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Enhanced gene transfer activity of peptide-targeted gene-delivery vectors

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Abstract

We have evaluated the capacity of the cell-binding heptapeptide SIGYPLP to enhance transgene expression using non-viral and viral gene delivery vectors. Targeted polyplex based vectors showed good levels of DNA uptake in freshly isolated human umbilical vein endothelial cells (HUVECs) compared to untargeted controls, whilst displaying only modest increases in reporter gene activity. The targeted polyplexes showed reduced levels of DNA uptake in cells of a none endothelial origin although they mediated higher levels of transgene expression. The enhanced efficiency of transgene expression may relate to the more rapid rate of cell division. However, since *in vivo* application of polyplexes is compromised by instability to serum proteins, serum-resistant polyplexes (surface modified with multivalent reactive hydrophilic polymers based on poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA)) were also evaluated for their ability to mediate transgene expression. Surface modification of polyplexes with pHPMA ablates non-specific cell entry, reducing levels of transgene expression, whilst the incorporation of the SIGYPLP peptide into the hydrophilic polymer resulted in restored transgene expression in all formulations tested. The technology of surface modification using pHPMA can also be applied in the context of viruses, masking receptor-binding epitopes and enabling the linkage of novel cell targeting ligands, enabling construction of a virus with receptor-specific infectivity. Retargeting of adenovirus based vectors using the same polymer-peptide construct enhanced levels of transgene expression in HUVECs to greater than 15 times that observed using parental (unmodified) virus, whilst restoring levels of transgene expression in non-endothelial cell lines tested. The use of constructs based on conjugates between hydrophilic polymers and small receptor-binding oligopeptides as agents for retargeting viral or non-viral vectors to cellular receptors represents a simple alternative to the use of antibodies as targeting ligands for cell specific gene delivery.

Keywords: *Peptide, receptor-targeted, transfection, vector, gene therapy*

Introduction

Gene therapy promises elegant cures for serious diseases, including cancer, yet clinical efficacy is continually thwarted by the poor target selectivity of the administered vectors. Although this can be addressed in part by using tumor-associated promoters to regulate transgene expression such as CEA (Schrewe et al. 1990) or AFP (Godbout et al. 1986), such promoters are often too weak for effective gene-directed enzyme prodrug therapy (GDEPT) (reviewed by Nishi 2003). In addition, transduction of non-target cells depletes the pool of vector available for tumor targeting, simultaneously promoting unwanted toxicities that could be dose limiting.

A more rational approach therefore is to identify suitable targeting ligands that bind receptors unique or upregulated within the tumor endothelium and microenvironment. Incorporation of such targeting ligands into gene delivery vectors, coupled with preclusion of non-specific interactions with non-target cells and blood serum components could enable

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receptor-mediated uptake exclusively into tumor endothelial cells following intravenous administration.

A number of receptors have been identified which appear to be upregulated within the tumor endothelium, and typically these tend to be markers of angiogenesis, such as the receptors for FGF and VEGF. However, the efficacy of corresponding ligands is likely to be limited by a number of factors. They tend to be large, bioactive molecules that are readily denatured, expensive to mass produce, and often of a charged and/or hydrophobic nature. Such traits are likely to promote non-specific interactions with components of the blood serum resulting in rapid clearance. Their incorporation might also decrease the dose limiting toxicity through interaction with the body's natural vasculature.

Due to such practical limitations, considerable attention has been engaged on identifying small receptor-binding peptides that could be used for cell targeting both *in vivo* and *in vitro*. Theoretically, oligopeptides such as those iterated using phage display technology, could be readily mass produced, since they require minimal post-translational modification, and are robust to chemical modification, permitting their incorporation into gene delivery vectors, thus enabling the production of receptor-specific gene delivery vectors. Arap et al. were among the first to demonstrate that small peptides identified in this manner could be used to direct agents specifically to the tumor vasculature *in vivo*, demonstrating selection of the integrin binding motif RGD and the aminopeptidase N binding motif NGR, considered to be upregulated in the proliferating endothelium (Arap et al. 1998). Since then, considerable attention has been focused upon the deployment of gene delivery vectors targeted with integrin receptor-binding oligopeptides, with noteworthy success. RGD-targeted vectors have been shown to be extremely efficient for gene delivery to cell lines *in vitro* (Colin et al. 1998), G_0 arrested, terminally differentiated corneal cells *ex vivo* (Collins and Fabre 2004) and have even been shown to inhibit tumor growth *in vivo* when coupled to a cDNA encoding TNFα (Zarovni et al. 2004). Indeed, RGD targeting strategies have been so successful that some groups have even speculated that RGD-coupled oligolysines may even facilitate nuclear translocation of exogenous DNA (Colin et al. 2001).

In this study, we have evaluated another peptide isolated using phage display technology for its ability to bind with high specificity to slowly dividing Human Umbilical Vein Endothelial Cells (HUVECs) *in vitro* (Nicklin et al. 2000). Our initial studies focused on how this peptide, SIGYPLP could be used to enhance the uptake and transfection activity of simple polyelectrolyte based complexes *in vitro* in both HUVECs and in non-endothelial cell lines. However, the application of simple polyelectrolyte based systems *in vivo* is known to be severely limited by protein instability, resulting in a rapid blood clearance profile due to hepatic accumulation by Kupffer cells (Dash et al. 1999). Therefore, we evaluated the activity of "laterally-stabilized complexes" coated with the multivalent hydrophilic polymer poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA), modified to incorporate the SIGYPLP targeting agent. Additionally, we have applied the same technology in the context of retargeting adenoviral vectors to test whether the same polymer-peptide conjugate could redirect adenoviral cell entry from its orthodox means of cell entry (via cellular coxsackievirus and adenovirus receptor (CAR), reviewed by Nemerow 2000) to alternative cellular receptors. In this context, polymer coated adenovirus has been show to exhibit an extended plasma retention period following intravenous administration (compared to unmodified virus), offering a window of opportunity for systemic targeting (Green et al. 2004). Polymer modified virus has also been shown to greatly reduce antibody binding and neutralization of the viral particle, and permits efficient retargeting of the viral particle via the linkage of appropriate targeting ligands (Fisher et al. 2001).

Materials and methods

Sources of peptides

Synthesis and purification by HPLC techniques of the SIGYPLPGGGS$(K)_{16}$ and $(K)_{16}$GACDCRGDCFCA peptide described in this study was performed by Severn Biotech Limited (Kidderminster, Worcester, UK). The peptide SIGYPLGGGSK was obtained from Alta Biosciences (Edgbaston, Birmingham, UK). In both cases, the peptides were supplied as a trifluoroacetate (TFA) salt, in the form of dry powder, and stored desiccated at -35°C

Monitoring particle formation by inhibition of EtBr/DNA fluorescence

The EtBr/DNA fluorescence ($\lambda_{ex} = 510$ nm, $\lambda_{em} = 590$ nm) of plasmid DNA (20 μg/ml) in ultrapure water containing EtBr (400 ng/ml) was measured and set to 100% using a fluorimeter (Perkin-Elmer LS50B), with EtBr fluorescence (without plasmid DNA) set to 0%. Aliquots of pLL or SIGYPLPGGGS$(K)_{16}$ were added sequentially and the fluorescence was measured after each addition until minimal further change in fluorescence was observed.

Transmission electron microscopy (TEM)

An aliquot (approx. 10 μl) of solution containing polyplexes was added to 200 mesh carbon coated copper grids and left to air dry. Uranyl acetate stain

(10 μl, 0.5% in water) was added for 1 min at room temperature. The strain was drawn off using filter paper, the grid washed by bathing in distilled water and left to air dry. Complexes were viewed in a JOEL 1200EX transmission electron microscope.

