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Comparison between the interactions of adenovirus-derived peptides with plasmid DNA and their role in gene delivery mediated by liposome–peptide–DNA virus-like nanoparticles

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Previously we have described the development and applications of an important new platform system for gene delivery known as liposome–mu–DNA (LMD), prepared from cationic liposomes (L), plasmid DNA (D) and the μ (M) peptide derived from the adenovirus core. In an attempt to improve upon mu, an alternative peptide (pepV) derived from the adenovirus peptide/protein–DNA core complex was identified, synthesised and studied alongside mu using a number of biophysical techniques including gel retardation, ethidium bromide exclusion, CD binding titration, DNA melting, and plasmid protection assays. PepV binds to pDNA less efficiently than mu but is able to charge neutralise and condense pDNA into negatively charged pepVD particles comparable in dimension to MD particles. The results of CD studies and plasmid protection assays suggest that peptide–DNA interactions are likely to cause pDNA condensation by a combination of charge neutralisation, base pair tilting, double helix destabilisation and the induction of pDNA superfolding. Data suggest that pepVD particles may be formulated with cationic liposomes to give defined LpepVD particles that appear to transfect HeLa cells with marginally more efficiency than LMD particles suggesting that pepV may have some effect on the pDNA transcription process. Although pepV harbours a nuclear–nucleolar localisation sequence (NLS), transfection data show that this capacity is not being appropriately harnessed by the current LpepVD formulation. Further improvements may be required in terms of optimising LpepVD formulations – for instance, to ensure the integrity of the peptide–DNA complexes following cell entry – in order to fully exploit the full NLS capacity of the peptide, thereby facilitating the transfection of slowly dividing or quiescent cells.

Introduction

Gene therapy may be described as the delivery of genes to a patient by means of a vector for some therapeutic purpose. Apart from a few notable exceptions, gene therapy has not yet proved successful in the clinic, primarily due to limitations with the current vector systems. Viral vector systems have dominated much of gene therapy research both in the laboratory and clinic,[1] but there has been a substantial interest in non-viral approaches as well.[2–5] Non-viral vectors are considerably less efficient than viral systems at mediating gene delivery and expression (transfection). In spite of this, these systems have many potential advantages over their viral counterparts, including lower toxicity/immunogenicity, significantly lower oncogenicity, size independent delivery of different varieties of nucleic acids (from oligonucleotides to artificial chromosomes), simpler quality control, and less onerous pharmaceutical and regulatory requirements. However, the development of non-viral vector systems suitable for clinical use has been severely hampered by persistent technical problems. These have included the ability to obtain reproducible and scalable formulations with plasmid DNA (pDNA)† and other nucleic acids, stability towards aggregation in solution, reproducible transfection outcomes (*in vitro*, *ex vivo* and *in vivo*) and stable long-term storage. Recently, we described the development of a new ternary synthetic non-viral vector system known as liposome–mu–DNA (LMD) wherein these basic technical problems were addressed.[6] Ternary synthetic non-viral vector systems such as LMD have been described by several other research groups including primarily the group of Huang and co-workers,[7–11] although others have made important contributions as well.[12–24] Given current reported data, LMD systems also appear to represent a sound platform upon which to develop clinically useful non-viral vectors by a process of modular upgrading. Clinically useful non-viral vector systems will need to be triggerable, that is stable and non-reactive in extracellular fluids but unstable once recognised and internalised by target cells in an organ of choice. Furthermore, once internalised by cells, nucleic acids such as plasmid DNA should remain sufficiently condensed and compact so as to enter cell nuclei efficiently but, once inside, become adequately unencumbered for active transcription to take place. The biological availability of plasmid DNA after delivery represents the main starting point for the work described here.

In our laboratory, LMD systems are prepared from cationic liposomes, plasmid DNA and the μ (mu) peptide derived from the adenovirus core. Adenoviruses are icosahedral, non-enveloped, particles enclosing a linear double stranded DNA genome which is covalently attached to the virus-encoded ter-

† *Abbreviations:* pDNA or D, plasmid DNA; NLS, nuclear localisation signal; Mu or M, adenovirus core peptide μ; pepV, 23 amino acid peptide containing a nuclear and nucleolar localisation and retention sequence (derived from adenovirus protein V); L, cationic liposomes; CD, circular dichroism; EtBr, ethidium bromide; ITC, isothermal titration calorimetry; PCS, photon correlation spectroscopy; DC-Chol, 3β-[*N*-(*N′*,*N′*-dimethylaminoethane)carbamoyl]cholesterol; DOPE, dioleoyl L-α-phosphatidylethanolamine; CDAN, N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; Lipof, lipofectamine, formulation of 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1–propanaminium trifluoroacetate (DOSPA) and DOPE (3 : 1 w/w).

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minal protein at the 5′-ends and non-covalently associated with two viral core proteins, protein V and protein VII, together with the mu peptide.[25] The 19 amino acid mu peptide is analogous to protamine found in sperm and is believed to play an integral role in compaction of virus genomes within particle cores.[26] In our hands, mu peptide has been found to be an efficient template for the binding and condensation of plasmid DNA.[6,27] At this point in time, the precise arrangement of the viral genome and core proteins inside the adenovirus particle is unclear, as are the relative contributions of the core components to the process by which the viral genome is delivered to cell nuclei and expressed (transduction).[28] Nevertheless, recent evidence suggests that protein V is integral to the delivery of the viral genome to the cell nucleus. For instance, protein V has been shown to interact with cellular proteins that shuttle between nucleus and mitochondria and is imported to the nucleus very shortly after viral infection along with the viral genome.[29,30] Furthermore, protein V contains multiple nuclear and nucleolar targeting sequences.[31] In particular one region (amino acid residues 315–337) from protein V of human adenovirus type 2 was found to be especially potent at mediating nuclear–nucleolar localisation.[31] Accordingly, we considered that peptide fragments of protein V harbouring a nuclear–nucleolar localisation sequence (NLS) could represent not only an alternative and/or addition to mu peptide as a means to bind and condense plasmid DNA, but could also provide the means to efficiently deliver pDNA to the nucleus, hence promoting efficient gene expression. Here we describe the preparation of pepV, the results of biophysical comparisons between the interactions of mu and pepV with plasmid DNA, the formulation of liposome–pepV–DNA (LpepVD) systems and the results of comparative transfection studies. PepV appears to be a useful surrogate of mu peptide but the NLS capacity of the peptide remains to be harnessed adequately.

