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$(LYS)_{16}$-based reducible polycations provide stable polyplexes with anionic fusogenic peptides and efficient gene delivery to post mitotic cells

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Abstract

Extracellular stability, endocytic escape, intracellular DNA release and nuclear translocation of DNA are all critical properties of non-viral vector/DNA particles. We have evaluated a $(Lys)_{16}$-based linear, reducible polycation (RPC) in combination with an acid-dependent, anionic fusogenic peptide for gene delivery to dividing and post-mitotic cells. The RPC was formed from $Cys(Lys)_{16}Cys$ monomers. Molecular weight was 24,000 Da, corresponding to an average of 10.5 peptide monomers per RPC. Non-reducible polylysine (PLL) (27,000 Da) and monomeric $(Lys)_{16}$ peptide were evaluated for comparison. $(Lys)_{16}$/DNA particles were disrupted at fusogenic peptide concentrations well below those used for gene delivery. By contrast, RPC/DNA an PLL/DNA particles were stable in the presence of high concentrations of the anionic peptide. Addition of 10% serum virtually abolished the transfection ability of $(Lys)_{16}$/DNA/fusogenic peptide particles, but had little effect on RPC/DNA/fusogenic peptide particles. RPC/DNA/fusogenic peptide particles were highly effective for gene delivery to both cell lines and post-mitotic corneal endothelium. PLL/DNA/fusogenic peptide particles were moderately effective on cell lines, but gave *no* gene delivery with corneal endothelial cells. We conclude that $(Lys)_{16}$-based RPC/DNA/fusogenic peptide particles provide a gene delivery system which is potentially stable in the extracellular environment and, on reductive depolymerisation, can release DNA plasmids for nuclear translocation.

Keywords: Reducible polycation; Fusogenic peptide; Post-mitotic cell; Lysine; Nuclear translocation; Non-viral gene delivery

1. Introduction

The development of vector/DNA particles which are stable in vivo, but nevertheless efficiently release DNA once inside the cell, is a major challenge for non-viral gene therapy. An interesting idea in this regard is the formation of large cationic structures composed of smaller cations linked by disulphide bonds, termed reducible polycations (RPCs) [1–3]. RPCs are constructed from synthetic peptides containing multiple lysines for electrostatic binding of DNA, and a minimum of two cysteines for disulphide-mediated polymerisation. RPCs provide high avidity electrostatic attachment to DNA, forming tightly condensed polycation/DNA particles resistant to salt and polyanion destabilisation. However, following endocytosis into the reducing intracellular environment, reduction of the disulphide bonds releases the individual cationic subunits. This decreases the strength of polycation/DNA interaction and facilitates release of DNA, an essential step for effective gene delivery [4].

In the original study by Trubetskoy et al. [1], the cationic monomer peptide had two cysteine residues (one at each terminus). Polymerisation was allowed to proceed *after* peptide/DNA complex formation, referred to as template polymerisation. These RPC/DNA complexes were effective for gene delivery to cell lines when used with cationic lipids to permit endocytic escape. McKenzie et al. [2] evaluated peptides with 2–4 cysteine residues, again using template polymerisation. Whereas two cysteines per monomer form linear polymers, three or more cysteines permit cross-linking of chains, producing "caged" DNA. These RPCs were all effective for gene delivery to cell

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lines, using chloroquine for endocytic escape. The linear RPCs were the most effective, possibly because the more extensive cross-linking hampered DNA release. Certainly, caged DNA particles formed with non-reducible bonds have excellent physical properties but poor effectiveness for gene delivery [5,6]. Receptor targeting moieties and hydrophilic polymers have been added to the cysteine containing monomeric peptides prior to template polymerisation, to form more sophisticated gene delivery vehicles [7].

More recently, RPCs have been formed from $Cys(Lys)_{10}Cys$ monomers *before* complexing with DNA. This requires high concentrations of the monomer to favour inter-peptide disulphide bond formation for polymerisation [3,8]. These RPC/DNA particles have excellent physical stability and gene delivery properties when used in vitro with chloroquine [3] or when >50% histidine residues are incorporated into the monomer [8] for endocytic escape via the "proton sponge" effect [9].

Our interest in RPCs is twofold. Firstly, we have been evaluating anionic fusogenic peptides as endocytic escape agents. These peptides are based on the fusogenic amino terminus of the HA2 moiety of influenza virus haemagglutinin [10]. Several homologous peptides have been shown to mediate acid-dependent lysis of membranes for endocytic escape of vector/DNA particles [11–14]. This approach has many advantages over the use of chloroquine and fusogenic lipids [15]. We have been studying the 20 amino acid peptide of Gottschalk et al. [14]. $(Lys)_{16}$ peptide/DNA particles plus the fusogenic peptide have provided effective gene delivery in a variety of contexts [16–18] especially to post-mitotic corneal endothelial cells [19]. Surprisingly, however, the anionic fusogenic peptide (net charge only −5) disrupts $(Lys)_{16}$/DNA particles at very low concentrations. Essentially all the DNA is displaced by the anionic peptide under conditions where transfection rates of >95% of target cells are nevertheless obtained [18]. The small amount of DNA still associated with the $(Lys)_{16}$ and fusogenic peptides might represent an especially effective combination for gene delivery. We were therefore interested to see if RPC/DNA particles were less susceptible to disruption by anionic fusogenic peptides, and provide even more effective gene delivery agents.

Secondly, there is strong evidence that oligolysines such as $(Lys)_{16}$ [18–21], but not high molecular weight polylysines [22], have nuclear translocating capacity. Translocation of DNA expression plasmids from the cytosol into the nucleus is a key issue for the clinical application of gene therapy, because most clinically important target cells are either non-dividing (e.g. neurons, skeletal and cardiac myocytes, corneal endothelial cells) or very slowly diving (e.g. hepatocytes, vascular endothelial cells). We therefore wished to see if RPCs based on $(Lys)_{16}$ subunits retain nuclear translocating capacity, a property which would depend on the degree of intracellular reduction of the RPC into smaller subunits.