Analysis of particle size by photon correlation spectroscopy (PCS)

Light scattering analysis was performed using a Malvern Instruments light scattering spectrophotometer in order to determine the size of DNA/polycation complexes. Measurements were taken at 25°C at an angle of 90°to the incident light using a disposable poly-methacrylate cuvette. To obtain accurate readings, buffers were degassed and filtered before use. The machine was calibrated using latex spheres (204 nm) before and after experimental samples using contin software with monomodal deconvolution for analysis.

Labelling of plasmid DNA

Plasmid DNA was radiolabeled by random prime incorporation of ^{32}P-dCTP using a Ready-to-Go DNA Labeling Kit (Amersham Pharmacia, UK).

The reagents in the Ready-to-Go kit were reconstituted by the addition of 20 μl of water (without mixing) and then left on ice for 5–30 min. The DNA (50 ng in 25 μl) was denatured by heating for 5 min at 95–100°C and then placed on ice for 2 min before being briefly centrifuged. The DNA was added to the reaction mixture, along with 5 μl of [α-^{32}P]-dCTP (50 μCi) and distilled water to make the volume up to 50 μl. The labeling reaction was started by mixing the components by gentle pipetting, followed by a 15 min incubation at 37°C. After this time the reaction was stopped by the addition of 5 μl of 0.2 M EDTA (pH 8.0).

The DNA was purified away from unincorporated nucleotides using MicroSpin DNA purification columns (Amersham Pharmacia, UK). The columns were pre-equilibrated in TE buffer (pH 7.6) and pre-packed with Sephacryl S-200 resin. The resin was re-suspended by vortexing briefly, and placed in a microcentrifuge tube and spun for 1 min at 3000 rpm in order to remove the TE buffer and avoid any dilution of the sample. After centrifugation the column was placed in a new microcentrifuge tube and the labeled plasmid sample added to the top of the column. The column was then centrifuged for 2 min at 3000 rpm; the purified sample eluted into a microcentrifuge tube with unincorporated oligos and oligonucleotides retained in the resin. Finally, the purity of the labeled DNA was checked following agarose gel electrophoresis and quantitative analysis with a Typhoon Fluorimager (Molecular Dynamics, UK).

Plasmid DNA labeled with the cyanine dimer nucleic acid stain YOYO-1 (Molecular Probes, Eugene, USA) was prepared by reaction of 20 μg of plasmid DNA with 1 μl of 100 μM YOYO-1 (in 10 mM HEPES, pH 7.4) in the dark for 5 h on ice in a total volume of 10 μl. The volume was increased to 1 ml and free YOYO-1 was removed by dialysis overnight against 10 mM HEPES, pH 7.4 at 4°C, protected from light, in snake-skin dialysis tubing with a molecular weight cut-off of 10 kDa (Pierce and Warriner, Cheshire, UK).

Formation of peptide/DNA nanoparticles

For calculation of peptide/DNA charge ratio (N/P ratio), an average mass per phosphate group (P) of 325 Da was used for DNA (hydrochloride salt), whilst the average mass (including TFA salts) per amino group (N) was calculated for each peptide individually. For poly(L-lysine) (pLL) solutions (hydrobromide salt), an average mass per charge of 209 was calculated, thus to achieve a theoretical charge ratio of 1:1 between pLL and DNA, a weight/weight (w/w) ratio of 1:0.64 is required.

Formation of peptide/DNA nanoparticles for uptake and transfection studies was achieved by the addition of the relevant volume of peptide or pLL (dissolved to a concentration of 2.5 mg/ml in 10 mM HEPES, pH 7.4) to a DNA solution (20 μg/ml DNA in 10 mM HEPES, pH 7.4) to a charge ratio of 2 or 4. The resulting solution was mixed thoroughly and left to stand at room temperature for 1 h.

Uptake of ^{32}P labeled pLL/DNA complexes

Cells were plated out in 24-well plates (50,000 cells/well, in 1 ml/well DMEM supplemented with 2 mM L-glutamine and 10% FCS). The following day, ^{32}P labeled polyplexes were incubated on cells (in DMEM) in the presence or absence of 100 μM chloroquine for 4 h (37°C, 5% CO_2). Following incubation, the media was discarded, and the cells washed with acid saline (150 mM NaCl, pH 3) to remove surface bound complexes, and finally dissolved in NaOH (0.5 ml, 2 M). The solutions were diluted in Ultima Flo AF scintillation fluid (4 ml, Packard) and assayed for radioactivity in a Packard 1900TR liquid scintillation analyser. Known quantities of ^{32}P labeled DNA were also assayed to provide a standard curve, and the results corrected for background radioactivity.

For analysis of uptake of YoYo-1 labeled DNA, plasmid DNA was pre-labeled with YoYo-1 (as described earlier) prior to complex formation. Polyplexes were then added to cells (70–80% confluent) in a 6-well plate and allowed to endocytose for 4 h at either 4°C or 37°C. Media containing residual polyplexes was then discarded and the cells washed twice with PBS. Cells were then removed from

the plate using trypisin/EDTA solution (400 μl/well) and subsequently fixed using 2% (w/v) paraformaldehyde solution (in PBS). The cells suspensions were analysed for fluorescence using a Perkin-Elmer Coulter XL flow cytometer.

Transfection of cell lines in vitro

Cells were plated out into 48 well plates on the day prior to transfection (20,000 cells/well in 500 μl of DMEM supplemented with 2 mM L-glutamine and 10% FCS) and incubated at 37°C overnight. Media were discarded and replaced with 175 μl of fresh DMEM in the presence or absence of FCS (10%) and also in the presence or absence of chloroquine (100 μM). Polyplexes prepared and added to the cells with 500 ng/25 μl of plasmid DNA per well in a total volume of 200 μl. After 4 h, media were removed, the cells washed twice in PBS, and replenished with fresh DMEM supplemented with 2 mM L-glutamine and 10% FCS and incubated at 37°C, 5% CO_2 for a further 20 h. Following incubation the media were discarded, the cells washed twice in PBS, and the cell lysates harvested by incubation of cells for 30 min at room temperature in 200 μl of 1 × lysis reagent (Promega, Madison, WI, USA). Of the remaining lysate 50 μl was assayed for luciferase expression by the following method: Luciferin (500 μl of a 10 mM stock solution consisting of: 10 mg beetle luciferin, 0.47 ml of 1 M glycoglycine pH 8.0, 15.3 ml water) was diluted into 10 ml of luciferase reaction buffer (20 mM glycoglycine, 1 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 0.5 mM ATP, 0.27 mM coenzyme A lithium salt). One hundred microlitres of this luciferin/luciferase reagent was added to 50 μl of the cell lysate, and the resulting luminescence integrated over 10 s in a Lumat LB 9507 (EG and G Berthold, Bundoora, Australia). Results are expressed as relative light units (RLU) per mg of cell protein, determined by BCA assay (Pierce, Chester, UK).

Production of peptide-pHPMA conjugate

SIGYPLP peptide (3 mg) was dissolved in 5 μl of anhydrous dimethylformamide and was added to 35 mg of pHPMA in 200 μl dimethylformamide. Diisopropylethylamine (1.9 μl) was then added, and the solution was stirred for 5 h at room temperature. The polymer was then precipitated into 20 ml of acetone–diethylether (1:1) mixture. Precipitated polymer was isolated by filtration and dried *in vacuo* overnight. The amount of remaining reactive groups was quantified from absorbance at 274 nm using molar extinction coefficient 9600.