Experimental

Chemicals

Plasmid pUMVC1 (formerly known as pNGVL1-nt-beta-gal) (7.53 kbp) was obtained from the National Gene Vector Laboratory at Michigan University, USA. Purified plasmid was purchased from Bayou Biolabs, Harahan, LA, USA. The pellet was redissolved in 4 mM HEPES pH 7.0 and frozen in aliquots at −80 °C. The concentration of plasmid DNA was determined by A_{260} according to standard protocols (using average nucleotide MW of 329 Da),[32] the concentration of the three peptides was determined by weight. All chemicals including the restriction endonuclease Taq 1 (10.000 units ml^{-1}) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) were purchased from Sigma-Aldrich, Poole, Dorset, UK. HEPES free acid was purchased from Anachem Ltd., Luton, Beds, UK. Unless otherwise stated, all experiments were performed at 20 °C in 4 mM HEPES, adjusted to pH 7.0 at room temperature with sodium hydroxide. Lipofectamine and all growth media were purchased from Invitrogen Ltd, Paisley, UK. The cytofectins 3β-[-*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol) and *N*^{1}-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine (CDAN) were prepared as described previously.[33]

Peptide synthesis

Mu peptide was prepared as described previously.[34] PepV was synthesised using Fmoc–Arg(Pbf)-Wang resin (100 mg, 0.50 mmol g^{-1}, 0.05 mmol) with double coupling for Arg(4)–Arg(7), Thr(11), Arg(13)–Arg(15), and Arg(20)–Pro. Extended coupling times were employed for the region Thr(16)–Arg(19). After post-synthetic *N*-terminal deprotection, the resin was washed with dichloromethane (30 ml) and methanol (30 ml) then dried under vacuum. The dry support was cleaved using 3 ml of ice-cooled cleavage mixture. The suspension was stirred for 5 h and the peptide was precipitated by filtration into ice-cold MTBE (15 ml) and repeatedly washed The pellet was dried *in vacuo* and subsequently purified by reversed-phase semi-preparative chromatography, eluting at 33% MeCN. The identity of both peptides (mu, MW 2440 Da; pepV, MW 3036 Da) was confirmed by MALDI-TOF and their purity confirmed by analytical HPLC on a Vydac RP C4 214TP54 column.

Agarose gel electrophoresis and enzyme digestion assays

1% or 2% agarose gel electrophoresis was performed according to standard protocols.[32] For gel retardation assays (1% agarose), aliquots of pDNA (20 μl, 0.2 mg ml^{-1}) were rapidly mixed with peptide from concentrated peptide stock solutions at ratios as described in the text. For pDNA digestion experiments (2% agarose), peptide–DNA complexes were digested with Taq 1 at 37 °C. PCR marker containing 8 double-stranded recombinant DNA fragments between 50 and 2000 bp (Sigma P9577) was used to visualise the bands appearing on the gels.

CD spectroscopy and UV spectroscopy

For peptide–DNA binding studies, CD spectra were recorded on a Jasco J-720 spectropolarimeter at 20 °C in a 0.1 cm path length quartz cell between 320 and 220 nm. Temperature unfolding experiments were performed by recording series of scans between 20 °C and 95 °C using the same 0.1 cm quartz cell. Only data in the relevant temperature range between 50 and 95 °C are shown. After each scan, the temperature was raised by 1 °C min^{-1} in steps of 5 °C or 2 °C, followed by an equilibration time of 300 s. The appropriate buffer background was subtracted from each spectrum. None of the peptides contributed significantly to the CD signal, rather the peptides proved to be completely unstructured (data not shown). The melting profiles were obtained as a direct plot of the absorbance values recorded simultaneously with the CD. T_m values were obtained from the respective derivative plots using Grafit (Erithacus Software).[35]

Fluorescence spectroscopy

Fluorescence intensity measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer with a thermostatically controlled cuvette assembly at 20 °C. A 10 mm × 4 mm quartz cuvette was used. Solutions of plasmid DNA were titrated with mu or pepV by adding small volumes of concentrated peptide stock solutions. After each addition, the solution was mixed thoroughly and incubated for 2 min after which the fluorescence emission intensity at 590 nm was recorded for 1 min (excitation at 260 nm, band widths 3 nm and 5 nm for excitation and emission, respectively). The data are expressed as the average of each measurement, the standard deviations of these measurements were less than the point size.

Isothermal titration calorimetry

ITC experiments were performed on a VP-ITC MicroCalorimeter at 20 °C (MicroCal Inc. Northampton, MA, USA). Aliquots of 5 μl of peptide (365 μM mu or 265 μM pepV) were added to pDNA (140 μg ml^{-1}, 30.7 nM) over a period of 10 s at 240 s intervals. Data were analysed with Microcal Origin (v. 6.0).

Preparation of liposome–peptide–pDNA complexes

Complexes of cationic liposome DC-Chol–DOPE (3 : 2 molar ratio, 4.7 mg ml^{-1}), pDNA and peptide:DNA (0.6 w/w of peptide to DNA) were formulated as described previously.[6] Briefly, LMD, LpepVD and LD describe particles prepared from preformed complexes of pDNA with mu (MD), pepV

Table 1 Amino acid sequences of mu and pepV and their condensation efficiencies as determined by EtBr exclusion. Peptide was titrated into pDNA (24 μg ml^{-1}) thereby displacing pDNA-bound EtBr. See also Fig. 2

Peptide	Sequence	IC_{50} (w/w)	IC_{50} (+/−)
Mu	MRRAH HRRRR ASHRR MRGG	0.55	0.66
PepV	RPRRR ATTRR RTTTG TRRRR RRR	0.65	0.96

(pepVD), and without peptide (D) respectively, then formulated with cationic liposomes at a ratio of DC-Chol/DOPE to peptide to pDNA of 12 : 0.6 : 1 (w/w/w) or DC-Chol/DOPE to pDNA of 12 : 1 (w/w). LMpepVD particles were prepared by rapid mixing of mu to pDNA (0.3 w/w), then pepV (0.3 w/w, to form MpepVD), followed by suspension in DC-Chol/DOPE (12 : 0.3 : 0.3 : 1 w/w/w). All samples containing liposome were prepared so as to achieve a concentration of 0.1 mg ml^{-1} pDNA in 10% sucrose and their complex sizes measured by photon correlation spectroscopy (PCS). CDAN–DOPE (3 : 2 molar ratio) liposomes were prepared analogously.