2. Materials and methods

2.1. Synthetic peptides

$Cys(Lys)_{16}Cys$, $(Lys)_{16}$ and the fusogenic peptide NH_2-Gly-Leu-Phe-Glu-Ala-Leu-Leu-Glu-Leu-Leu-Glu-Ser-Leu-Trp-Glu-Leu-Leu-Leu-Glu-Ala-CO_2H [14] were synthesized and purified by Cambridge Research Biochemicals, Northwich, Cheshire, UK. They were supplied as trifluoroacetate salts in the form of a dry powder, and stored dessicated at −35°. Quantities of 1–2 mg were dissolved in phosphate-buffered saline (PBS) with Ca^+ and Mg^+ (Gibco BRL, Renfrewshire, UK), 5% dextrose buffered to pH 7.4 in 10 mM Tris (dextrose/Tris), or pure water, and stored in small aliquots at −35 °C.

The fusogenic peptide is poorly soluble in pure water. The pH drops to ~5, resulting in protonation of some of the gamma carboxyls, and consequently greater hydrophobicity. This peptide must be dissolved in solutions buffered to pH ~7.

A poly(L-lysine) preparation of average molecular weight 27,000 Da (pLL) and a poly(L-aspartate) preparation of molecules weight 15,000 to 50,000 Da were purchased from Sigma-Aldrich, Dorset, UK. These were supplied as dry powders, and stock solutions were prepared in PBS as described above.

2.2. Production and characterisation of RPC

This was performed essentially as described by Oupicky et al. [23]. Fifteen mg of the $Cys(Lys)_{16}Cys$ peptide was dissolved in 150 μl of 0.5× PBS (100 mg/ml), and 50 μl of dimethyl sulphoxide (DMSO) was added as the oxidising agent. Oxidative polymerisation was left to proceed at room temperature, with progress monitored by measuring the size of the resulting polymer by gel permeation chromatography. Two μl of the reaction mixture was removed at various times, diluted to a final volume of 150 μl in eluting buffer (0.3 M NaCl, 0.1% v/v TFA), and 50 μl was loaded on to a CATSEC-300 column (Eprogen Inc., Illinois, USA). This was eluted at 0.25 ml/min and the optical density of the fractions measured at 220 nm (Kontron Instruments, California, USA). At 96 h, a small amount (25 μl) of 1 M aminoethanethiol was added to the reaction mixture to react with the free thiols at the ends of RPC molecules without disrupting the structure. The RPC was purified from low molecular weight contaminants using Centricon centrifugal concentrators with a molecular weight cut-off of 10,000 Da (Millipore, Massachusetts, USA).

2.3. Measurement of concentration of RPC

This was performed by assaying free amino groups (overwhelmingly the epsilon amino groups of the lysines). Peptide samples were diluted in 100 mM disodium tetraborate, pH 7.3. Thirty μl of 1 M 2, 4, 6- trinitrobenzenesulphonic acid (TNBS) (Sigma Aldrich, Dorset, UK) in water was added to 970 μl of the tetraborate buffer, and 25 μl of this solution was added to each peptide sample. After 30 min at room temperature, absorbance of the samples was measured at 420 nm. The concentration of RPC was determined by comparison with a standard curve generated with commercial polylysine.

2.4. DNA plasmids

The pGL3 plasmid (Promega, Madison, WJ, USA) carrying the firefly luciferase gene was used at a final concentration of 2.5 μg/ml unless otherwise stated. For the cornea transfections, the CMVβ plasmid (Clontech, Palo Alto, CA, USA) carrying the *Escherichia coli* β galactosidase gene was used at 10 μg/ml. Plasmids were propagated in *E. coli* DH5α and prepared from overnight cultures by alkaline lysis followed by column purification under endotoxin-free conditions (Qiagen Ltd., Dorking, UK). They were stored in aliquots at 1 mg/ml in pure water at −35 °C.

2.5. Formation of peptide/DNA complexes

Charge ratios were calculated on the basis of an average of 325 Da per phosphate group on the DNA, and 209 Da per epsilon amino group on commercial polylysine (which is a hydrobromide salt). Thus a weight:weight ratio of DNA to polylysine of 1:0.64 is required for a 1:1 charge ratio. For the synthetic $(Lys)_{16}$ peptides (which are TFA salts) there are 243 Da per amino group, and thus a weight:weight ratio of DNA to $Lys)_{16}$ peptide of 1:0.75 is required for a 1:1 charge ratio.

The required volume of stock DNA at 1 mg/ml in water was added to PBS, Dulbecco's modified Eagles medium (DMEM, Invitrogen, Renfrewshire, UK) without supplements, or dextrose/Tris in a 15-ml tube (Helena Biosciences,

Sunderland, Tyne and Wear, UK). The required volume of lysine peptide at a concentration of 1 mg/ml was added dropwise to the DNA solution while vortexing. This was allowed to stand for 15 min before the required volume of fusogenic peptide at 1 mg/ml was added dropwise while vortexing. The samples were allowed to stand for a further 15 min before beginning measurements, adding to target cells for gene delivery, or beginning electrophoresis. Where RPC/DNA particles were reduced, a solution of 1 M dithiothreitol (DTT) (Sigma-Aldrich, UK) in pure water was prepared fresh, and the appropriate volume added to RPC/DNA particles to 20 mM DTT, 15 min after particle formation. After a further 15 min, the fusogenic peptide was added. Where particles were exposed to poly (L-aspartic acid), the appropriate volume of a stock solution at 1 mg/ml in PBS was added to RPC/DNA particles. Electrophoresis was performed 15 min later.

2.6. Other vector/DNA complexes

Polyethlyenimine (PEI)/DNA complexes were formed by the dropwise addition of 3 μl of branched PEI 25,000 (10 mM amine nitrogen) (Sigma Aldrich, Dorset, UK) per μg of DNA while vortexing, as recommended by the manufacturers. This corresponds to an amine to phosphate (N:P) ratio of 10. The samples were allowed to stand for 30 min before adding to target cells.