Formation of pHPMA surface modified pLL/DNA complexes

Polyplexes were formed between pLL and DNA to a charge ratio of 2 at a DNA concentration of 26 μg/ml. Subsequent surface modification of the polyplexes was achieved by buffering the solution by the addition of HEPES pH 7.8 to a final concentration of 50 mM before addition of poly N-(2-hydroxypropyl)methacrylamide, (pHPMA-ONp) (prepared by the Institute for Macromolecular Chemistry, Prague) to achieve a final concentration of between 0.1 and 10 mg/ml. The polymer was left to react with the polyplexes for 12 h at room temperature, after which any remaining reactive esters were removed by aminolysis using 0.01% amino ethanol. Production of SIGYPLP targeted complexes was achieved by prederivatising the pHPMA-ONp to incorporate the SIGYPLP peptide (see above), and then surface modifying polyplexes as described above.

Analysis of complex stability to polyanion mediated dissociation

To assess complex stability to polyanions, polyplexes were formed between poly(L-lysine) and plasmid DNA (N/P = 2). Polyplex containing solution was subsequently subdivided and subject to surface modification with pHPMA or SIG-pHPMA (0.2 mg/ml). The following day, each solution was further subdivided and exposed to increasing concentrations of the polyanion poly(L-aspartic acid) (pLAA; 0–1000 μg/ml) for 1 h at room temperature. Complex stability to polyanions was assessed by examining the electrophoretic mobility of the plasmid DNA on agarose gels (1%, 100 V, 45 min).

Production of polymer modified virus

The virus used in these studies was the E1 deleted adenovirus vector Ad5/$\beta Gal_{\Delta E1}$. All virus was propagated and purified according to previously published protocols (Stallwood et al. 2000).

Frozen stocks of adenovirus were thawed at room temperature, vortexed and centrifuged for 1 min (5000 rpm). Virus particles (10^9–10^{10}) in 25 μl were adjusted to pH 7.8 by addition of 5 μl of 0.4 M HEPES pH 7.8. The desired amount of polymer (pHPMA) or polymer-peptide conjugate (SIG-pHPMA) was weighed out and diluted in distilled water to obtain a final concentration of 20 mg/ml pHPMA. Ten microlitres of the polymer solution was then added to the virus, vortexed and spun at 1000 rpm for 1 min to obtain a final pHPMA concentration of 5 mg/ml. The reaction was allowed to proceed at 4°C for 12 h to form polymer coated adenovirus (pHPMA-Ad5). The reaction was terminated by the addition of aminoethanol (final concentration 0.01% v/v) to effect aminolysis of remaining reactive ester groups.

For preparation of FGF targeted Ad5/$\beta Gal_{\Delta E1}$ bFGF was added to the reaction mixture 2 h after the initiation of the coating reaction to a final concentration of 80 μg/ml, and incubation continued for

a further 10 h, before termination of the reaction with aminoethanol.

Infection protocol using Ad5/β-gal

For assays of gene expression, 96-well plates were seeded at a density of 1×10^4 cells/well. Cells were infected with polymer modified, retargeted or parental Ad5/β-gal at 1×10^4 particles per cell (ppc) for 2 h in DMEM supplemented with 2 mM L-glutamine and 2% FCS. Expression of the β-Gal transgene was quantified 48 hours post-infection.

Analysis of β-Gal transgene expression using Ad-5/βgal

Following infection of cells using Ad5-βgal, expression of the transgene was analysed using the Galacto-Light Plus chemiluminescent assay system (Tropix, Perkin-Elmer, Massachusetts, USA).

Cells were washed briefly twice with PBS before the addition of 200 μl of 1 × promega lysis buffer to each well. The Galacton-Plus substrate was diluted 50-fold with Galacto-Light reaction buffer and warmed to room temperature. Samples of cell lysate (5–50 μl) were placed in a luminometer tube and 200 μl of the reaction buffer was added and gently mixed before being incubated at room temperature for 60 min. Three-hundred microlitres of accelerator was added prior to measurement of β-gal expression in the luminometer.

Results

Assembly of SIGYPLPGGGS(K)$_{16}$/DNA polyplexes

The peptide SIGYPLPGGGS(K)$_{16}$, comprising the SIGYPLP receptor binding heptapeptide linked to the DNA condensing polycationic 16 mer of Lysine through the GGGS spacer region (to mimic the spacer present on the phage particle) was synthesized by Severn Biotech (Kidderminster, UK). This peptide was studied for its ability to condense plasmid DNA as measured by the ethidium bromide inhibition assay. Peptide was added sequentially to plasmid DNA (20 μg/ml) in water containing EtBr (0.4 μg/ml) in 0.2 N:P charge ratio increments and changes in the DNA/EtBr fluorescence monitored using a fluorimeter. A sudden fall in DNA/EtBr fluorescence occurred over the N:P ratio of 0.8–1 (Figure 1(a)) thought to correspond to hydrophobic collapse of the DNA into nanoparticles (Parker et al. 2002). A similar profile of EtBr/DNA fluorescence inhibition was observed when the plasmid DNA was condensed with a control 3.4 kDa poly(L-lysine), comprising an average of 16 positively charged amino groups per molecule. The presence of the charge neutral targeting and spacer regions in the SIGYPLP-GGGS(K)$_{16}$ appeared, therefore, to not affect the ability of the polycationic component to condense plasmid DNA into nanoparticles. The complexes formed between SIGYPLPGGGS(K)$_{16}$ and plasmid DNA were between 90 and 100 nm in diameter as shown by PCS, and of almost identical size to those formed between 3.4 kDa pLL and DNA. The size and monodispersity of the complexes formed between SIGYPLPGGGS(K)$_{16}$ and plasmid DNA was also confirmed by transmission electron microscopy (Figure 1(b)).

Uptake of SIGYPLP targeted polyplexes in vitro

^{32}P-dCTP trace labeled DNA was condensed into nanoparticles at an N:P ratio of 2 using either SIGYPLPGGGS(K)$_{16}$, untargeted 3.4 kDa pLL or with the RGD bearing positive control (K)$_{16}$GACD-CRGDCFCA, previously shown to internalize via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Carlisle et al. 1999). Complexes were then incubated on cells (500 ng DNA/well) at 37°C for 4 h, in the presence or absence of the endosomal disrupting agent chloroquine (100 μM). Two point four- and 1.25- fold higher levels of uptake of SIGYPLPGGGS(K)$_{16}$/DNA complexes was observed in HUVEC (Figure 2(a)) compared to B16F10 cells (Figure 2(b)) in the absence and presence of chloroquine, respectively. Complexes formed between (K)$_{16}$GACDCRGDCFCA and DNA also showed good levels of uptake into HUVECs compared to B16F10 cells, showing 1.3- and 1.7-fold higher uptake in the absence and presence of chloroquine. Conversely, untargeted complexes showed low levels of uptake in both cell lines tested, showing uptake levels of less than 20 ng DNA per well in both cell lines tested. In HUVECs, SIGYPLP targeted polyplexes resulted in 10.4- and 9.0-fold higher levels of uptake than untargeted polyplexes with similar increases observed with the RGD targeted polyplexes. Similar levels of enhancement were observed in B16F10 cells, with 9.1- and 12.7-fold enhancement of uptake observed with SIGYPLP targeted polyplexes, compared to increases of 16.3- and 12.2-fold observed with polyplexes formed of the RGD bearing polycation.

