained from Corning-Costar (High Wycombe, UK). Dulbecco's Modified Eagle's Medium (DMEM 25 mM HEPES), foetal bovine serum, penicillin–streptomycin solution and trypsin–EDTA solution 1X were from Life Technologies (Paisley, UK)

2.2. Methods

2.2.1. Preparation of complexes

DOTAP:pDNA complexes were prepared by adding extruded DOTAP liposomes (1 mg/ml stock in sterile purified water, approximate mass median aerodynamic diameter (MMAD) of 120 nm) to pEGFP-N1 plasmid (1 mg/ml stock in sterile purified water) at a mass (w/w) ratio of 3:1, 6:1 and 12:1, gently mixing and incubating at room temperature for 20 min. LPD complexes were prepared by sequential addition (with 10 min incubation at each step) of firstly protamine (1 mg/ml stock in sterile purified water) and then extruded DOTAP liposomes to pDNA to achieve a lipid:peptide:DNA of 3:2:1 (w/w). All complexes were freshly prepared and maintained on ice (not exceeding 60 min) prior to aerosolisation.

2.2.2. Nebulisation of complexes

Solutions (3 ml) of complexes at a pDNA concentration of 50 μg/ml were placed into the reservoir of a Pari LC Plus nebuliser and jet nebulised for a 10 min period at a flow rate of 5 l/min. The Pari LC Plus jet nebuliser was selected as it has been shown relatively efficient at generating respirable aerosols of concentrated active cationic lipid:pDNA complexes (Eastman et al. 1998). Samples were taken from the nebuliser reservoir prior to and post nebulisation and from the aerosolised mist collected in ice cold glass tubes.

2.2.3. Microelectrophoresis and size analysis

The zeta potentials of the DOTAP:pDNA and DOTAP:protamine:pDNA complexes were determined by microelectrophoresis (Malvern Zeta-Sizer 3, Malvern Instruments, Malvern, UK). All complexes were formed in distilled, degassed water and consecutively measured ten times with instrument calibration prior to each series of measurements. The diameters of the complexes were measured by photon correlation spectoscopy (PCS) at 25°C using a Coulter N4 Plus (Coulter Electronics, Luton, UK) with a 10 mW laser and a scattering angle of 90°. The diameter of the complexes was determined in triplicate and expressed in unimodal and Size Distribution Processor (SDP) mode. Prior to use all glass and plasticware was prewashed with filtered water to minimise particulate contamination. Results are expressed as mean ± SD.

2.2.4. Ethidium bromide gel exclusion assay

The nature and relative concentrations of DNA remaining after the nebulization procedure were estimated by a standard gel based assay exploiting DNA intercalation of the fluorescent probe, ethidium bromide (EtBr) (Tomlinson and Rolland 1996). Complexes were nebulised as described above and samples from the nebulizer resevoir (pre- and post-nebulisation) and from the aerosol mist were collected. To release DNA, the samples were digested (100 mM NaCl, 10 mM Tris–Cl pH8, 25 mM EDTA pH 8, 0.5% sodium dodecyl sulphate, 0.1 mg/ml proteinase K) for 35 mins at 50°C. The liberated DNA was purified (Wizard® DNA Clean-Up System, Promega, Madison, USA) and a 25 μl aliquote (equivalent to 2.5 μg of pre-nebulised pDNA) was then loaded onto a 1% agarose gel. The gel was placed in an electrophoresis tank containing 0.5 μg/ml EtBr in $0.5 \times$ TBE buffer, run at 100 volts for 1 h, removed from the tank and visualised under UV light with quantitation by Molecular Analyst® software (Bio-Rad Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA).

2.2.5. Negative stain transmission electron microscopy

The method used to visualise the lipid–DNA complexes was adapted from Zabner et al. (1995). Freshly prepared complexes were placed onto 100 mesh nickel grids. After 3 min the excess solution was wicked off with filter paper and replaced with freshly filtered and centrifuged 2% aqueous uranyl acetate for 30 s. The grids were washed twice with distilled water and allowed to dry. The grids were imaged using a Philips 208 transmission electron microscope.