Lipofectamine 2000™ (Invitrogen, Paisley, UK) at 1 mg/ml was added to 10% of the transfection volume of DMEM, to a concentration of 25 μg/ml. After 5 min, plasmid DNA at 1 mg/ml in water was added to a final concentration of 25 μg/ml while vortexing, to give a 1:1 weight:weight ratio of DNA: lipofectamine, as recommended by the manufacturers. The samples were allowed to stand for 30 min before addition of DMEM to give 2.5 μg/ml of DNA, vortexed briefly and added to the target cells.

2.7. DNA electrophoresis

Samples containing 0.2 μg of DNA were transferred to 1% agarose gels containing 0.1 μg/ml of ethidium bromide for electrophoresis (45 min, 100 V). DNA in the gel was visualised on an ultraviolet transilluminator.

2.8. Analysis of particle size by dynamic light scattering

Dynamic light scattering (DLS) measurements were performed on a Zetasizer 3000 HS (Malvern Instruments Ltd., Malvern, UK). Data analysis used the non-negatively constrained least squares (NNLS) method, via software provided by Malvern Instruments. The hydrodynamic radius (Zav) and polydispersity index of the peptide/ DNA nanoparticles were measured at least 3 times and the average of these measurements is reported.

2.9. Cell lines

The HUH7 cell line (a kind gift of Dr. David Crabb, Indiana University Medical Centre, Indianapolis, USA) and the HeLa cell line (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) are adherent lines originally derived respectively from a human hepatocyte carcinoma and a cervical carcinoma. They were maintained under mycoplasma-free conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM glutamine and 1× non-essential amino acids (Invitrogen, Renfrewshire, UK), referred to as culture medium.

2.10. Transfection of cell lines

Cells were harvested from tissue culture flasks using trypsin/EDTA (Invitrogen, Renfrewshire, UK), pelleted by centrifugation, and resuspended to 5×10^4 cells/ml in culture medium. One ml of the cell suspension was added to each well of a 24-well plate (Helena BioSciences), and cultured overnight at 37 °C in 95% air/5% CO_2. At this stage, the cells were ~60% confluent. The wells were washed twice with PBS, and 0.3 ml of the vector/DNA complexes in DMEM without supplements was added. This was incubated for 4 h at 37 °C and then 0.3 ml of DMEM medium containing 20% FCS, 4 mM glutamine and 2× non-essential amino acids was added to each well. After overnight culture, the medium was replaced with 1 ml of normal culture medium. After a further 24 h of culture, the wells were assayed for reporter gene expression. In some experiments, RPC/DNA/fusogenic peptide and $(Lys)_{16}$/DNA/ fusogenic peptide particles in DMEM were made up to 10% FCS 15 min before adding to the target cells.

2.11. Luciferase reporter gene activity

The cells were washed twice with PBS and 200 μl of lysis buffer (Promega, Southampton, UK) was added to each well and incubated at room temperature on a rocker for 30 min. The cells were dislodged by scraping, and quickly frozen at −80 °C. For assay, the cell lysate was thawed and centrifuged at 16,000×*g* for 5 min at 4 °C to remove cell debris, and 20 μl of the supernatant was analysed for luciferase activity using the luciferase activity kit (Promega, Madison, WI, USA). Total light emission was measured for 10 s on a Luminometer (Anthos Lucy 1 Luminometer, Labtech International Ltd., Uckfield, E. Sussex, UK). The protein concentration of each sample was determined with a protein assay reagent (Biorad, Hercules, CA). Luciferase enzyme activity was expressed as relative light units (RLU) per mg of total protein ±SE of the mean.

2.12. Transfection of rabbit corneas

Eyes were obtained from male New Zealand White rabbits of 4.5 kg weight (Harlan Sera-Lab Ltd., Loughborough, Leicestershire), placed in sterile, ice-cold saline for transportation on ice, and used within 4 h of death. The cornea was removed with a thin rim of sclera and cut with a sharp scalpel blade into six pieces. These were transferred into individual wells of 24-well plates containing 1 ml of minimal essential medium (MEM; Invitrogen, Paisely, UK) supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml penicillin, 100 units/ml streptomycin and 2.5 μg/ml amphotericin B. They were cultured overnight at 37 °C in an atmosphere of 95% air/ 5% CO_2.

For gene delivery, the CMVβ plasmid was used. The pieces of cornea were washed twice with PBS at room temperature, and 500 μl of vector/DNA complexes at a DNA concentration of 10 μg/ml in dextrose/Tris, or culture medium without additives, was added. The corneas were incubated for 4 h at 37 °C in 95% air/ 5% CO_2. The transfection medium was then drained and replaced with 2 ml of fresh culture medium. The corneas were cultured for a further 48 h prior to reporter gene analysis.

Enzymatic analysis was carried out using the Galactostar kit (Applied Biosystems, Boston, MA, USA) according to the manufacturer's instructions. The corneas were washed twice with PBS and then 500 μl of lysis buffer was added to each well and incubated at room temperature on a rocker for 1 h. The lysate was removed, and frozen at −80 °C. On thawing, the lysate was centrifuged at 16000×*g* for 5 min at 4 °C to remove cell debris, and 20 μl of the supernatant was analysed for luminescence using the Galactostar reagent and the Anthos Lucy 1 Luminometer (Labtech International Ltd., Uckfield, East Sussex, UK), as described above for luciferase activity.

2.13. Adenoviral gene delivery to corneal endothelial cells

The adenovirus vector (a kind gift from Dr. S. Salehi) was an E1-deleted adenovirus serotype 5 (AdEasy, Q Biogene, Cambridge, UK) carrying the *E. coli β*-galactosidase gene driven by the cytomegalovirus (CMV) promoter. The adenovirus was diluted to 10^9 pfu/ml in DMEM, and 0.5 ml was used to transfect the pieces of cornea, as described above. This corresponds to a multiplicity of infection of ~200 with regard to the corneal endothelial cells.