To assess the dependence of temperature on uptake activities of polyplexes formed with the SIGYPLP targeted polycation, plasmid DNA was labeled with fluorophore YoYo-1. Complexes were formed between the YoYo-1 labeled DNA and either 3.4 kDa pLL or SIGYPLPGGGS(K)$_{16}$ at an N:P ratio of 2. The resulting nanoparticles were then incubated on B16F10 cells at either 4°C or 37°C. Fluorescence microscopy of the labeled complexes appeared to indicate a perinuclear distribution of SIGYPLP-GGGS(K)$_{16}$/DNA complexes (data not shown), whilst minimal detectable signal could be observed from the cells which had been exposed to untargeted (K)$_{16}$/DNA complexes. Cells were subsequently trypsinised and fixed using 2% paraformaldehyde, then subject to FACS analysis (Figure 2(c)). Whilst complexes formed of the 3.4 kDa pLL showed uptake

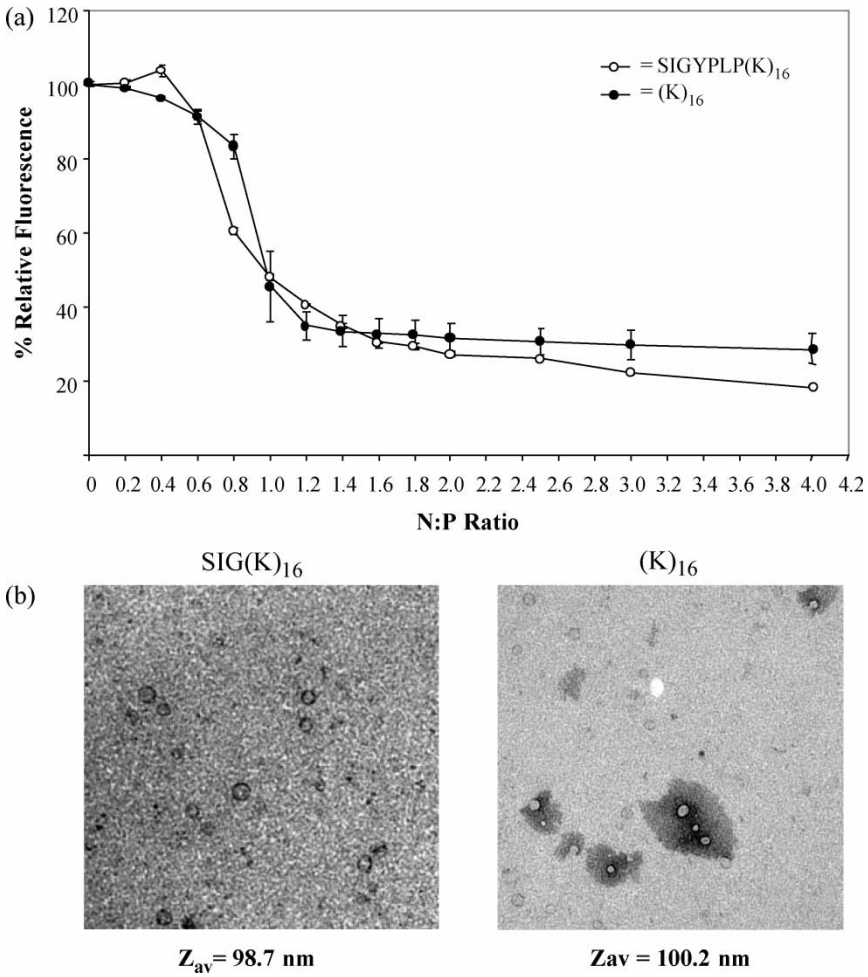

Figure 1. *Formation of SIGYPLP targeted polyplexes*. Formation of nanoparticles in water was monitored by the ethidium bromide exclusion assay (a) and by transmission electron microscopy (b). The size and polydispersity of the resulting nanoparticles was quantified by photon correlation spectroscopy.

of less than 2% positive cells at either 4°C or 37°C, complexes formed of the SIGYPLPGGGS$(K)_{16}$ showed 75.3% cells positive for YOYO-1 DNA at 37°C, with levels reduced to less than 2% at 4°C, suggesting that cellular uptake of such complexes is energy dependent, which would be consistent with a receptor-mediated means of cell entry.

Transfection activity of SIGYPLP targeted polyplexes in vitro

To investigate whether the SIGYPLPGGGS$(K)_{16}$ polycation could promote transgene expression in *in vitro* transfections, complexes were formed using 3.4 kDa pLL, SIGYPLPGGGS$(K)_{16}$ or $(K)_{16}$GAC-DCRGDCFCA and pGL3 plasmid DNA, encoding the luciferase reporter gene under the control of the SV40 promoter. Complexes were formed to N/P ratios of 2 and 4 and subsequently used in a transfection assay using a number of different cell lines, namely HUVEC (Figure 3(a)), 911 (Figure 3(b)), SKOV-3 (Figure 3(c)) and B16F10 (Figure 3(d)) for 4 h at 37°C in serum free media in the presence or absence of 100 μM chloroquine. Levels of reporter gene expression were assayed 24 h post-transfection. Transfection of HUVECs resulted in disappointingly poor levels of gene expression using all the complexes tested, and could not be substantially enhanced by the presence of 100 μM chloroquine in the transfection media. Since considerable levels of DNA uptake was achievable using SIGYPLP targeted complexes in HUVECs (Figure 2(a)), but only very low levels of transgene expression could be achieved, indicating levels of transgene expression are most likely limited by access to the transcriptional machinery within the nucleus (i.e. the efficiency of nuclear uptake of exogenous DNA is extremely low). This most likely reflects the fact that freshly isolated HUVECs are very slowly dividing cells, with the vast majority of cells in G_0/G_1 phase of the cell cycle (Bettinger et al. 2001), with minimal disassembly of the nuclear membrane occurring to allow access of exogenously applied DNA. However, levels of transgene expression were significantly higher using the targeted complexes in