In vitro transfection experiment

HeLa cells were seeded in Dulbecco's Modified Eagle Medium (DMEM) (1X) containing 10% foetal calf serum (FCS) and 10000 units ml^{-1} penicillin plus 10000 μg ml^{-1} streptomycin (1 : 100 dilution) in eight 48-well plate culture plates and grown to 40% or 90% confluence at 37 °C (5% CO_2). Cells were washed by brief exposure first to growth medium and then to neat OptiMEM. Cells were treated with solutions containing D, LD, LMD, LpepVD and LMpepVD (4 wells each). An equivalent volume of 4 mM HEPES pH 7.0, 10% sucrose, was added to control cells. All samples were prediluted with OptiMEM, and cells were transfected at 37 °C with a DNA dose of 0.5 or 1.0 μg per well. Following transfection, cells were washed and then incubated in growth medium (10% FCS). Levels of transfection were determined by chemoluminescent β-Gal Reporter Gene Assay purchased from Roche Diagnostics Ltd., Lewes, East Sussex, UK. BCA Protein Assay Reagent Kit (Pierce) was purchased from Perbio Science UK, Cheshire, UK.

Cell viability studies

HeLa cells were prepared in 48-well plates as described above and exposed for 30 min at 37 °C to various samples in OptiMEM (as described in the text). This was followed by incubation in growth medium (10% FCS). All experiments were performed in triplicates or quadruplets. Cell viabilities were determined by CellTiter Solution Cell Proliferation Assay (Promega). The total cell protein was determined as described above.

Photon correlation spectroscopy

The size of peptide : DNA particles was measured using dynamic light scattering on a Coulter N4 plus. All measurements were performed with equilibrated samples of approximately 30 μg ml^{-1} pDNA (4 mM HEPES pH 7.0) and varying amounts of peptide at 20 °C at an angle of 90° with a run time of 300 s. The results of three runs were averaged. DC-Chol/DOPE liposome : peptide : DNA (12 : 0.6 : 1 w/w/w) particles sizes were measured analogously on equilibrated samples of approximately 3 μg ml^{-1} pDNA.

Results

Cationic peptides

The sequences of mu (19-mer, MW 2440 Da) and pepV (23-mer, MW 3036 Da) are described in Table 1. Both peptides were prepared by solid phase peptide synthesis but with the application of frequent double coupling and/or the use of pseudo-proline diamino acid residue building blocks in order to maximise the yield of purified peptide.[6,27,34] The formation of mu–DNA (MD) and pepV–DNA (pepVD) complexes was studied by means of gel retardation assays, ethidium bromide exclusion assays, circular dichroism (CD), DNA melting studies, photon correlation spectroscopy (PCS), isothermal titration calorimetry (ITC) and plasmid protection assays.

Gel retardation and ethidium bromide exclusion assays

The ability of the peptides to form complexes with pDNA was studied by agarose gel electrophoresis. This technique has been used commonly to characterise DNA and peptide–protein interactions.[12,14,27,34,36] Results obtained when a fixed quantity of pDNA was combined with increasing amounts of pepV are shown in Fig. 1. The migration of pDNA through a gel is retarded by the introduction of cationic peptides owing to the formation of peptide–pDNA complexes and negative charge neutralisation.[37,38] Retardation is complete when charge neutralisation is complete. Both peptides were found to bring about almost complete retardation of both supercoiled and relaxed forms of pDNA at 0.7–0.8 : 1 (w/w), suggesting that both may have similar capacities to neutralise the charge of pDNA and form complexes with both supercoiled and relaxed forms. At the highest pepV–DNA ratio of 1.2 : 1 (w/w) complexes could not be visualised with ethidium bromide (EtBr) (Fig. 1, lane 10) consistent with complete masking of EtBr binding sites in pDNA by bound peptide.

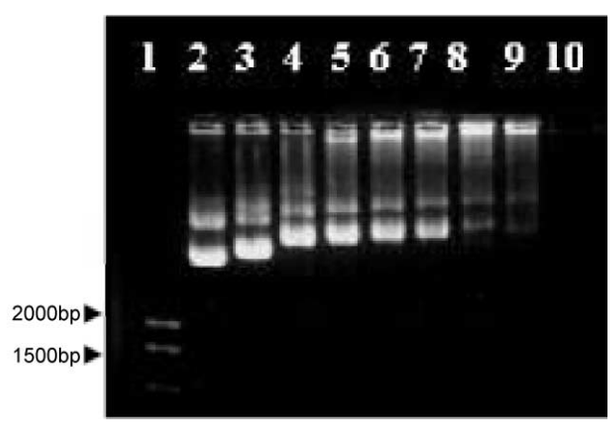

Fig. 1 Gel retardation assay of DNA complexed with pepV showing that peptide binding to pDNA neutralises DNA charge. DNA marker (lane 1), free DNA (lane 2), pepVD complexes at ratios of peptide to DNA of 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 and 1.2 (w/w) (lanes 3–10); 2 μg of DNA were loaded onto each lane. The complexes were run on a 1% agarose gel.

In light of these data, the ability of peptides to form complexes with pDNA was also studied by ethidium bromide exclusion assays. This assay procedure has been used on a number of occasions to study DNA–cationic peptide and DNA–cationic liposome interactions.[8,12,38] EtBr is an intense fluorophore (I_{590}) following intercalation between DNA base pairs. When DNA–cationic peptide and/or DNA–cationic liposome interactions lead to charge neutralisation and condensation of DNA, EtBr is excluded leading to a corresponding drop in fluorescence intensity. At ratios below charge neutralisation, mu was noticeably more efficient than pepV at EtBr exclusion when a fixed quantity of pDNA was combined with increasing quantities of either peptide (Fig. 2), suggesting