2.14. Confocal microscopy

The pGL3 plasmid was labelled with rhodamine using the TM-rhodamine labelling kit (Mirus, WI, USA). The labelled plasmid retained ~50% of its gene delivery activity for HUH7 cells, in comparison with unlabelled pGL3. $(Lys)_{16}$/ DNA, RPC/DNA and PLL/DNA particles were formed at 5 μg/ml DNA, and a ± charge ratio of 3:1. Varying proportions of labelled:unlabelled DNA, from 5:0 to 1:4 were used.

The HUH7 cell line (grown in glass chamber slides) (Labtek, VWR International, Leicestershire, UK) and rabbit corneas were exposed to rhodamine-labelled peptide/DNA particles precisely as for the gene delivery

studies. At 4 h and 24 h, the medium was removed and the tissues fixed with 10% formalin for 15 min at room temperature. The HUH7 cells were then mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Peterborough, UK) and kept in the dark at 4 °C for up to 3 days before examination on a Zeiss LSM510 Meta confocal microscope (Zeiss, Welwyn Garden City, UK). The corneas were either mounted as whole pieces in the mounting medium, or frozen in liquid nitrogen. In the latter case, 20 μ sections were cut and examined in the mounting medium.

3. Results

3.1. Formation of the reducible polycation

Polymerisation of the Cys(Lys)$_{16}$Cys monomer proceeded rapidly during the first 24 h of the reaction, and was more-or-less complete at 48 h (Fig. 1a). The reaction was stopped at 96 h. Analysis of the purified RPC by gel permeation chromatography showed a single peak (Fig. 1b) with a retention time corresponding to a molecular weight of 23,900 Da. This is equivalent to an average of 10.5 monomers or ~160–175 lysines per RPC. On the basis of recovered amino groups, approximately 25% of the original monomers were found in the final RPC preparation.

Destabilisation by reduction is a typical property of RPCs. Fig. 1c demonstrates that reduction caused the ~80–90 nm RPC/DNA particles to aggregate into larger particles of ~130 nm. Polylysine/DNA particles, formed with non-reducible polylysines of comparable molecular weight (~27,000 Da), were unaffected by reduction.

3.2. Susceptibility of nanoparticles to disruption by anionic peptides

Cationic vector/DNA particles are known to be disrupted by polyanions, such as glycosaminoglycans and polyaspartate, and also by high salt concentrations, e.g. [24]. Fig. 2a demonstrates that even low concentrations of fusogenic peptide (2.5 μg/ml, corresponding to a DNA:(Lys)$_{16}$ peptide:fusogenic peptide w/w ratio of 1:2:0.5) release substantial amounts of DNA from (Lys)$_{16}$/DNA particles. At a DNA:(Lys)$_{16}$ peptide:fusogenic peptide w/w ratio of 1:2:1 (corresponding to the 5 μg/ml row in Fig. 2a), the bulk of the DNA is released from the particles even though these are the optimal ratios for gene delivery [19]. In contrast the RPC/DNA particles showed only low levels of DNA release at the highest fusogenic peptide concentration tested (20 μg/ml), corresponding to a DNA:RPC:fusogenic peptide w/w ratio of 1:1.3:4 (Fig. 2b). The studies in Fig. 2a,b were performed with a cationic peptide:DNA ±charge ratio of 3:1. When RPC/DNA particles are formed at a ±charge ratio of 6:1 (that used in our transfection studies), no DNA is released even at a DNA:RPC:fusogenic peptide w/w ratio of 1:2.6:10 (corresponding to 50 μg/ml of fusogenic peptide in Fig. 2c). If the RPC/DNA particles (at a ±charge ratio of 6:1) are reduced with 20 mM DTT prior to addition of the fusogenic peptide, DNA begins to be released at 10 μg/ml of the fusogenic peptide, and is more marked at 50 μg/ml (Fig. 2d). Another measure of particle stability is resistance to disruption by the polyanion poly (L-aspartate acid) (24). Fig. 2e and f demonstrates that this polyanion at 2.5 μg/ml disrupts RPC/DNA particles (at a ±charge ratio of 6:1) only in the presence of 20 mM DTT.