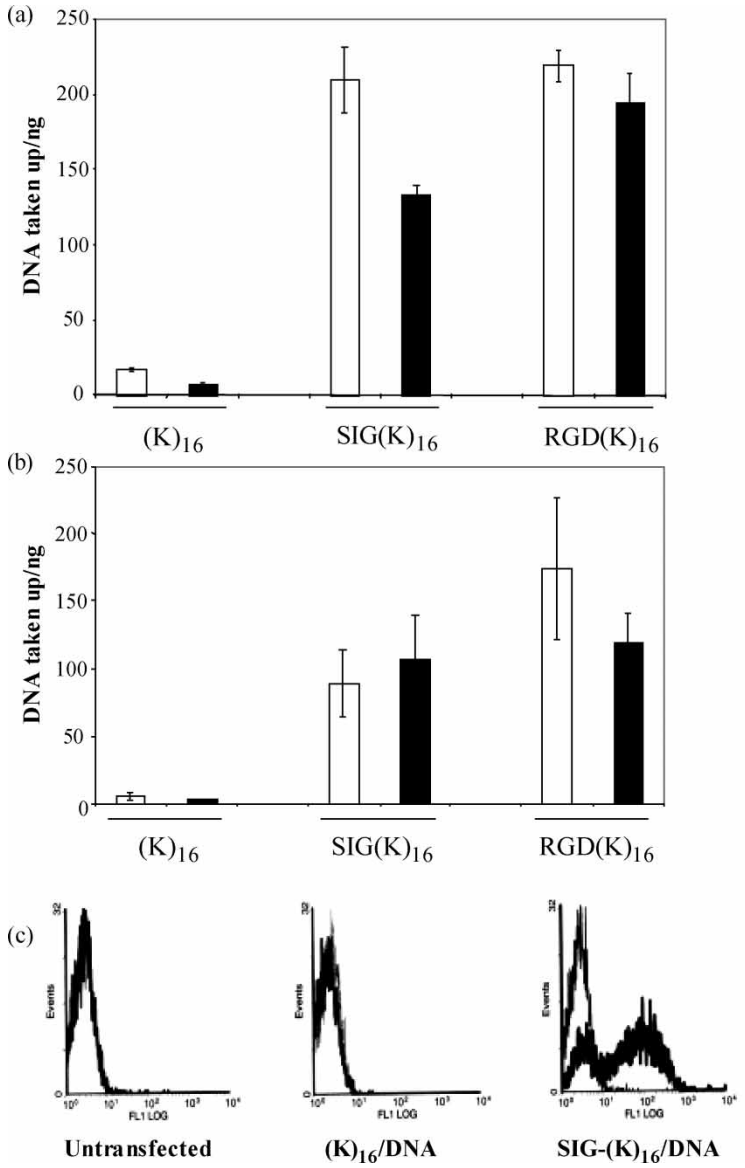

Figure 2. *Uptake of ^{32}P dCTP labeled complexes in vitro.* Polyplexes were formed between $(K)_{16}$, SIGYPLP$(K)_{16}$ and $(K)_{16}$GACDCRGDCFCA and plasmid DNA trace labeled with ^{32}P dCTP. Complexes incubated on HUVEC (a) or B16F10 (b) cells (20,000 cells/well) in serum free media for 4 h in the presence (black bars) or absence (white bars) of 100 μM chloroquine. Unassociated polyplexes were removed and the cell-associated radioactivity quantified using Packard 1900TR liquid scintillation analyser. To examine the effect of temperature on polyplex uptake, plasmid DNA was labeled with the YoYo-1 fluorophore prior to complex formation with either 3.4 kDa pLL or SIGYPLPGGGS$(K)_{16}$ to an N:P ratio of 2. Complexes were then incubated on B16F10 murine melanoma cells (70–80% confluent in 6 well plates) for 4 h at either 4°C (thin line) or 37°C (wide line), the cells were subsequently removed from the wells by trypsinisation and subject to FACS analysis using a Perkin-Elmer Coulter XL (c).

the other cell lines tested, all of which were more rapidly dividing. In all cases, levels of transgene expression were further augmented typically 1–2 logs by the presence of 100 μM chloroquine in the transfection media. Greater levels of transgene expression were observed in B16F10 cells with complexes targeted with the SIGYPLP peptide than with the RGD bearing positive control (although this was dependent upon the presence of chloroquine in the transfection media), whilst the opposite was true in SKOV-3 and 911 cells. The highest levels of transgene expression was observed for all complexes in B16F10 cells, possibly reflecting the fact that these cells were the most rapidly dividing with significant numbers of cells at any given point at G2/M phase in the cell cycle, undergoing mitosis and nuclear disassembly.

Production of targeted, serum stable polyplexes

The transfection activity of non-viral gene delivery vectors based on polycation/DNA complexes or cationic lipids/DNA complexes show extreme sensitivity to the presence of even low concentrations of

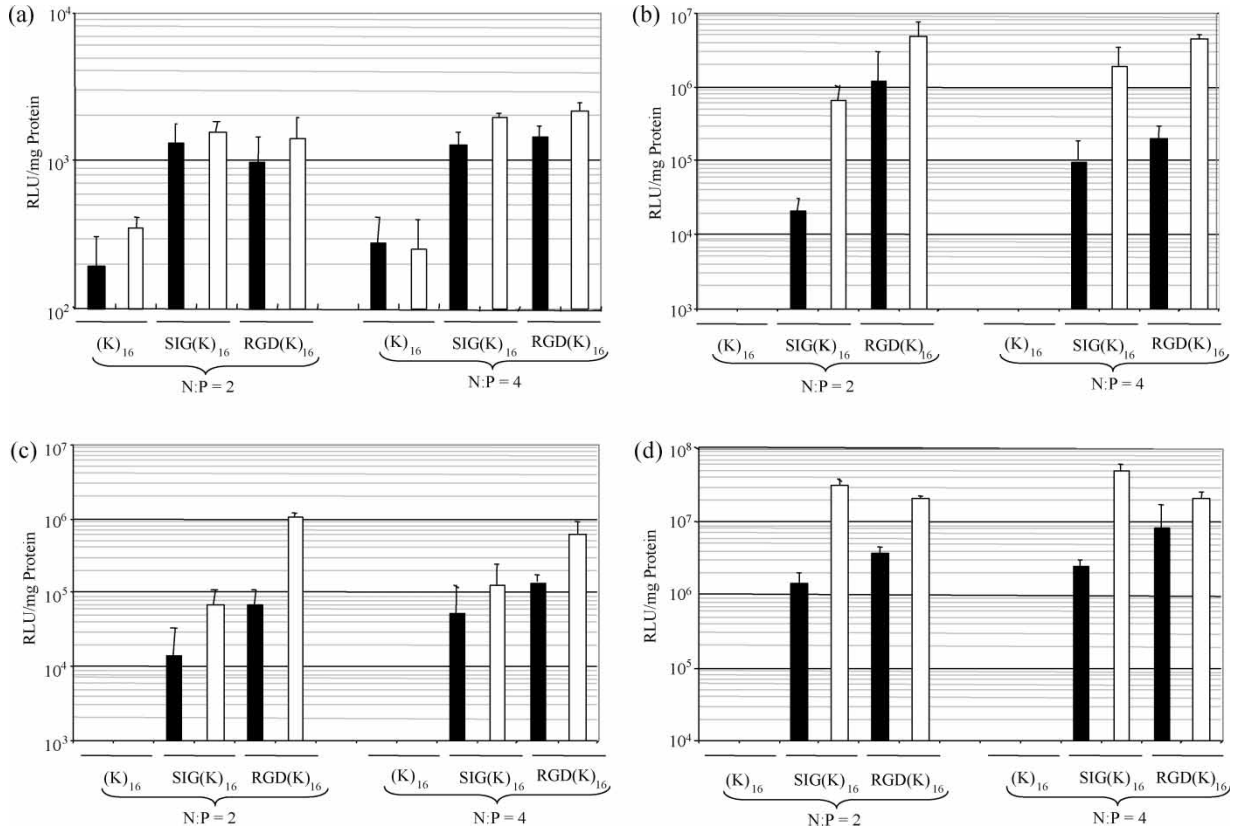

Figure 3. *Transfection activity of polyplexes targeted with SIGYPLP in (a) HUVE (b) 911 (c) SKOV-3 and (d) B16F10 cells*. Complexes were formed in 10 mM HEPES pH 7.4 between pGL3 plasmid DNA and either 3.4 kDa poly(L-lysine) ($(K)_{16}$), $SIGYPLPGGGS(K)_{16}$ or $(K)_{16}GACDCRGDCFCA$ to N/P ratios or 2 and 4. The resulting complexes were used in transfection assays against a range of cell lines. Transfections were performed in the absence of serum and in the absence (black bars) or the presence (white bars) of 100 μM chloroquine. Levels of transgene expression were quantified 24 h post-transfection.

serum in the transfection media. Typically, in the presence of 10% serum, the efficiency of transgene delivery is reduced 10–50-fold (data not shown). This is believed to result from negatively charged serum components such as albumin binding electrostatically to the complexes and inactivating them by blocking interactions with the cellular membranes.

Polyelectrolyte complexes can be protected against disruption by serum and polyanions by coating their surfaces with hydrophilic polymers. This provides steric stabilization, and can be combined with surface crosslinking to provide also lateral stabilization (Oupicky et al. 2002). Previous studies have shown that complexes surface modified with pHPMA (2 mg/ml) demonstrate enhanced stability with respect to salt induced aggregation, and improved pharmacokinetics *in vivo* (Oupicky et al. 2002). However, pHPMA coated complexes show very low levels of transgene expression, possibly reflecting loss of positive charge on the polyplex surface decreasing electrostatic interaction with negatively charged cell membranes and resulting in low basal transduction levels. However, this does provide a window of opportunity for the incorporation of targeting ligands onto the polymer to promote specific, receptor mediated uptake. Whilst levels of transgene expression are significantly lower following the surface modification of complexes, they are unaffected by the presence or absence of serum in the transfection media, presumably due to the enhanced stability of the resulting complexes.