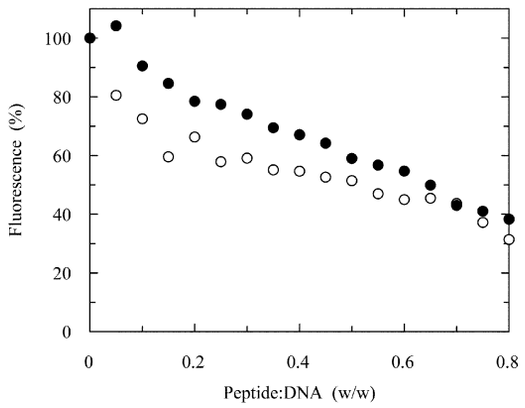

Fig. 2 Peptide binding displaces EtBr from pDNA. Increasing amounts of mu (○) or pepV (●) were added to DNA (24 μg ml^{-1}) and their ability to bind to pDNA was assessed by monitoring the fluorescence of EtBr at 590 nm. Results are expressed as a percentage of the fluorescence increase observed on binding of EtBr to free pDNA. Error bars were smaller than the point size. See also Table 1.

that mu was somehow more efficient at charge neutralisation and condensation of pDNA than pepV. The ability of mu and pepV to charge neutralise and condense pDNA was quantified by means of IC_{50} values corresponding to the amount of each peptide required to reduce EtBr fluorescence intensity to 50% of the initial intensity (Table 1). The IC_{50} value of mu, expressed as the N–P charge ratio (*i.e.*, +/−), was approximately 70% the value of pepV, confirming the greater efficiency of mu peptide as an agent of pDNA charge neutralisation and condensation. At the highest pepV–DNA ratio of 1.2 : 1 (w/w) complexes, only basal EtBr fluorescence was observed (results not shown) consistent with previous gel retardation data (see above).

Isothermal titration calorimetry and photon correlation spectroscopy

Previously, isothermal titration calorimetry (ITC) was used to quantify the affinity of mu peptide for pDNA and make an estimate of binding stoichiometry at saturation.[27] A macroscopic dissociation constant, K_d, of 0.6 ± 0.1 μM was determined with approximately 1300 mu peptides binding per pDNA (7.5 kbp) at saturation (n). This corresponds to a mu–pDNA ratio of approximately 0.6 : 1 (w/w) and a charge ratio of 0.7 (+/−). The K_d value describes the strength of binding of n peptides to a plasmid molecule. Correspondingly, the true, microscopic, dissociation constant, κ_d, for the binding of a single peptide was calculated to be 780 μM ($n \times K_d$) assuming mutual binding site independence. In this study, ITC was used similarly to quantify pepV binding to the equivalent pDNA molecule (Fig. 3). The value of K_d was found to be 0.9 ± 0.7 μM with a stoichiometry of approximately 1020 pepV peptides binding per pDNA at saturation. This corresponds to an essentially identical pepV–pDNA ratio of 0.6 : 1 (w/w) but a slightly higher charge ratio of 0.9 (+/−) (pepV has 14 cationic arginine residues as compared to mu which has 9; see Table 1). In this instance, κ_d for pepV was calculated to be approximately 920 μM, a value that is significantly greater than that for mu peptide. The interaction of pepV with pDNA was found to be driven by a negative enthalpy change, ΔH°_{bind}, of −3.3 ± 0.1 kcal mol^{-1} and a positive entropy change, ΔS°_{bind}, of 20 cal mol^{-1} K^{-1}, figures that agree closely with those calculated for the interaction between mu and pDNA.[27]

Previously, mu peptide has been shown by photon correlation spectroscopy (PCS) to form discrete, essentially mono-disperse mu–pDNA (MD) particles when combined with pDNA at the saturating ratio of 0.6 : 1 (w/w).[6,27] PepV was found to behave identically in the formation of pepV–DNA (pepVD) particles (Table 2). Moreover, given the fact that the charge ratio was less than unity at a pepV–DNA ratio of 0.6 (w/w), we deduced that pepVD particles formed under these conditions should have an overall negative charge and hence be able to form liposome–pepV–DNA (LpepVD) particles, equivalent to LMD particles, when formulated with cationic liposomes using the same procedure used to prepare LMD particles.[6] Gratifyingly, this is precisely what was observed when pepVD particles were combined in suspension with extruded cationic liposomes formulated from cytofectin DC-Chol and the neutral lipid DOPE at their routine molar ratio of 3 : 2 (m/m) (Table 2).

Table 2 PCS measurements

Peptide	Peptide–DNA size/nm[a]		Liposome–peptide–DNA size/nm[b]	
Mu	MD	123 ± 38	LMD	151 ± 46
PepV	PepVD	138 ± 37	LpepVD	174 ± 48
Mu/pepV[c]	MpepVD	144 ± 60	LMpepVD	158 ± 46
(No peptide)	D	—	LD	213 ± 57

[a] Measurements were performed at a ratio of peptide to DNA of 0.6 (w/w). [b] Measurements were performed at a ratio of DC-Chol/DOPE liposome–peptide–DNA of 12 : 0.6 : 1 (w/w). Error bars refer to the unimodal standard deviation. [c] Equal amounts of mu and pepV of 0.3 (w/w). Liposomes only: 101 ± 33 nm.

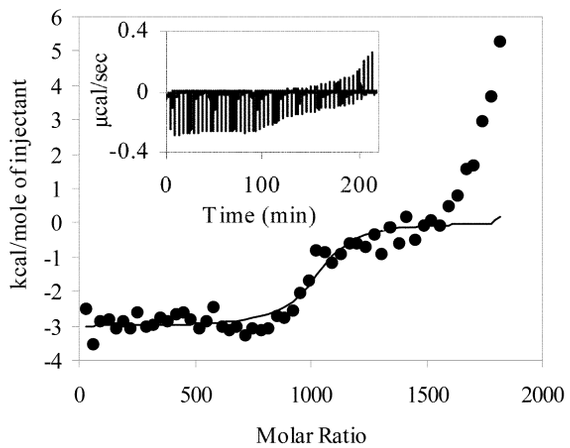

Fig. 3 Thermodynamic profile obtained from isothermal titration calorimetry (ITC) studies of the binding of pepV to pDNA. The figure shows the curve fitting of the experimental data to a single binding site model using the least squares method.