Table 1
Theoretical charge ratio and zeta potential of lipid:pDNA and lipid:polycation:pDNA complexes

Constituent ratio (w/w)	Theoretical charge ratio (±)	Zeta potential (mV)
DOTAP:DNA 3:1	1.4:1	-8.9 ± 0.8
DOTAP:DNA 6:1	2.8:1	0.183 ± 0.1
DOTAP:DNA 12:1	5.7:1	19.517 ± 0.8
DOTAP :protamine:DNA 3:2:1	4.1:1	7.767 ± 1.7

2.2.6. In vitro transfection efficiency of complexes

A549 (human lung epithelial carcinoma) cells (European Collection of Animal Cell Cultures, Salisbury, UK) were cultured in 24-well format with media comprising DMEM, 10% foetal bovine serum, and the antibiotics penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were grown to 85% confluency at 37°C in a humid atmosphere at 95% air/5% CO_2. For transfection, cells were washed twice with PBS and, in the absence of serum, exposed to equivalent volumes of pre-nebulised suspension (equivalent to 5 μg of pDNA per well or per million cells) and aerosol mist. The cells were incubated at 37°C for 6 h then surface rinsed thoroughly and fed with 1 ml of culture medium. The cells were returned to the incubator for a further 42 h to allow intracellular expression of the plasmid (pEGFP-N1) to proceed. The percentage of cells showing Green Fluorescent Protein (GFP) associated fluorescence (FL1-H) was quantified by flow cytometry (FACScan, Beckton Dickinson Immunocytometry Systems, San Jose, CA).

3. Results and discussion

DOTAP:pDNA complexes were prepared at mass ratios of 3:1, 6:1 and 12:1 w/w and the lipid:peptide:pDNA (LPD) complexes containing DOTAP, protamine and pDNA were prepared at a mass ratio of 3:2:1 w/w. The theoretical charge ratio, i.e. the ratio of cationic amine groups contributed by each lipid and protamine molecule to anionic phosphate groups contributed by each nucleotide, and the zeta potential, as determined by microelectrophoresis, of each of the complexes are shown in Table 1. Although it is apparent that not all the cationic and anionic groups will be available for electrostatic interaction the theoretical and actual surface charge estimations of the complexes reflect their constituent ratios with the DOTAP:pDNA 12:1 w/w complex showing the highest charge excess.

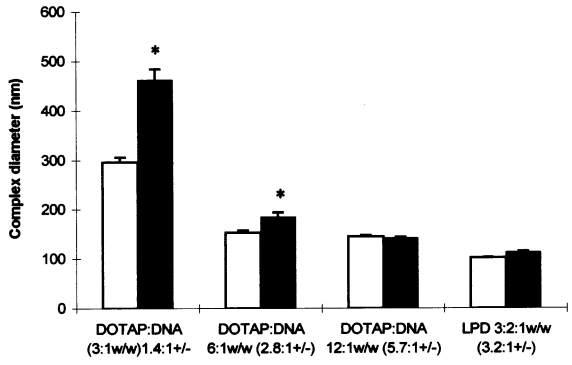

Fig. 1. Diameter of pDNA complexes pre- (white bars) and post- (black bars) nebulisation. Complexes were measured by photon correlation spectoscopy (PCS) using a Coulter N4 Plus. Data represented as mean unimodal diameter $\pm$ SD, $n = 3$. Statistical analysis by One-Way Analysis of Variance and Duncan's multiple range test, showing differences between pre- and post-nebulisation data. Significance level $P < 0.05$. *, Statistically significant difference compared to pre-nebulisation diameters.