To produce a targeted, serum stable polyplex, the peptide targeting ligand SIGYPLP was synthesized with the amino terminus acetylated (to prevent unwanted linkage through the amino terminus) (Alta Biosciences, Birmingham, UK) coupled via a GGGS spacer to a single, carboxy terminal lysine providing a single amino group for covalent linkage to the reactive ester groups on the pHPMA (Figure 4(a)). The SIGYPLPGGGSK was chemically linked to the pHPMA reactive polymer at a molar ratio of approximately 1 ligand per reactive polymer. Complexes formed by surface modification of pLL/DNA complexes with the polymer-peptide conjugate were found slightly larger than those formed with unmodified pHPMA, as gauged by PCS (Z_{av} pHPMA-pLL/DNA = 227.6 ± 12.4 nm; Z_{av} SIG-pHPMA-pLL/DNA = 246.1 ± 11.5 nm).

To address whether the presence of the targeting peptide in the reactive polymer might abolish the capacity of the polymer to protect the polyplex

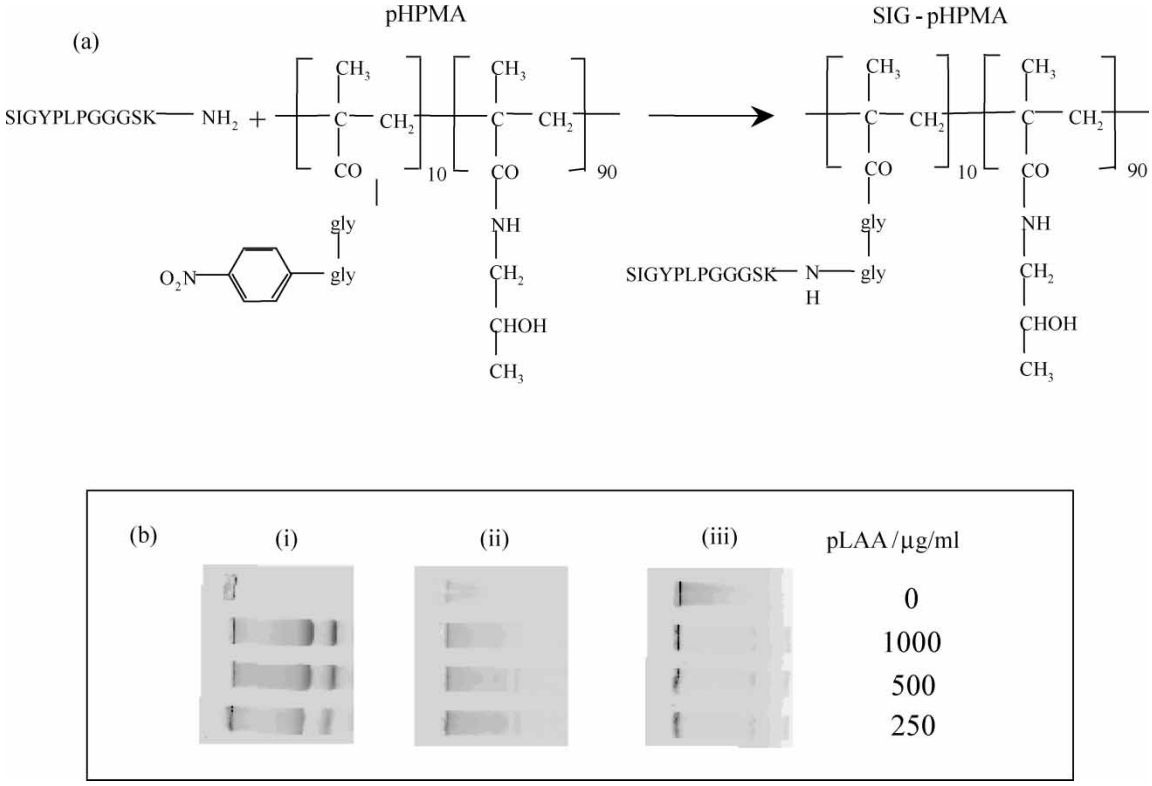

Figure 4. *Production of a targeted, serum stable polyplex*. The polymer pHPMA was prederivatised to incorporate the targeting peptide SIGYPLP. The SIGYPLP-pHPMA conjugate was subsequently used to surface modify pLL/DNA complexes. The stability of unmodified polyplexes (i) or polyplexes surface modified with pHPMA (ii) or SIG-pHPMA (iii) to polyanion-mediated dissociation was subsequently analysed following polyanion challenge with increasing concentrations of pLAA by agarose gel electrophoresis (c).

against polyanion mediated dissociation (a factor that has proved to be crucial in determining the *in vivo* pharmacology (Oupicky et al. 2002)), polyplexes were formed between poly(L-lysine) (29.3 kDa, N:P = 2) and pGL3 plasmid DNA, then surface modified using either unmodified pHPMA (200 μg/ml), or the pHPMA prederivatised to incorporated the SIGYPLP peptide (SIG-pHPMA). Complexes were then challenged with increasing (0–1000 μg/ml) concentrations of the polyanion poly(L-aspartic acid) for 1 h. The resulting mixtures were subject to electrophoresis and planar imaging to detect EtBr/DNA fluorescence (Figure 4(b)). Simple polyplexes complexes were readily dissociated by polyanions, and hence the plasmid DNA migrated freely into the gel. However, complexes which had been subject to surface modification with either pHPMA or SIG-pHPMA were resistant to pLAA mediated dissociation at all concentrations tested, indicating that the polymer-peptide conjugate retains its ability to stabilize polyplexes to polyanions mediated dissociation.

Transfection efficiency of SIG-pHPMA coated polyplexes in vitro

To address whether the incorporation of the targeting peptide into the hydrophilic polymer could enhance levels of transgene expression *in vitro*, complexes were formed between plasmid DNA and pLL 29.3 kDa. Complexes were subsequently subdivided and surface modified with either pHPMA or SIG-pHPMA at concentrations of either 2 mg/ml or 0.2 mg/ml. The resulting complexes were used in a transfection assay using B16F10 cells (20000 cells/well) in the presence or absence of 100 μM chloroquine (Figure 5). In the absence of serum, simple polyplexes mediated high levels of transgene expression through non-specific electrostatic interaction with the cellular membrane, with their efficiency of transgene expression improved up to 100-fold in the presence of chloroquine. As previously demonstrated, surface modification with pHPMA reduces the efficiency of transgene expression in a dose dependent manner, although the low levels of transgene expression could still be augmented by the presence of chloroquine in the transfection media. Prederivatisation of the polymer to incorporate the SIGYPLP targeting peptide resulted in increased levels of transgene expression in all the formulations tested compared with non-targeted controls, with levels of gene expression enhanced 60.3 times and 3.2 times for complexes coated at 2 mg/ml in the presence and absence of chloroquine respectively, and 8.4 times and 4.2 times for complexes formed at 0.2 mg/ml in the presence and absence of chloroquine, respectively.