Circular dichroism spectroscopy of peptide–DNA complexes

Circular dichroism (CD) spectroscopy is routinely used as an experimental technique to monitor conformational changes in DNA.[39–43] CD titration experiments of pDNA with peptides were performed in an attempt to describe the complexes in terms of their macromolecular structure. Free pDNA exhibited a conservative spectrum typical of B-form DNA, the dominant form of DNA under physiological conditions (Fig. 4, bold lines).[40,44] In the presence of pepV or mu significant changes in ellipticity were observed. When a fixed concentration of pDNA was titrated with either pepV or mu, there was a slight decrease in positive ellipticity near 275 nm that was accompanied by a 20 nm red shift, and a substantial increase in negative ellipticity at 245 nm accompanied by a 10 nm red shift. In both cases, an isodichroic point was established at 288 nm suggesting that pDNA was undergoing a two-state macromolecular transition from B-form DNA to a form of DNA wherein base pairs are tilted with respect to the main double helical axis as a result of interaction with either mu or pepV. Broadly similar CD spectral changes have been observed when DNA was titrated with other known DNA binding proteins or peptides and these changes have been attributed to the induction of pDNA superfolding and/or supercoiling.[39,40,43,45,46] By plotting $\Delta\Delta OD_{260}$ as a

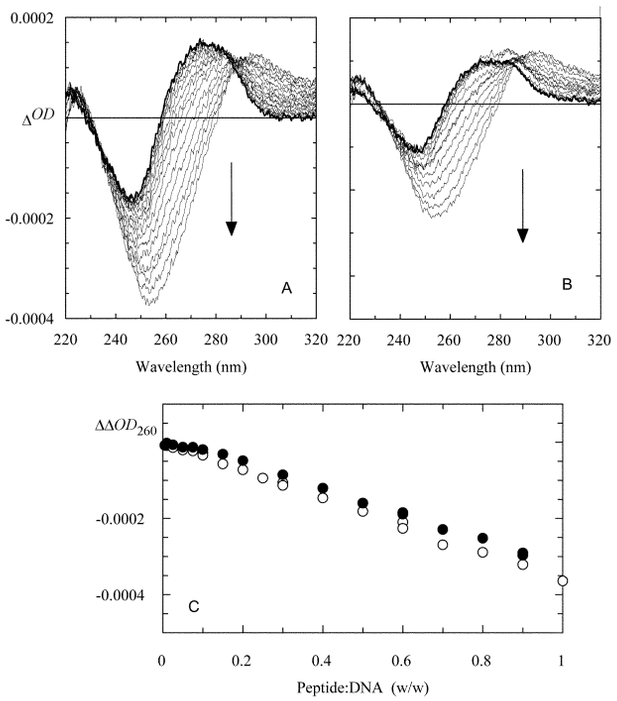

Fig. 4 CD spectra of pDNA on interaction with (A) mu and (B) pepV. Plasmid DNA (140 μg ml^{-1}, bold lines) was titrated with increasing amounts of cationic peptide (fine lines, 0.05 to 1.0 w/w). The arrow indicates the direction of increasing peptide concentration. (C) Binding isotherms of peptides to DNA. Shown are the ellipticities at 260 nm after subtraction of the contribution of free DNA ($\Delta\Delta OD$) for mu (○) and pepV (●).

function of peptide concentration, binding isotherms were generated (Fig. 4C). Neither isotherm appeared to saturate at a peptide–DNA ratio of 0.6 : 1 (w/w) in apparent contradiction to the ITC data described above. However, above this saturation binding point, endothermic spikes were observed in the ITC data both here and previously,[27] suggesting that higher-order peptide–DNA particle association processes and aggregation were beginning to take place. These processes offer an explanation as to why saturation was not achieved in the CD titration experiments. This also suggests that conformational changes associated with DNA condensation and aggregation are very similar on a macromolecular level.

Temperature denaturation and plasmid protection assays

Temperature denaturation experiments were carried out alongside the binding titration experiments described above in order to determine whether the formation of pepVD particles would result in double helix destabilisation as has been suggested for MD particle formation.[27] Other DNA binding proteins or peptides have also been shown to destabilise the double helix so this proposition seemed reasonable.[47–49] Temperature denaturation experiments were carried out by preparing mixtures of pDNA with mu or pepV at a peptide–pDNA ratio of 0.4 (w/w) and then monitoring the absorbance at 260 nm as a function of temperature (Fig. 5). For free DNA (Fig. 5, ▲), sigmoidal changes in absorbance were observed characteristic of a single pDNA melting transition. The melting temperature (T_m) was found to be 83.5 °C under these experimental conditions. By contrast, when temperature experiments were performed with the pepVD and MD particle mixtures, two sigmoidal transitions were observed giving rise to two T_m values in each case, one higher and one lower than the single pDNA melting transition. In consideration of our previous data and that of others,[27,47–49] these two T_m values were thought to reflect the development of regions of both lower and higher double helix stability within the pDNA as a consequence of

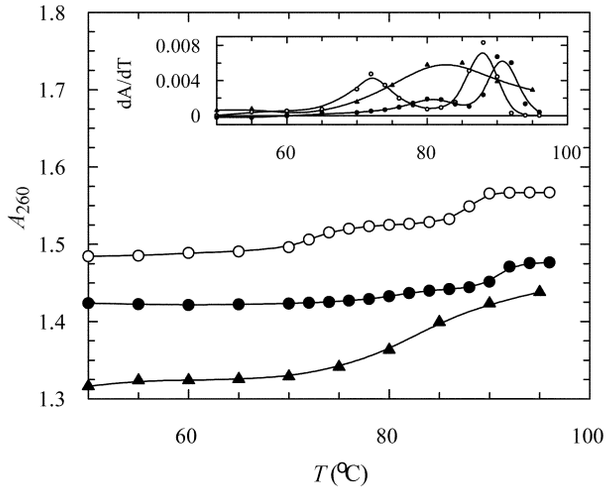

Fig. 5 Representative melting profiles of free pDNA (▲), mu–DNA (○) and pepV–DNA (●) at a ratio of 0.4 (w/w) in both cases. The profiles were obtained by plotting the absorbance at 260 nm *versus* the temperature (T). The inset shows the respective derivate plots from which values for T_m were obtained (see text). Data points were connected by spline functions.

peptide binding. PepV binding appeared to be slightly less effective at developing regions of lower double helix stability than mu peptide (lower T_m for MD and pepVD were 73 °C and 79 °C, respectively).