Maintaining the size of lipid:DNA complexes is considered critical in order to retain their transfection efficiency (Anchordoquy et al., 1997) with a likely diminished cell internalisation of complexes of increased size (Mahato et al., 1997). Fig. 1 shows the effect of jet nebulisation on the MMAD of: (i) DOTAP:pDNA complexes prepared at mass ratios of 3:1, 6:1 and 12:1 (w/w) and a lipid:peptide:pDNA (LPD) complex containing DOTAP:protamine:pDNA prepared at a mass ratio of 3:2:1 (w/w). The LPD formulation produced particles of the smallest size with pre- and post-nebulisation diameters remaining statistically indistinguishable ($P > 0.05$) at 102 ± 1 and 113 ± 2 nm, respectively. The diameter of the DOTAP:pDNA complex formulated at a lipid:DNA mass ratio of 12:1 (w/w) was also observed to remain unaffected by the nebulisation procedure, although the pre-nebulisation diameter of this complex (145 ± 3 nm), as for all the DOTAP:pDNA complexes, was significantly ($P < 0.05$) larger than that of the pre-nebulised LPD complex (for clarity symbols defining this latter statistical significance are not shown in Fig. 1). As the relative amount of cationic lipid in the DOTAP:pDNA complexes was reduced to mass ratios of 6:1 and 3:1 (w/w) there was increasing susceptibility of the complex to increase in size following the nebulisation procedure, e.g. the diameter of DOTAP–pDNA complexes prepared at 3:1 (w/w) increased from a pre-nebulisation diameter of 296 ± 7.9 to 463 ± 17 nm following nebulisation. More detailed analysis of the data (Size Distribution Processor (SDP) mode) revealed that the size distribution of pre-nebulised DOTAP-pDNA 3:1 (w/w) complexes was bimodal, consisting of a population of complexes of 226 ± 9 nm (approximately 70% of total population) and a population of aggregates with MMAD of 1143 ± 156 nm (30%), with nebulisation causing an increase (to 45%) in the proportion of particles represented by the larger diameter. DOTAP–pDNA 6:1 and 12:1 (w/w) complexes possessed unimodal size distributions before nebulisation. However, whereas the 12:1 (w/w) complexes remained unimodal following aerosolisation, the size distribution of the 6:1 (w/w) complexes was bimodal, with 25% of the total population forming aggregates of 806 ± 414 nm. Intriguingly, SDP analysis of the LPD complexes revealed a bimodal distribution prior to nebulisation, with 30% of the population having diameters of 62 ± 1 nm and 70% having diameters of 158 ± 37 nm. Following aerosolisation the LPD complexes adopted a unimodal size distribution with a mean particle diameter of 131 ± 19 nm.

Agarose gel electrophoresis was used to determine the degree of protection afforded to the plasmid by complexation with either lipid alone or with combined lipid and polycationic peptide (Fig. 2). Samples of the pDNA complexes were removed from the nebuliser reservoir prior to and post-nebulisation. In addition, the generated aerosol mist was collected for analysis. All samples were incubated with digestion buffer to liberate free pDNA and the purified extracts were analysed by gel electrophoresis with ethidium bromide staining. The fluorescent bands corresponding to pDNA fell in the linear response range of gel documentation software (Molecular Analyst, Bio-Rad Laboratories, Hercules, CA) with band intensities being reflective of the relative concentrations of pDNA in the nebuliser solutions and collected aerosol mist. The gel analysis revealed that the bands for pDNA, when complexed with

DOTAP liposomes at lipid:pDNA ratios of 3:1 and 6:1 (w/w), was almost totally lost during the nebulisation process. Specifically, the band associated with the supercoiled pDNA recovered from the nebuliser reservoir after 10 min aerosolisation was, for the 3:1 (w/w) ratio only 5% (lane 2) of its pre-nebulisation value (lane 1). For the 6:1 (w/w) ratio the respective value was 8% (lane 5) of its pre-nebulisation control (lane 4). In either case the nature of the degradative process was more severe than simple induction of DNA nicks, as evidenced by the parallel loss of band intensity for both supercoiled and open circular forms of pDNA. For both the 3:1 and 6:1 (w/w) complexes the band intensity for supercoiled pDNA collected from the aerosol mist (lanes 3 and 6, respectively) was less than 1% of its respective pre-nebulisation value.

A significant retention of pDNA signal in the reservoir post-nebulisation was observed (Fig. 2) for the LPD complex and for the DOTAP complex formulated at 12:1 (w/w). For the lipid:DNA