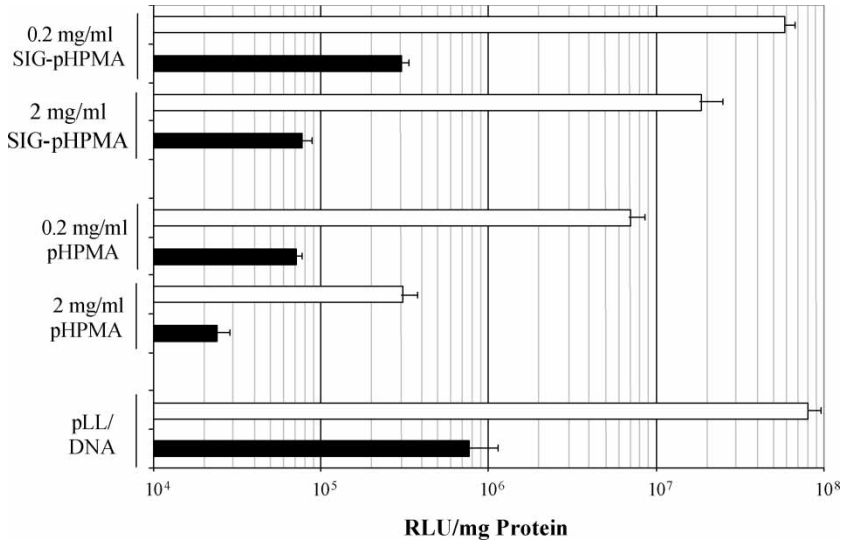

Figure 5. *Transfection efficiency of SIG-pHPMA coated complexes in vitro.* Complexes were formed between pGL3 plasmid DNA and pLL 29.3 kDa (N/P = 2), subdivided and surface modified with either pHPMA or SIG-pHPMA (2 or 0.2 mg/ml). Complexes were used in a transfection assay using B16F10 cells (20,000 cells/well) in the presence of 10% serum, except for pLL/DNA complexes, which were transfected in the absence of serum (to provide a 100% value for non-specific, polyplex mediated gene delivery). Transfections were performed in the presence (white bars) or absence (black bars) of 100 μM chloroquine in the transfection media.

Retargeting adenovirus using SIG-pHPMA

The promising receptor-binding activity of SIGYPLP seems difficult to apply successfully in the context of surface modified polyplexes because of their intrinsically low transgene expression in slowly dividing endothelial cells. However, similar retargeting strategies can be applied for adenovirus, a very potent gene delivery vector (Fisher et al. 2001). Accordingly, to evaluate the capacity of SIGYPLP to retarget viral vectors, the Ad5-βGal vector was used to infect freshly isolated HUVECs (Figure 6(a)) or the cell lines A549 (Figure 6(b)) and SKOV-3 (Figure 6(c)). Polymer modification of the virus with pHPMA (5 mg/ml) resulted in a substantial reduction of β-Gal expression in all cell lines tested, since polymer coating appears to ablate all forms of receptor-mediated uptake. Incorporation of bFGF onto the surface of pHPMA coated Ad5 vectors restores levels of β-Gal expression to similar levels of the parental virus in A549 and SKOV-3 cells, whilst in HUVECs, bFGF retargeted Ad-5 showed levels of β-Gal expression approximately 20 times that of the parental virus (reflecting the low levels of the CAR receptor on these cells). Interestingly, expression of β-Gal was also restored to levels similar to that of the parental Ad5-β-Gal virus when SIG-pHPMA was used to surface modify the Ad5-β-Gal vector in A549 and SKOV-3 cells, whilst levels of β-Gal expression were enhanced to more than 15- fold that of the parental virus in HUVECs. The observation that SIGYPLP appears to be able to mediate efficient retargeting of viral vectors but not so non-viral vectors is consistent with the major limiting factor in mediating transgene expression in HUVECs being the efficiency of cytoplasmic to nuclear transfer.

Discussion

Using small receptor-binding oligopeptides to confer tropism to gene or drug delivery agents offers considerable advantages to the use of larger targeting ligands — they can be mass produced, are less prone to denaturing, and robust to chemical incorporation into delivery constructs (Erbacher et al. 1999). They are generally less likely to stimulate cell signalling pathways following receptor binding than naturally occurring ligands, improving their suitability for use in cancer treatment, although some studies have shown activation of caspase signalling pathways following peptide/integrin interactions (Buckley et al. 1999).

Here, we evaluated the activity of a putative endothelial-cell binding peptide SIGYPLP, identified by phage display (Nicklin et al. 2000), to enable receptor-mediated transgene expression in a variety of cell lines. Simple polyplexes formed between the DNA condensing peptide SIGYPLPGGGS$(K)_{16}$ and plasmid DNA demonstrated higher levels of uptake in endothelial cells (HUVEC) than in epithelial cell lines, but conversely, much lower levels of transgene expression. Release of DNA from endosomes is unlikely to represent the major limiting factor in endothelial cells, since transfection studies performed in the presence of the endosomolytic agent chloroquine showed greatly enhanced levels of trangene expression in epithelial cells, but not in HUVECs. It is much more likely that the most significant barrier limiting transgene expression in freshly isolated endothelial cells is the difficulty of cytoplasmic to nuclear transfer of exogenous DNA. Disassembly of the nuclear membrane during the G_2-M stage of the cell cycle is known to play a major role in the levels of

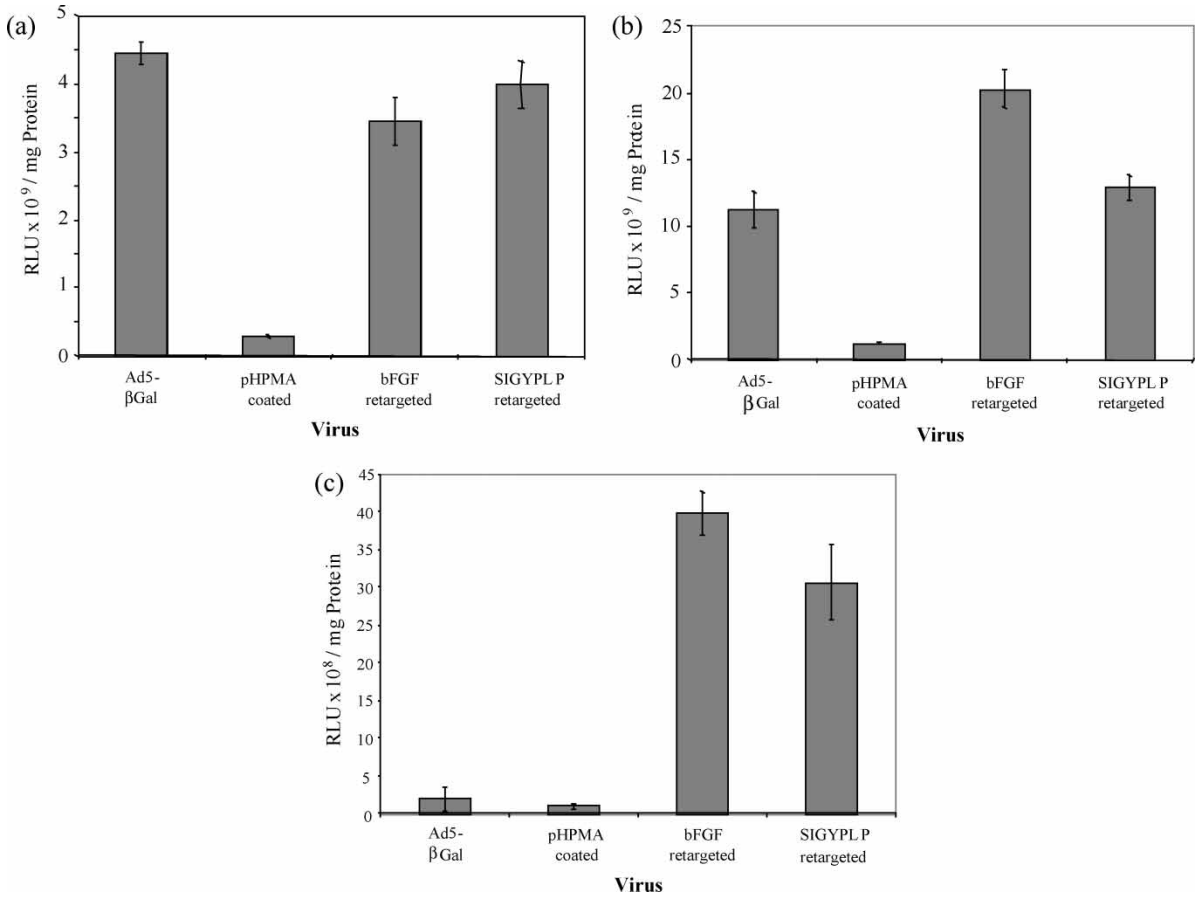

Figure 6. *Effect of surface modification and retargeting on Ad5 mediated β-gal transgene expression*. Ad5-βgal was used to infect A549 (a), SKOV-3 (b) and HUVE cells (c) (50,000 cells per well) following surface modification with pHPMA (5 mg/ml; ± 100 μg/ml bFGF for retargeting) or SIG-pHPMA. Cells were infected at a multiplicity of infection (moi) of 100, and β-gal activity was quantified 48 h post-infection.