This interpretation was corroborated by the results of plasmid protection assays. Studies such as these are performed commonly on peptide–DNA complexes and cationic liposome–DNA complexes (lipoplexes, LD) in order to study the extent of DNA protection afforded as a result of the formation of peptide–DNA or lipoplex particles.[7,16,50,51] The enzyme DNase I is customarily used for this type of experiment,[10,12,14,22,24,37,52] but in our case we chose to use Taq1 restriction enzyme. Taq1 was determined to cut our pDNA (pUMVC1) into 18 distinct fragments between 1100 and 10 base pairs giving a defined restriction ladder covering a wide molecular weight distribution. Using this enzyme, pDNA digestion was followed as a function of time and compared with pDNA digestion in the presence pepV (Fig. 6). Virtually identical results were obtained using the mu peptide (not shown). The extent of pDNA digestion was significantly enhanced in the presence of both peptides compared with pDNA alone, a result that is entirely consistent with the possibility that peptide–DNA interactions were in some way destabilising regions of pDNA double helix, thereby introducing some hydrolytic lability into pDNA structure. By constrast, pDNA was resistant to digestion at mu–DNA or pepV–DNA ratios > 1.0 (w/w) (data not shown). Furthermore, pDNA resistance to digestion was also complete when either MD or pepVD particles (peptide–DNA ratio 0.6 : 1 w/w) were encapsulated by means of cationic liposomes to form either LMD or LpepVD particles respectively (results not shown). There is a possibility that our pDNA digestion data may be compromised by direct peptide mediated inhibition. However, such a problem has not been observed by others using DNase I,[7,16,50,51] and in any event our digestion and temperature denaturation data are in agreement. Accordingly, this possibility is unlikely in our view.

Cell viability studies

A colorimetric cell proliferation assay was used to determine the number of metabolically active cells following exposure to various components of LMD or LpepVD systems. Cell viability was plotted as a function of the amount of peptide or DNA per well (Fig. 7). *In vitro* gene transfection assays are usually performed at 0.5–1.0 μg pDNA per well; corresponding to 0.3–0.6 μg peptide per well and 6–12 μg lipid per well.[6,34,38]

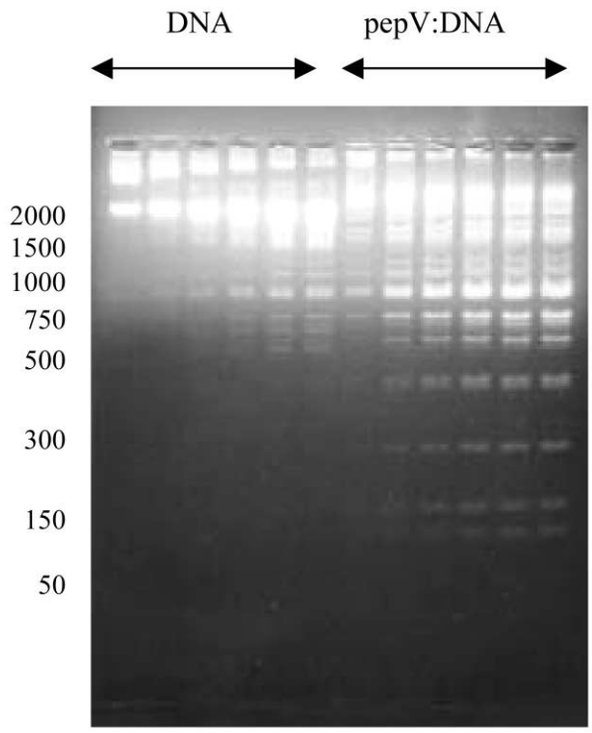

Fig. 6 Cationic peptides make DNA more susceptible to Taq1 digestion at low ratios. Time course of digestion of pDNA, free and in complex with pepV. Lanes 1–6, t = 0, 10, 20, 30, 40, 50, 60 min. Lanes 6–12, same as with free pDNA but using pepV–DNA at a ratio of 0.3 (w/w). PCR markers are indicated (in bp).

Therefore, cell viability tests were performed with components at these levels and above to assess general toxicity levels of each component *in vitro*. Cell viability was found to be robust with peptides and/or pDNA (Figs 7A and 7C), but cationic liposomes (DC-Chol/DOPE, CDAN/DOPE and lipofectamine) proved to be toxic at levels substantially above 12 μg of lipid per well (Fig. 7B). Fortunately, cell viabilities were reasonably robust in the presence of LMD, LpepVD or hybrid LMpepVD systems (Fig. 7D). Interestingly, lipofectamine, a commercially available cationic lipid transfecting agent which served as a control reagent in this study proved to be the most challenging agent; at concentrations used for actual transfection experiments, however, all samples behaved analogously. The analysis of the total cell protein revealed similar results (not shown), suggesting that any observed reductions in cell viability could be correlated directly with the number of surviving cells rather than with specific effects of the components on cell metabolism. A study is under way that aims to determine in more detail the effects of our agents with respect to the up- or down-regulation of specific genes as a result of exogenous gene transfer.

In vitro transfection assays

HeLa cell transfection experiments were performed with LMD, LpepVD or hybrid LMpepVD systems in order to determine if pepV containing samples were able to confer any improvements on cationic liposome-mediated transfection efficiency over and above mu peptide (Fig. 8). Transfection by the above systems was also compared to free DNA (D) and cationic liposome–pDNA (LD) systems prepared from DC-Chol/DOPE cationic liposomes and pDNA in the lipid–DNA ratio of 12 : 1 (w/w). In all cases transfection times were 30 min or 1 h, the dose of pDNA per well was either 0.5 or 1 μg, and serum was absent during the transfection process. The pDNA used in all these transfection experiments, pUMVC1, was the same as for the biophysical experiments described above. This plasmid harbours a β-galactosidase (β-gal) transgene and successful transfection results in elevated levels of β-gal activity in trans-