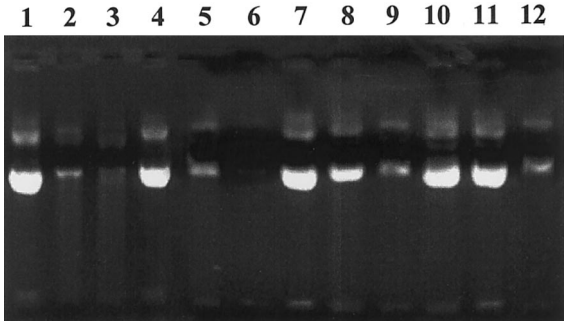

Fig. 2. Ethidium bromide (EtBr) exclusion gel. The amount of intact DNA was estimated by the accessability of the plasmid to the intercalating dye EtBr. Lane 1, DOTAP:pDNA 3:1 (w/w) before nebulisation; Lane 2, DOTAP:pDNA 3:1 (w/w) from nebuliser reservoir upon completion of nebulisation; Lane 3, DOTAP:pDNA 3:1 (w/w) in aerosol mist; Lane 4, DOTAP:pDNA 6:1 (w/w) before nebulisation; Lane 5, DOTAP:pDNA 6:1 (w/w) from nebuliser reservoir upon completion of nebulisation; Lane 6, DOTAP:pDNA 6:1 (w/w) in aerosol mist; Lane 7, DOTAP:pDNA 12:1 (w/w) before nebulisation; Lane 8, DOTAP:pDNA 12:1 (w/w) from nebuliser reservoir upon completion of nebulisation; Lane 9, DOTAP:pDNA 12:1 (w/w) in aerosol mist Lane 10, LPD before nebulisation; Lane 11, LPD from nebuliser reservoir upon completion of nebulisation; Lane 12, LPD in aerosol mist.

12:1 (w/w) complex the band for supercoiled pDNA recovered from the nebuliser reservoir following 10 mins aerosolisation was 35% (lane 8) of its pre-nebulisation value (lane 7), while for the LPD complex an even greater proportion, i.e. 90% (lane 11), of pre-nebulisation level (lane 10) was retained. Clearly the levels of pDNA remaining in the reservoir may reflect to some extent a concentrating effect of the nebulisation procedure arising from preferential aerosolisation of the solvent (Eastman et al., 1998). Nevertheless, it is evident that the LPD complex afforded significantly more protection to pDNA than even the 12:1 (w/w) DOTAP complex. This is surprising given the high ratios of lipid to pDNA under study, and that DOTAP liposomes at these ratios have been assessed for their protective effect on pDNA following jet nebulisation (Crook et al., 1996) and subsequently been employed as gene therapy vectors in clinical aerosolisation studies (Porteous et al., 1997). However, despite the significant amount of pDNA retained in the nebuliser reservoir with the LPD complex, both the LPD complex and the 12:1 (w/w) DOTAP complex resulted in similar mass output of pDNA with the aerosol mist, i.e. respectively, 12 and 10% of the pre-nebulisation concentration (Fig. 2). The relatively low level of pDNA output in the aerosol mist is not representative of any further degradation beyond that encountered in the nebuliser reservoir itself, but is a combined function of the inefficiencies of the nebulisation process (Eastman et al., 1998) and the physico-chemical characteristics of pDNA particulates at increasingly elevated concentrations. Indeed, corroborative data for this is provided by the lipid:pDNA nebulisation experiments of Stern et al. (1998) demonstrating equivalent pDNA aerosol mass output for complexed pDNA spanning a sixfold range of reservoir concentrations, and reflecting a reduced relative efficiency of output as reservoir concentrations increase.

The structures formed between DOTAP liposomes, protamine sulphate and pDNA were visualised by negative stain transmission electron microscopy (EM). In all cases the size of the majority of lipid:pDNA and LPD complexes visualised by EM was representative of the diameters

measured by PCS. The relatively large un-complexed pDNA structures observed using this procedure (Fig. 3A) appear to result from aggregation of the negatively charged plasmid in the presence of the cationic uranyl (UO_2^{2+}) ion (Harris 1991) as these large pDNA aggregates are also observed with samples pre-filtered (400 nm pore size) prior to EM staining. Fig. 3B shows the appearance of the small (approximately 100 nm) unilamellar DOTAP liposomes used in this study. Fig. 3C shows the appearance of complexes prepared between these DOTAP liposomes and the pEGFP-N1 formulated at the maximum lipid:pDNA weight ratio of 12:1 (w/w) (5.7:1 ±