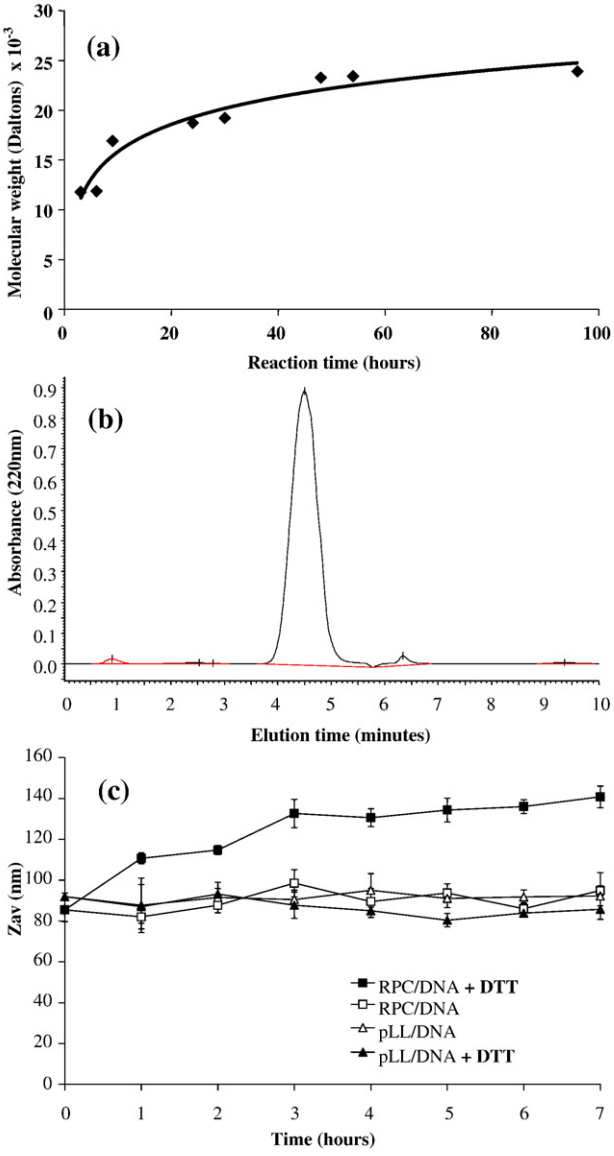

Fig. 1. Properties of Cys (Lys)$_{16}$ Cys-based reducible polycations. (a) At the time points indicated, aliquots of the Cys(Lys)$_{16}$Cys plus DMSO reaction mixture were subjected to CATSEC-300 HPLC gel permeation chromatography. The size of the RPC was determined by comparison with commercially available polylysines of various molecular weights. (b) The RPC mixture at 96 h was purified from low molecular weight components using centrifugal concentrators, and a sample was analysed by gel permeation chromatography as in (a) above. (c) RPC/DNA and non-reducible polylysine (PLL)/DNA particles were formed at a ±charge ratio of 6:1 and a DNA concentration of 2.5 μg/ml in 20 mM HEPES, pH 7.4. Particle size (Zav) was assessed by Dynamic Light Scattering after 30 min (time 0). At that point, dithiothreitol was added to 20 mM, as indicated (+DTT), and particle size measured hourly.

3.3. Gene delivery to cell lines

In view of the resistance of RPC/DNA particles to fusogenic peptide-mediated disruption, the gene delivery properties of these particles was of particular interest. The results in Fig. 3 demonstrate that RPC/DNA/fusogenic peptide particles are ~5–

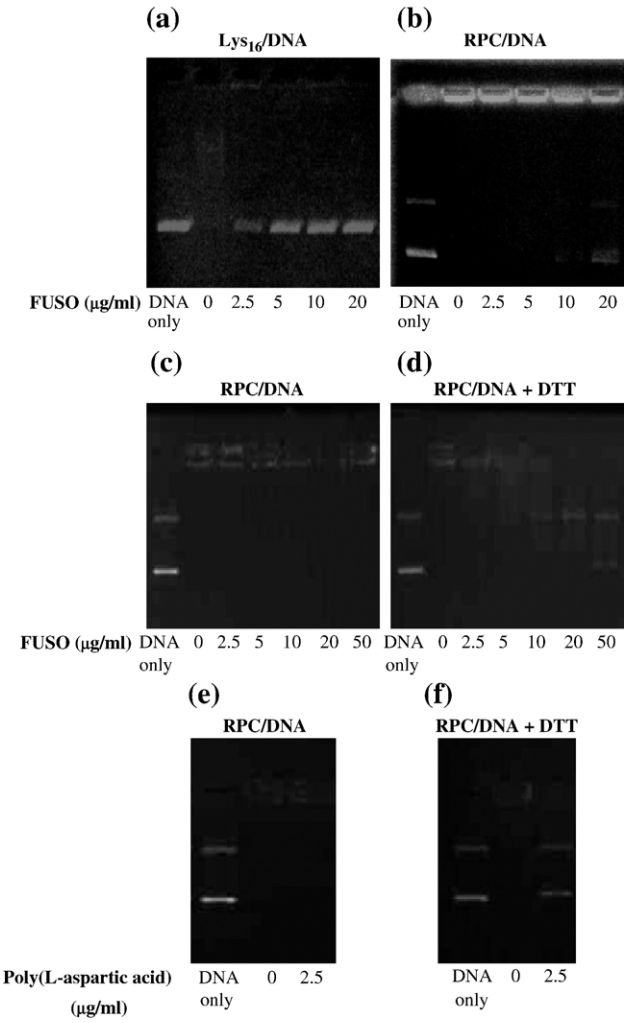

Fig. 2. Disruption of peptide/DNA nanoparticles by anionic peptides. $(Lys)_{16}$/DNA (a) and RPC/DNA (b) particles were formed at a ±charge ratio of 3:1 and a DNA concentration of 5 μg/ml in PBS. This represents a DNA:peptide w/w ratio of 1:2 for the synthetic $(Lys)_{16}$ peptide (which is a TFA salt) and 1:1.3 for the RPC (which is a hydroxyl salt). After 15 min, the fusogenic peptide was added to the particles at the concentrations indicated. After a further 15 min, the samples were electrophoresed on agarose gels and the DNA visualised under UV light. In c to f, RPC/DNA particles were formed at a ±charge ratio of 6:1. In d and f, 20 mM DTT was added to the particles 15 min after particle formation. The fusogenic peptide (c,d) or poly (L-aspartic acid) (e,f) were added to the concentrations indicated after a further 15 min. Electrophoresis was performed 15 min later.

10-fold better than PEI 25000 and Lipofectamine 2000, and ~200-fold better than $(Lys)_{16}$/DNA/fusogenic peptide particles. Very similar results were obtained with the HeLa cell line.

In view of the much greater stability of the RPC/DNA particles in the presence of the fusogenic peptide and polyanions (Fig. 2) and also in the presence of high NaCl concentrations (24 and our unpublished data) we evaluated the efficacy of these particles in the presence of serum. Fig. 4 demonstrates that the efficacy for gene delivery of RPC/DNA/fusogenic peptide particles is only marginally affected by 10% serum (79% of value without serum), whereas the efficacy of $(Lys)_{16}$/DNA/fusogenic peptide is virtually abolished (2.9% of value without serum).