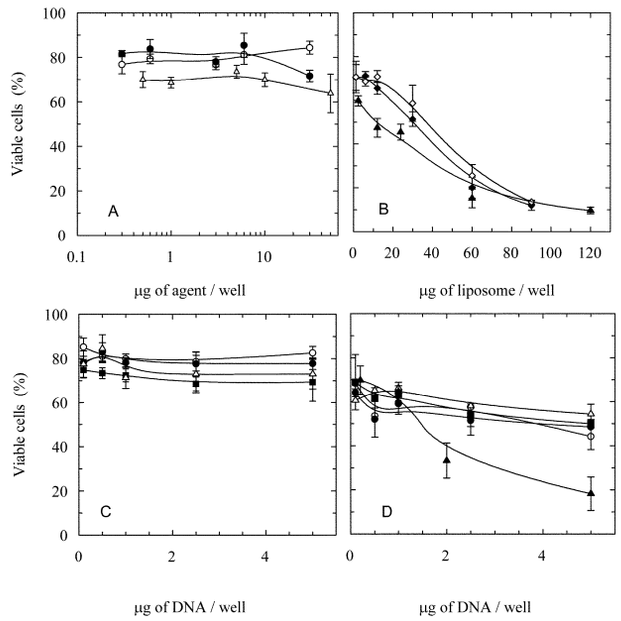

Fig. 7 HeLa cell viability following exposure to peptide, DNA and cationic liposomes. HeLa cells were exposed to samples containing pDNA, peptide and/or cationic liposomes for 30 min and then incubated for 24 or 48 h. Remaining cell viabilities are expressed in % of the viability of untreated cells. Data points were connected by spline functions. *In vitro* gene transfection assays are usually performed at 0.5–1.0 μg DNA per well. This corresponds to 6–12 μg liposome and 0.3–0.6 μg peptide. (A) mu (○), pepV (●), pDNA (△). (B) DC-Chol/DOPE liposomes (◇), CDAN/DOPE liposomes (◆), Lipof. (▲). (C) MD (○), pepVD (●), MpepVD (■), D (△). Peptide–DNA complexes were used at the ratio of 0.6 (w/w). (D) liposome–peptide–pDNA complexes were used at a ratio of 12 : 0.6 : 1 (w/w/w). LMD (○), LpepVD (●), LMpepVD (■), LD (△ 12 : 1 w/w), Lipof.D (▲, 12 : 1 w/w).

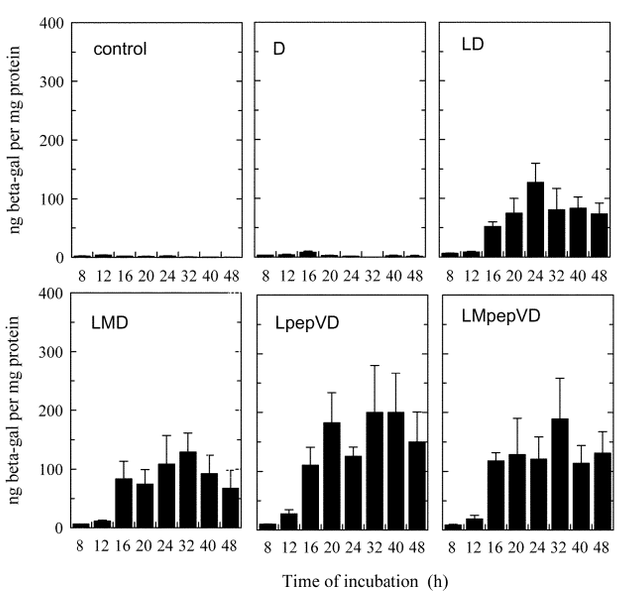

Fig. 8 Expression levels in HeLa cells following exposure to samples of D, LD, LMD, LpepVD and LMpepVD (prepared as in Fig. 7). The dose was 0.5 μg of DNA per well; the transfection time was 1 h. Following transfection, samples were incubated for several hours, as indicated. Expression levels are given in terms of ng of β-Gal enzyme expressed as a result of transfection per mg of total cell protein. Error bars show the standard deviation of quadruplets. The control shows the background β-Gal levels in untreated cells. Liposome–peptide–pDNA complexes were prepared at cationic liposome to peptide to pDNA ratio of 12 : 0.6 : 1 (w/w/w). Lipoplexes (LD) were prepared at cationic liposome to pDNA ratio of 12 : 1 (w/w).

fected cells. For all experiments, the transfection efficiency was plotted as a function of cell growth time post-transfection. Results are shown for the case when the transfection time was 1 h and the pDNA dose per well was 0.5 μg, the trends in other data sets closely agreed with these results (Fig. 8). Transfection by the LpepVD system was clearly a little more efficient than the other systems suggesting that pepV was providing some additional facilitation to the transfection process over and above any capacity of the mu peptide. This is also consistent with the LMpepVD hybrid sample which was found to result in transfection efficiencies that were between those of LpepVD and LMD. Serum-free transfection of LMD and LD systems were otherwise very similar to each other. Previously, we demonstrated that LMD transfections are significantly more dose and time efficient than LD systems which does not appear to be the case here. However, our previous comparative results were obtained with LMD systems formulated in optimal conditions and for the transfection of non-HeLa cells.[6] Furthermore, transfection experiments of this study were performed in serum-free medium. Typically, the improvement of LMD over LD systems is reflected in their stability in normal growth medium (containing 10% serum) where LMD transfect significantly better than LD.

Discussion

The results described above and in our previous work demonstrate that the mu peptide is a sound template for pDNA charge neutralisation and condensation, capable of forming well-defined MD particles that may be formulated into well-defined LMD particles.[6,27] The pepV appears to behave similarly although it seemed a little less efficient at pDNA condensation (Figs 2 and 3, Table 1). Nevertheless, pepVD complexes may be formed and convincingly formulated into LpepVD and LMpepVD particles (Table 2). Hence, in terms of biophysical properties pepV appears to be a convincing surrogate for mu peptide. The interactions between DNA and proteins–peptides predominantly involve charge interactions but also hydrogen bonding and stacking interactions between the aromatic moieties of DNA bases and amino acid residues,[53–57] wherein differences in the molecular recognition and binding of pDNA by cationic peptides are subject to amino acid residue composition. Although both mu and pepV are similarly composed of extensive numbers of arginine residues, the fact that mu is more efficient at pDNA condensation is, perhaps a result of greater peptide conformational flexibility. PepV has a proline residue that can dampen conformational flexibility as we have noted previously.[27] It is also possible that their slightly different behaviour accrues from the presence of basic residues in mu (H) and polar side chains in pepV (T). In any case, arginine-rich peptides such as pepV and mu appear to be substantially more effective than lysine-rich peptides at pDNA charge neutralisation and condensation according to the ethidium bromide exclusion assay data described here and data described previously by Schwartz *et al.*,[12] further underlining the importance of arginine residues.