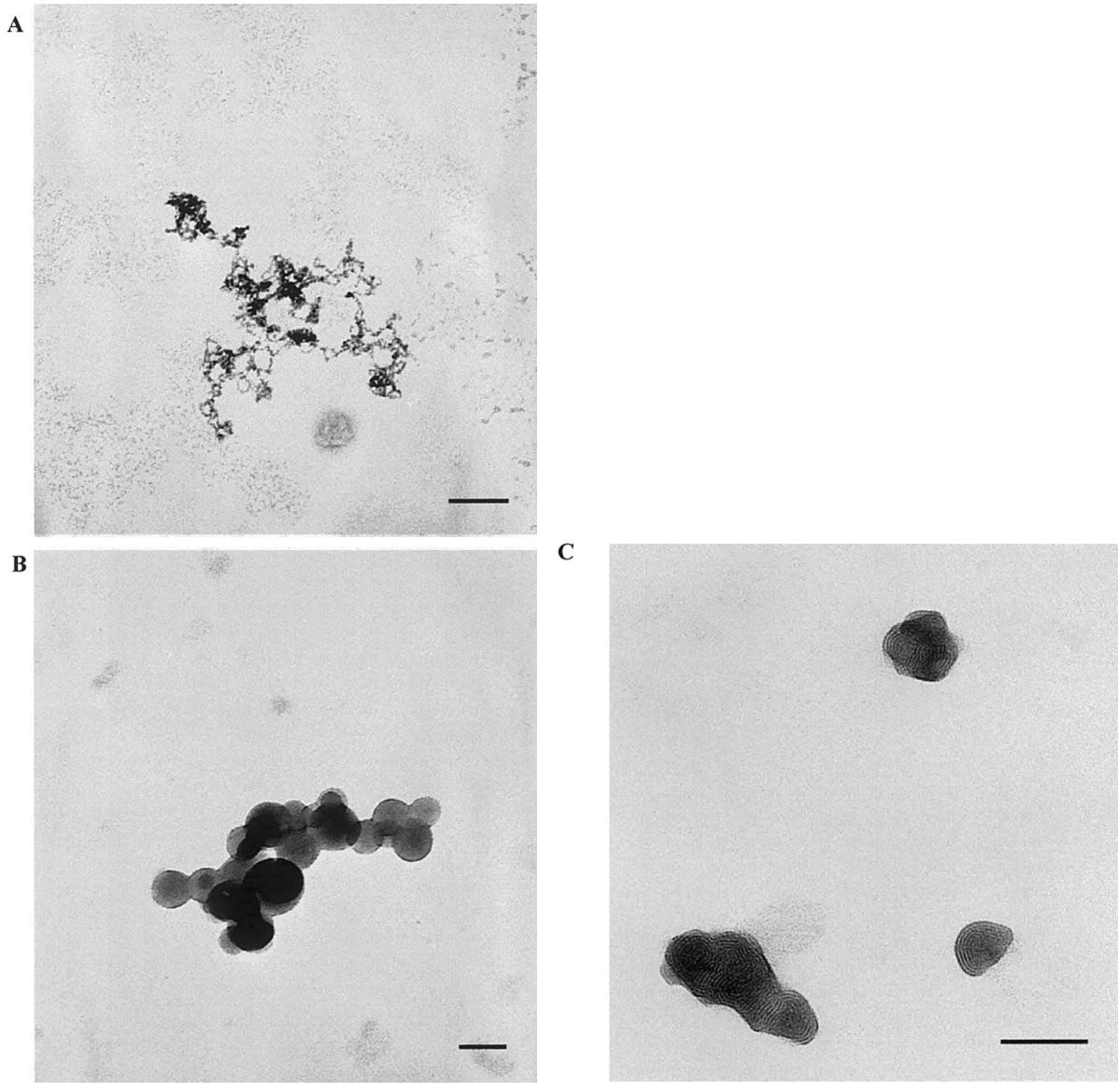

Fig. 3. Negative stain transmission electron microscopy of complexes. The method used to visualise the lipid–DNA complexes was adapted from Zabner et al. (1995). pEGFP-N1 (A), DOTAP (B), DOTAP:pDNA 12:1 (w/w) before nebulisation (C), LPD 3:2:1 (w/w) before nebulisation (D) and LPD 3:2:1 (w/w) in aerosol mist (E). Bar, 100 nm.