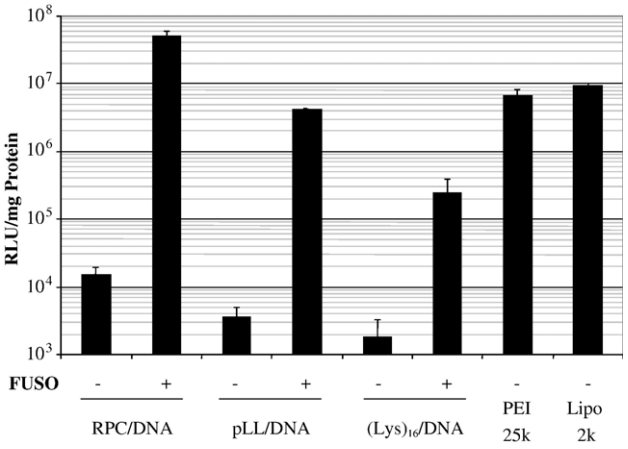

Fig. 3. Gene delivery to the HUH7 cell line. RPC (molecular weight ~24,000 Da), non-reducible polylysine (molecular weight ~27,000 Da) (PLL) and $(Lys)_{16}$ were used to form nanoparticles with the pGL3 plasmid at DNA concentration of 2.5 μg/ml in RPMI medium. A cationic peptide: DNA ±charge ratio of 6:1 was used. Fusogenic peptide at 5 μg/ml was included or not, as indicated. Complexes with PEI 25000 and Lipofectamine 2000 were formed as recommended by the manufacturers. Cells were analysed for luciferase expression after 48 h. Results are means±SE of triplicates, given as Relative Light Units (RLUs) per mg of protein. This experiment was repeated three times with similar results.

3.4. Gene delivery to Go arrested corneal endothelial cells

The non-reducible, high molecular weight polylysine (PLL) gave no gene delivery to corneal endothelial cells (Fig. 5), even though it gave good levels of gene delivery to cell lines (Fig. 3). This is consistent with the reported poor ability of high molecular polylysines to effect nuclear translocation [22]. Similarly, PEI 25000 and Lipofectamine 2000 gave excellent gene delivery to the cell lines (Fig. 3), but essentially background levels in the

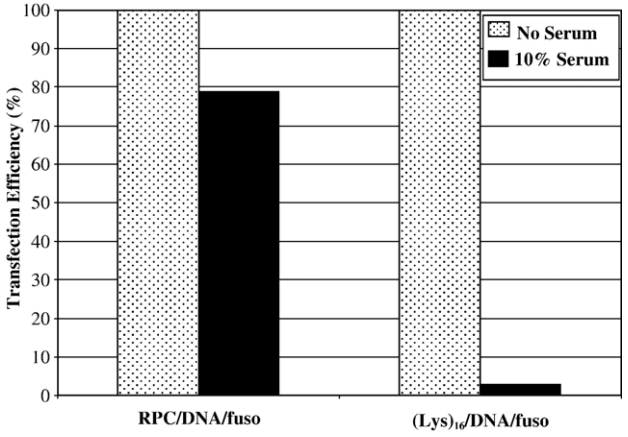

Fig. 4. Gene delivery in the presence of serum. RPC/DNA/fusogenic peptide and $(Lys)_{16}$/DNA/fusogenic peptide particles were formed as in the legend to Fig. 3. Fifteen minutes before addition to HUH7 cells, the peptide/DNA/fusogenic peptide particles were made up to 10% FCS (10% serum) or an equivalent volume of DMEM medium was added (no serum). Luciferase expression was measured after 48 h. For the RPC/DNA/fusogenic peptide particles without serum, the values were $135 \pm 9 \times 10^6$ RLU/mg protein, which is taken as 100%. For the $(Lys)_{16}$/DNA/fusogenic peptide complexes without serum, the values were $7.5 \pm 2.0 \times 10^6$ RLU/mg protein, which is taken as 100%. This experiment was repeated three times with similar results.

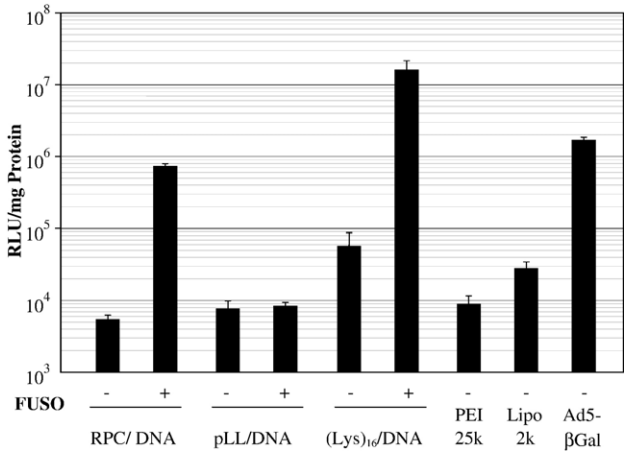

Fig. 5. Gene delivery to corneal endothelial cells. Conditions were similar to those in Fig. 3, except that the CMVβ plasmid was used, the transfection medium was 5% dextrose, 10 mM Tris, pH 7.4, and the DNA concentration was 10 μg/ml. An adenovirus 5 vector carrying the β galactosidase reporter gene from pCMVβ was used at 10^9 plaque-forming units/ml. This experiment was repeated three times with similar results.

corneal endothelial cells (Fig. 5). The RPC/DNA/fusogenic peptide particles gave good levels of gene delivery to corneal endothelium, comparable to that by the adenovirus vector at a multiplicity of infection of >100. Interestingly, however, the best gene delivery for corneal endothelium was seen with the $(Lys)_{16}$/DNA/fusogenic peptide particles, which were 10–15-fold better than the RPC and adenovirus vectors.

3.5. Confocal microscopy studies

$(Lys)_{16}$/DNA/fusogenic peptide, PLL/DNA/fusogenic peptide and RPC/DNA/fusogenic peptide particles were evaluated, using rhodamine-labelled pGL3 plasmid. The peptide/DNA/fusogenic peptide particles were easily seen as bright particles, positive for both rhodamine and DAPI. However, we were unable to demonstrate rhodamine staining of nuclei, at either four or 24 h, in either the HUH7 cell line or the corneal endothelial cells. Very occasionally, we saw with what looked like an entrapped peptide/DNA particle in the nucleus of an HUH7 cell.