The results of CD and pDNA digestion studies indicate something of the mechanism by which both mu and pepV may condense pDNA into defined nanometric MD and pepVD particles at a peptide–pDNA ratio of 0.6 : 1 (w/w) (Table 2). A number of experimental studies have revealed how DNA tertiary and secondary structures may be altered by DNA binding proteins and cationic ions,[39–43,45,46,58] with functional consequences for DNA replication and transcriptional activity. Peptide–pDNA interactions clearly give rise to base pair tilting with respect to the helical axis (Fig. 4) and in the process create regions of higher and lower double helical stability (Fig. 5), rendering the pDNA sensitive to enzymic digestion (Fig. 6), unless completely protected by excess peptide binding. Such double helical destabilisation also appears to result from interactions between non-histone chromosomal proteins and pDNA as well.[47–49] Protamine, an arginine-rich peptide similar in character and amino acid residue composition to mu and pepV, has been suggested to have a non-uniform interaction with the major and minor grooves of B-form DNA.[59] Therefore, mu and pepV are likely to interact with pDNA in a similar manner to protamine and such a non-uniform interaction mode would offer a reasonable starting explanation for the observed peptide–pDNA binding effects. In addition, Sato and Hosokawa have reported CD spectral changes when pDNA was titrated with proteins similar to our results obtained with peptides (Fig. 4), and proposed that these spectral changes were also indicative of a combined process of pDNA condensation and increased pDNA superfolding.[45] Results obtained from studies of EtBr interactions with pDNA have further indicated that superhelical turns are induced in closed circular pDNA in response to double helix destabilisation caused by EtBr intercalation.[32] Hence we would propose that binding of either mu or pepV to pDNA results in base pair tilting and the formation of regions of pDNA double helix instability that bring about condensation and collapse of pDNA by increasing pDNA superfolding. In our view, such a proposal would be completely consistent with combined data from melting profiles, pDNA digestion, gel retardation and ethidium bromide exclusion assays (Figs 1,2,5 and 6).

Our cell toxicity and transfection data clearly demonstrate that LMD, LpepVD and the hybrid LMpepVD systems are effective agents of transfection, and that pepV is a potential surrogate of mu peptide (Figs 7 and 8). Moreover, there is some suggestion that LpepVD is a marginally more effective transfection agent over time than the LMD system with the hybrid sample LMpepVD in between, at least in this HeLa cell line under these transfection conditions (Fig. 8). Therefore, there is at least some indication that pepV could provide some additional features to mu peptide by mediating intracellular trafficking of pDNA to the nucleus, and/or promoting gene transcription. The latter possibility appears to be more likely. The illustrated transfection data (Fig. 8) were obtained using actively dividing HeLa cells in culture. During cell division (M phase) the nuclear membrane is partially dismantled to allow cell division to take place, at which time epichromosomal, exogeneous pDNA is also able to enter the nucleus in preparation for transcription.[60] Therefore, any pepV-mediated enhancement in transfection efficiency is unlikely to be a consequence of enhanced pDNA nuclear entry rates. This suggestion is consistent with data from a second set of transfection experiments performed with near confluent HeLa cells (*i.e.*, cells grown to saturation and therefore suppressed in cell division). In this case, those samples that contained a nucleolar–nuclear localisation signal (LpepVD and LMpepVD)[31] were also not significantly different in their ability to transfect cells to any great degree (results not shown). This would be expected if the NLS capacity of the peptide was functional. This indicates that neither mu nor pepV were able to promote transfection under conditions where nuclear membranes remained largely intact during the transfection process. Therefore, we conclude that the current LpepVD and LMpepVD formulations appear to be unable to capture fully the NLS capacity of pepV in order to facilitate pDNA transport into cell nuclei in the absence of cell division.

There have been a number of significant efforts in the past to utilise peptides with NLS motifs in order to boost transfection efficiency[12,15,21,36,61–66] by seeking to harness the importin–transportin active transport system by which large molecules (>40–60 kDa) traverse the intact nuclear barrier at nuclear pore complexes.[67,68] NLSs interact with cytosolic factors, such as importin α and β or transportin, as a prelude to the import of large molecules into the nucleus through the nuclear pore complexes.[67,68] However, the results of transfection experiments using NLS containing peptides have usually been as dis-

appointing as they were here.[12,21,61,65] In our view, the NLS capacity of pepV has not been adequately harnessed in the current formulations for either or all of two main reasons. First, the peptide–pDNA interaction is not especially strong even when measured in ideal conditions such as those used in our experiments. Therefore, pepV (and mu) is likely to dissociate readily from pDNA once the particles enter cells. Second, once LpepVD (or LMpepVD) particles enter cells there is no guarantee that pDNA whether naked or in contact with pepV, mu or cationic lipids, is able to traffic freely to the nuclear barrier. Indeed, pDNA has already been shown independently to diffuse poorly in the cell cytoplasm.[65] Interestingly, polyethyleneimine, a cationic polymer, has been shown to undergo nuclear localisation both in isolation and complex with DNA.[20] Given this somewhat surprising result, an obvious step forward would be to mimic polyethyleneimine by concatenation of several NLS-containing peptide molecules, thereby creating a more "polyethyleneimine-like polypeptide polymer" with a potentially potent NLS capacity. Attempts to synthesise such a molecule are currently under way in our laboratory.

Alternatively further inspiration could be drawn from adenovirus to plot a future strategy for a more efficient non-viral vector system. Viruses have solved many problems of DNA transport, including mechanisms to target and translocate the viral genome towards and into the nucleus. Current evidence suggests that viruses use the cytoskeleton for transport of viral DNA to the nuclear pore complex, although the nature of the actual viral proteins that recruit or interact with the cytoskeletal transport complex is not always known.[69] In the case of adenovirus, however, hexon coat proteins appear to be involved in the transport of viral nucleocapsid to the cytoplasmic side of the nuclear pore complex.[28] These capsid proteins remain associated with the adenovirus core protein–DNA complex after virus particles have entered cells and exited endosome compartments. After docking with the nuclear pore complex, the nucleocapsid particle disassembles further, thereby releasing the core protein–DNA complex and allowing viral DNA to transport across the nuclear barrier in a process that presumably involves protein V.[28] Perhaps elements of the hexon protein could also be harnessed to facilitate non-viral vector mediated transfection.

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