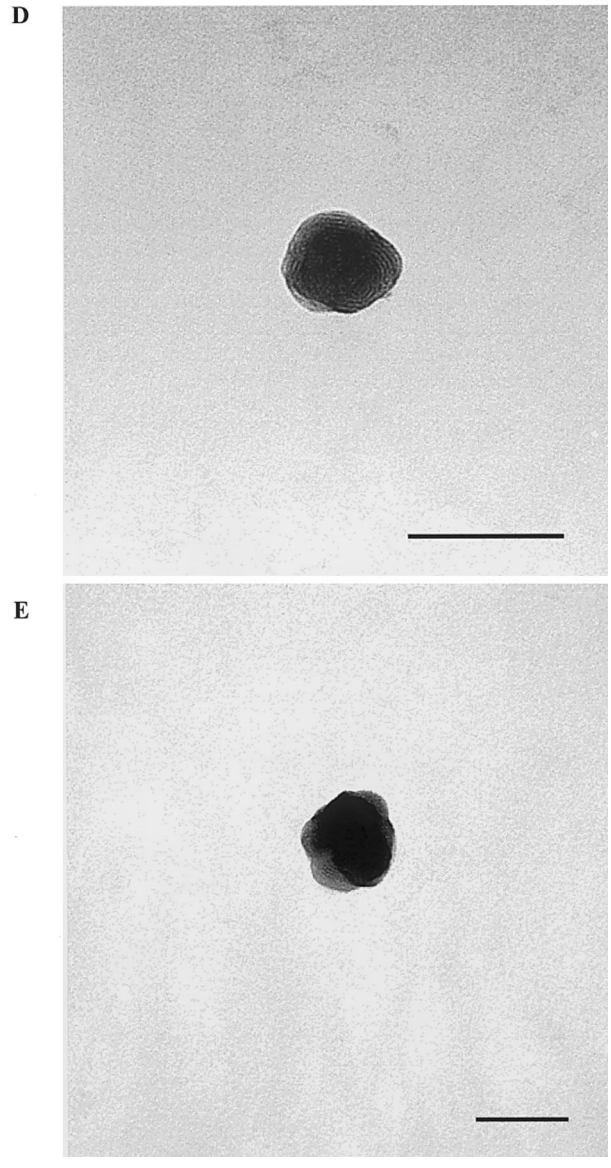

Fig. 3. (*Continued*)

charge ratio). The unilamellar liposomes appear rearrange in the presence of the DNA to form multilamellar lipid-coated particulates. These 'fingerprint-like' colloidal particles are the only structures apparent at positive lipid:pDNA charge ratios and have been reported by other investigators (Tomlinson and Rolland, 1996). LPD complexes were observed to form small, spherical, multilamellar particulates as shown in Fig. 3D, the structure of which was fully retained following nebulisation (Fig. 3E).

In vitro cell culture was used to determine whether the pDNA collected in the aerosol mist retained its transfection efficiency. Prior to nebulisation all of the complexes were able to mediate cell transfection with expression of Green Fluorescent Protein (GFP). As described previously (Gao and Huang, 1996; Li and Huang, 1997;