4. Discussion

DNA directly injected into the cytosol is usually inefficiently translocated into the nucleus [25]. Directional transport via the nuclear pore complex is an energy-dependent process mediated by more than 20 members of the karyopherin family [26]. Karyopherin α is an adaptor protein which binds the classical cationic nuclear localizing signal (NLS) of the SV40 large T antigen. It then binds to karyopherin β, which docks into the nuclear pore complex for translocation of the bound "cargo". These functions of NLS motifs in proteins can be replicated by free synthetic peptides [27]. Moreover, synthetic peptides corresponding to NLS motifs have been reported to effect nuclear translocation of DNA plasmids [28].

There is strong functional evidence that oligolysine sequences, such as $(Lys)_{16}$, are also effective for nuclear translocation of plasmids [18–21]. In our previous studies we have demonstrated that transfection of corneas using $(Lys)_{16}$ peptides as vectors is strictly restricted to the corneal endothelium [19,21] and also that (as expected) there is no mitosis of the corneal endothelial cells in the course of the gene delivery studies [18]. As translocation to the nucleus is a prerequisite for reporter gene expression, the fact that >95% of corneal endothelial cells express the reporter gene is clear functional evidence that the plasmid is translocated into the nucleus. It is possible that the $(Lys)_{16}$ peptide is simply permissive for DNA translocation, for example by more readily dissociating from the DNA plasmid and thereby allowing translocation via proteins already in the cytosol. However, DNA directly injected into the cytosol is generally poorly taken up into the nucleus [25]. Moreover, substituting the amino terminus of $(Lys)_{16}$ resulted in a peptide with excellent gene delivery properties for cell lines (thus demonstrating good delivery of the DNA to the cytosol) but with no ability *at all* for gene delivery to corneal endothelium [18], suggesting an active role for the $(Lys)_{16}$ in nuclear translocation. In addition, the fact that high molecular weight polylysine is effective for gene delivery to cell lines (Fig. 3), but totally ineffective for gene delivery to the cornea (Fig. 5) is consistent with the nuclear translocation data of Colin et al. [20] and points to an active role of low molecular weight (but not high molecular weight) polylysines in nuclear translocation. However, we were unable to demonstrate localization of fluorescent DNA in the nuclei of either cell lines or corneal endothelium using confocal microscopy. This is possibly because the DNA plasmids are huge ($\sim 5 \times 10^6$ Da) in comparison with the proteins normally translocated across the nuclear core complex, and that translocation is relatively inefficient. If only one or a few plasmids are translocated, this is sufficient for gene expression, but makes detection difficult.

$(Lys)_{16}$-based RPCs represent a potentially ideal non-viral gene delivery system. The high molecular weight RPCs form peptide/DNA particles which are stable *in vitro* to salt, polyanions and serum. They are therefore likely to be more stable in the extracellular environment *in vivo*. In addition, provided that the RPCs are reduced to sufficiently small components after internalization by the target cell, they enable nuclear translocation of DNA plasmids (a property of the $(Lys)_{16}$ monomer). In our studies, the non-reducible polylysine of 27,000 Da gave no gene delivery at all to the corneal endothelial cells, but good gene delivery to cell lines, whereas the RPC of 24,000 Da gave high levels of gene delivery to both targets. Thus, at least, in corneal endothelial cells *in vitro*, the RPC appears to be reduced to monomers or units sufficiently small to permit nuclear translocation of the plasmid. Interestingly, $(Lys)_{16}$/DNA particles were even more effective than RPC/DNA particles on corneal endothelium. This could be a consequence of incomplete reduction of the RPC, reducing the efficacy of nuclear translocation.

Following endocytosis, non-viral vector/DNA particles require assistance with endocytic escape for effective gene delivery. The use of chloroquine is severely limited in vivo by cardiovascular toxicity [29] and cationic lipids are also toxic in vivo [30]. The potential toxicities of histidine-containing

peptides have not been evaluated. However, histidine-containing RPCs must be used at high peptide:DNA w/w ratios [8] and, because of the protein sponge effect, are likely to be associated with severe vacuolation in cells, similar to chloroquine [15]. Acid-dependent fusogenic peptides are potentially a more elegant and safer approach [15], as they become membrane-active only *after* cellular internalization. However, their anionic nature complicates their use with non-viral cationic vector/DNA particles, because of the potential for particle disruption [24]. We report that (in contrast to monomeric $(Lys)_{16}$/DNA particles) the $(Lys)_{16}$-based RPC/DNA particles are highly resistant to fusogenic peptide-mediated disruption. Lack of RPC/DNA particle disruption, and consequent higher DNA content of the particles, might be the basis of the superior gene delivery properties of RPC/DNA/fusogenic peptide particles. We demonstrate that RPC/DNA particles plus fusogenic peptide form excellent gene delivery vehicles for both dividing and non-dividing cells.

References

[1] V. Trubetskoy, V. Budker, L. Hanson, P. Slattum, J. Wolff, J. Hagstrom, Self-assembly of DNA–polymer complexes using template polymerization, Nucleic Acids Res. 26 (1998) 4178–4185.

[2] D.L. McKenzie, K.Y. Kwok, K.G. Rice, A potent new class of reductively activated peptide gene delivery agents, J. Biol. Chem. 275 (2000) 9970–9977.

[3] M.L. Read, K.H. Bremner, D. Oupicky, N.K. Green, P.F. Searle, L.W. Seymour, Vectors based on reducible polycations facilitate intracellular release of nucleic acids, J. Gene Med. 5 (2003) 232–245.

[4] D.V. Schaffer, N.A. Fidelman, N. Dan, D.A. Lauffenburger, Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery, Biotechnol. Bioeng. 67 (2000) 598–606.

[5] V. Trubetskoy, A. Loomis, P. Slattum, J. Hagstrom, V. Budker, J. Wolff, Caged DNA does not aggregate in high ionic strength solutions, Bioconjug. Chem. 10 (1999) 624–628.

[6] R.C. Adami, K.G. Rice, Metabolic stability of glutaraldehyde cross-linked peptide DNA condensates, J. Pharm. Sci. 88 (1999) 739–746.