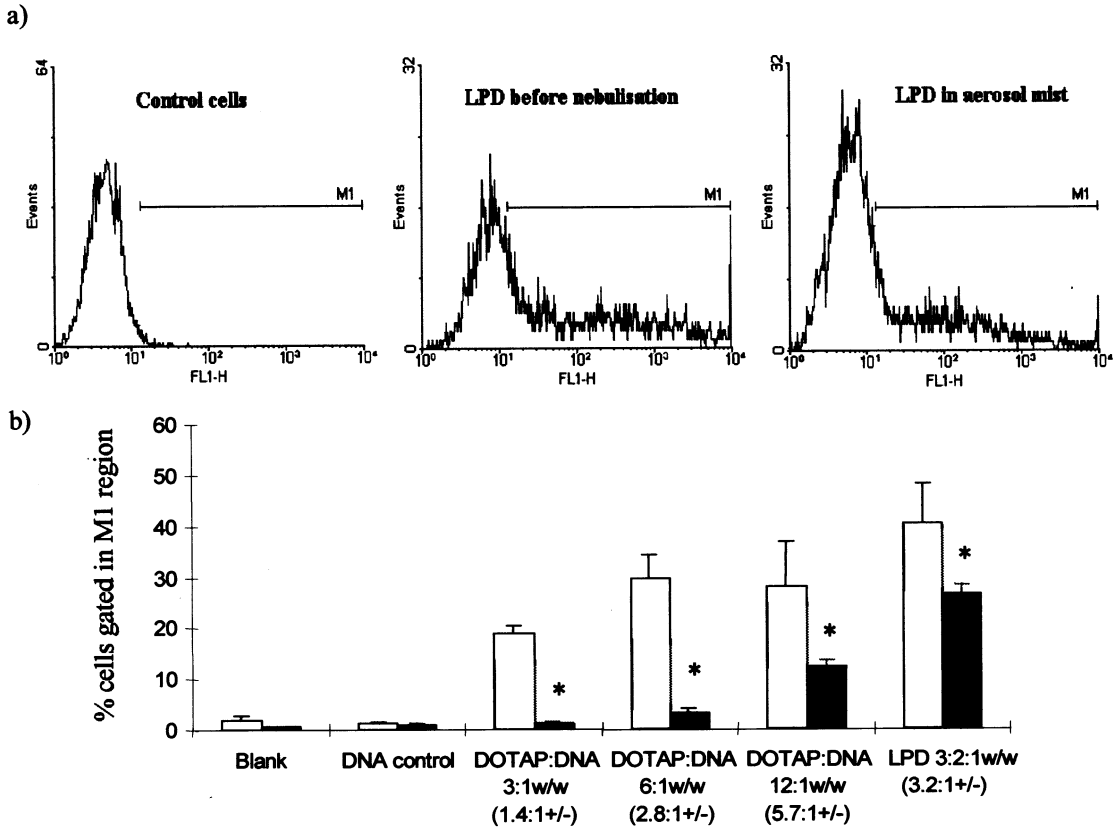

Fig. 4. In vitro transfection efficiency of complexes before (white bars) and after (black bars) nebulisation. The percentage of A549 cells showing Green Fluorescent Protein (GFP) associated fluorescence (FL1-H) was quantified by flow cytometry. A marked region (M1) was established containing 1% of the population of the control cells (a) and the percentage of cells moving into this marked region calculated for pre-nebulised samples (5 μg of pDNA per well) (b) and for samples collected in the aerosol mist following nebulisation for 10 min at 5 l/min (c). Data represented as mean ± SD, $n = 6$. Statistical analysis by One-Way Analysis of Variance and Duncan's multiple range test. Significance level $P < 0.05$. *, Statistically significant difference compared to pre-nebulisation diameters.

Sorgi et al., 1997), the addition of protamine enhanced the efficiency of cationic lipid mediated transfection with levels of GFP expression above control evident in 41% of the cell population treated with the LPD complex compared with approximately 29% for both the 12:1 and 6:1 (w/w) DOTAP complexes. For each treatment the transfection per unit volume of solution was reduced by nebulisation (Fig. 4). In the case of DOTAP complexes at 3:1 and 6:1 (w/w), transfection with complexes collected in the aerosol mist resulted in signal for GFP expression indistinguishable from background cellular autofluorescence. For the DOTAP:pDNA 12:1 (w/w) complex and the LPD complex, transfection by an equivalent volume of aerosolised mist was 43 and 65% of that attained with the pre-nebulisation solutions, respectively. This is intriguing given the relatively low levels of pDNA determined in the aerosol mist for these complexes (i.e. 10 and 12% of pre-nebulisation levels, respectively) and could reflect one of two possibilities. Firstly, a restructuring of the complex during nebulisation to generate more active transfection particles and/or elimination of those complexes that are less efficient at transfection (note the lower degree of variability in transfection data following nebulisation). Our observation of greater transfection efficiency per unit pDNA

with nebulised complexes is consistent with recent investigations demonstrating increased transfection efficiency of lipid-pDNA complexes in vitro (Eastman et al. 1997; Pouton et al. 1998) and in vivo (Eastman et al. 1998) following jet nebulisation. Secondly, it is conceivable that the percentage of cells moving into the M1 region may reach a plateau at 45% such that the apparent difference between pre- and post-nebulisation transfection efficiency is less pronounced. However, median fluorescence intensity data for the transfected A549 cells (not shown) paralleled the results based upon the percentage of cells transfected and data from our own laboratory (unpublished) has shown a consistent ability with optimised formulations to achieve greater than 60% of cells in the M1 region using the same analysis criteria. The increased expression per unit pDNA following nebulisation is intriguing and may argue for the exposure of all lipid-based pDNA complexes to such a treatment prior to administration by non-lung routes of administration.

In summary, this work represents initial studies exploring the use of peptide pre-condensation in conjunction with cationic lipid complexation to enhance the protection afforded to pDNA against the shearing forces encountered during jet nebulisation. The protection afforded by the LPD complexes, and indeed the physical characteristics of the LPD complexes themselves, were observed to be superior to that of lipid:pDNA complexes alone, even when the latter were utilised at very high lipid to pDNA mass ratios. The protection afforded by LPD complexation was achieved without the use of excess quantities of cationic lipid and indeed with particles that provide for a lower overall net positive charge. Incorporation of a peptide platform will also provide a scaffold for the covalent attachment of sub-cellular targeting 'signals' aimed at refining the intracellular trafficking of the plasmid.

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