[7] Y. Park, K.Y. Kwok, C. Boukarim, K.G. Rice, Synthesis of sulfhydryl cross-linking poly(ethylene glycol)-peptides and glycopeptides as carriers for gene delivery, Bioconjug. Chem. 13 (2002) 232–239.

[8] M.L. Read, S. Singh, Z. Ahmed, M. Stevenson, S.S. Briggs, D. Oupicky, L.B. Barrett, R. Spice, M. Kendall, M. Berry, J.A. Preece, A. Logan, L.W. Seymour, A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids, Nucleic Acids Res. 33 (2005) e86.

[9] P. Midoux, M. Monsigny, Efficient gene transfer by histidylated polylysine/pDNA complexes, Bioconjug. Chem. 10 (1999) 406–411.

[10] L. Vaccaro, K.J. Cross, J. Kleinjung, S.K. Straus, D.J. Thomas, S.A. Wharton, J.J. Skehel, F. Fraternali, Plasticity of influenza haemagglutinin fusion peptides and their interaction with lipid bilayers, Biophys. J. 88 (2005) 25–36.

[11] R.A. Parente, S. Nir, F.C. Szoka Jr., pH-dependent fusion of phosphatidylcholine small vesicles. Induction by a synthetic amphipathic peptide, J. Biol. Chem. 263 (1988) 4724–4730.

[12] E. Wagner, C. Plank, K. Zatloukal, M. Cotten, M.L. Birnstiel, Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin–polylysine–DNA complexes: toward a synthetic virus-like gene transfer vehicle, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7934–7938.

[13] P. Midoux, C. Mendes, A. Legrand, J. Raimond, R. Mayer, M. Monsigny, A.C. Roche, Specific gene transfer mediated by lactosylated poly-l-lysine into hepatoma cells, Nucleic Acids Res. 21 (1993) 871–878.

[14] S. Gottschalk, J.T. Sparrow, J. Hauer, M.P. Mims, F.E. Leland, S.L.C. Woo, L. Smith, A novel DNA–peptide complex for efficient gene transfer and expression in mammalian cells, Gene Ther. 3 (1996) 448–457.

[15] J.W. Fabre, L. Collins, Synthetic peptides as non-viral DNA vectors, Curr. Gene Ther. 6 (2006) 459–480.

[16] X. Zhang, L. Collins, J.W. Fabre, A powerful co-operative interaction between a fusogenic peptide and lipofectamine for the enhancement of receptor-targeted, non-viral gene delivery via integrin receptors, J. Gene Med. 3 (2001) 560–568.

[17] L. Collins, A.A. Asuni, B.H. Anderton, J.W. Fabre, Efficient gene delivery to primary neuron cultures using a synthetic peptide vector system, J. Neurosci. Methods 125 (2003) 113–120.

[18] A.L. Parker, L. Collins, X. Zhang, J.W. Fabre, Exploration of peptide motifs for potent non-viral gene delivery highly selective for dividing cells, J. Gene Med. 7 (2005) 1545–1554.

[19] L. Collins, J.W. Fabre, A synthetic peptide vector system for optimal gene delivery to corneal endothelium, J. Gene Med. 6 (2004) 185–194.

[20] M. Colin, S. Moritz, P. Fontanges, M. Kornprobst, C. Delouis, M. Keller, A.D. Miller, J. Capeau, C. Coutelle, M.C. Brahimi-Horn, The nuclear pore complex is involved in nuclear transfer of plasmid DNA condensed with an oligolysine-RGD peptide containing nuclear localisation properties, Gene Ther. 8 (2001) 1643–1653.

[21] L. Shewring, L. Collins, S.L. Lightman, S.L. Hart, K. Gustafsson, J.W. Fabre, A non-viral vector system for efficient gene transfer to corneal endothelial cells via membrane integrins, Transplantation 64 (1997) 763–769.

[22] S. Brunner, T. Sauer, S. Carotta, M. Cotten, M. Saltik, E. Wagner, Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus, Gene Ther. 7 (2000) 401–407.

[23] D. Oupicky, A.L. Parker, L.W. Seymour, Laterally stabilized complexes of DNA with linear reducible polycations: strategy for triggered intracellular activation of DNA delivery vectors, J. Am. Chem. Soc. 124 (2002) 8–9.

[24] A.L. Parker, D. Oupicky, P.R. Dash, L.W. Seymour, Methodologies for monitoring nanoparticle formation by self-assembly of DNA with poly(l-lysine), Anal. Biochem. 302 (2002) 75–80.

[25] M. Wilke, E. Fortunati, M. van den Broek, A.T. Hoogeveen, B.J. Scholte, Efficacy of a peptide-based gene delivery system depends on mitotic activity, Gene Ther. 3 (1996) 1133–1142.

[26] L.F. Pemberton, B.M. Paschal, Mechanisms of receptor-mediated nuclear import and nuclear export, Traffic 6 (2005) 187–198.

[27] D. Kalderon, B.L. Roberts, W.D. Richardson, A.E. Smith, A short amino acid sequence able to specify nuclear location, Cell 39 (1984) 499–509.

[28] W. Ritter, C. Plank, J. Lausier, C. Rudolph, D. Zink, D. Reinhardt, J. Rosenecker, A novel transfecting peptide comprising a tetrameric nuclear localisation sequence, J. Mol. Med. 81 (2003) 708–717.

[29] X. Zhang, G.J. Sawyer, X. Dong, Y. Qiu, L. Collins, J.W. Fabre, The in vivo use of chloroquine to promote non-viral gene delivery to the liver via the portal. vein and bile duct, J. Gene Med. 5 (2003) 209–218.

[30] J.D. Tousignant, A.L. Gates, L.A. Ingram, C.L. Johnson, J.B. Nietupski, S.H. Cheng, S.J. Eastman, R.K. Scheule, Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid: plasmid DNA complexes in mice, Hum. Gene Ther. 11 (2000) 2493–2513.