gene expression mediated by polyplexes (Brunner et al. 2000), presumably allowing non-specific nuclear uptake of complexes that have no intrinsic mechanism for accessing the nuclear transcription machinery. Previous studies have shown that in freshly isolated HUVECs, the cells exist in an essentially quiescent state, with almost 100% of the cells existing in G_1/G_0 (Bettinger et al. 2001). Even following extensive subculturing, cell division is infrequent. By contrast, the epithelial cell lines used in this study are much more rapidly dividing, and therefore undergo frequent disassembly of the nuclear membrane. Indeed, the highest levels of transgene expression observed using simple polyplexes was in the most rapidly dividing cell line B16F10. Cell cycle analysis of B16F10 cells has shown that at any given point, greater than 65% of cells exist in G_2/M (Bettinger et al. 2001), making it extremely likely that cell division will occur in the majority of target cells over the duration of a transfection experiment, facilitating nuclear transfer of DNA.

Lateral stabilization of polyplexes (using reactive polymers based on pHPMA) improves the physico-chemical properties and enables extended plasma circulation and receptor mediated targeting (Dash et al. 2000). The stability of pHPMA coated complexes can be assessed by studying polyelectrolyte exchange reactions. Here, we demonstrate that the chemical incorporation of SIGYPLP peptide into pHPMA did not sensitize the coated polyplex to polyanion-exchange reactions, demonstrating that small peptides can be incorporated into the protective polymer for targeting purposes, without compromising the ability of the polymer confer lateral stability to the vector.

Simple non-viral systems such as those based on polycations or cationic lipids exhibit sensitivity to serum in transfection assays. Surface modification of polyplexes with pHPMA protects them from destabilization by serum proteins, but also results in charge neutralization that leads to ablation of the non-specific interaction with negatively charged cellular membranes. Resultant complexes show vastly reduced levels of transgene activity in transfection assays. Significant restoration of transgene expression was observed when complexes were surface modified with the polymer-peptide construct (compared to pHPMA alone), indicating that interaction with the cell

membrane could be restored, thought to be mediated through the binding of the SIGYPLP peptide with cell surface receptors.

Polymer coating has recently also been evaluated in the context of adenoviral vectors. Polymer modification of adenovirus extends the plasma circulation of the vector (Green et al. 2004), decreases antibody-mediated neutralization of the vector (Stallwood et al. 2000), blocks CAR-mediated cell infectivity, and permits efficient retargeting via alternative receptors following the attachment of relevant targeting ligands (Fisher et al. 2001). In this study we have investigated the activity of adenovirus following polymer modification with pHPMA and retargeting by the subsequent linkage of FGF2 (a ligand that has previous shown excellent ability for retargeting adenovirus) or by polymer modification of the virus with SIG-pHPMA. The transgene expression activity of the unmodified virus was found to be lower in HUVECs than in the epithelial cell lines tested, possibly reflecting low level expression of CAR in HUVECs. Polymer modification of the virus resulted in a uniformly reduced activity of the virus in all cell lines tested, which was restorable through the linkage of FGF2 to the coated virus in SKOV-3 cells and A549 cells, and resulted in a 20-fold increase in gene expression in HUVECs. Similarly, retargeting of the adenoviral vector with SIG-pHPMA restored levels of gene expression to that of the parental virus in SKOV-3 and A549 cells, and resulted in approximately 15-fold greater activity in HUVECs, demonstrating the possibility for selective retargeting of gene therapy vectors using polymer-peptide conjugates.

Intriguingly, whilst SIG-pHPMA could enhance adenoviral gene delivery to HUVECs, the same polymer-peptide conjugate showed no significant increase of transgene expression when used to surface modify polyplexes (data not shown). There are several possible explanations for this apparent discrepancy. For example, whilst adenovirus utilizes cellular microtubules to overcome the barrier of the intact nuclear membrane and translocate across the NPC in an energy dependent manner (Trotman et al. 2001), polyplexes have no intrinsic nuclear uptake mechanism *per se*. The poor activity of SIG-pHPMA surface modified polyplexes to mediate transgene expression in HUVECs may reflect their inability to overcome this barrier. Alternatively however, this could reflect the inability of polyplexes to release DNA following cellular uptake. Confocal studies have confirmed the ability of adenoviral DNA to dissociate from pHPMA surface modified adenoviral particles, presumably dissociating when the adenoviral coat proteins are stripped from the virus during the infection process (Fisher et al. 2001), whilst similar studies have shown that plasmid DNA appears unable to dissociate from pHPMA coated polyplexes following cellular uptake (Fisher et al. 2000).

Initial studies by Nicklin *et al.* (2000) investigated the possibility of using SIGYPLP as a targeting agent to alter adenoviral tropism and suggested that the peptide was able to mediate efficient and specific retargeting of viral tropism to HUVECs. Data presented here suggest that whilst the SIGYPLP peptide is indeed capable of mediating efficient retargeting of adenoviral vectors to HUVECs, retargeting can also occur in cell lines of a non-endothelial origin. More recent data has demonstrated that the adenovirus vector AdKO1SIG, (comprising a SIGYPLP insertion into the HI loop of adenovirus fiber knob domain, in combination with point mutations that eliminate Ad-fibre CAR binding) efficiently transduced 6 out of 10 tumor cell lines tested (Nicklin et al. 2002), including the cell line A549, in agreement with data presented here. Taken together, these data suggest that whilst expression of the receptor for SIGYPLP is may be upregulated in endothelial cells, it might not in fact be unique, highlighting the need for additional studies to elucidate the as yet unidentified cellular receptor for this peptide.

Acknowledgements

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References

Arap W, Pasqualini R, et al. 1998. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380.

Bettinger T, Carlisle RC, et al. 2001. Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells. Nucleic Acids Res 29:3882–3891.

Brunner S, Sauer T, et al. 2000. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther 7:401–407.

Buckley CD, Pilling D, et al. 1999. RGD peptides induce apoptosis by direct caspase-3 activation. Nature 397:534–539.

Carlisle RC, Read ML, et al. 1999. Self-assessing poly(L-lysine)/DNA complexes capable of integrin-mediated cellular uptake and gene expression. Colloids and Surfaces B 16:261–272.

Colin M, Harbottle RP, et al. 1998. Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells. Gene Ther 5:1488–1498.

Colin M, Moritz S, et al. 2001. The nuclear pore complex is involved in nuclear transfer of plasmid DNA condensed with an oligolysine-RGD peptide containing nuclear localisation properties. Gene Ther 8:1643–1653.

Collins L, Fabre JW. 2004. A synthetic peptide vector system for optimal gene delivery to corneal endothetium. J Gene Med 6:185–194.

Dash PR, Read ML, et al. 1999. Factors affecting blood clearance and *in vivo* distribution of polyelectrolyte complexes for gene delivery. Gene Ther 6:643–650.

Dash PR, Read ML, et al. 2000. Decreased binding to proteins and cells of polymeric gene delivery vectors surface modified with a multivalent hydrophilic polymer and retargeting through attachment of transferrin. J Biol Chem 275:3793–3802.

Erbacher P, Remy JS, et al. 1999. Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. Gene Ther 6:138–145.

Fisher KD, Ulbrich K, et al. 2000. A versatile system for receptor-mediated gene delivery permits increased entry of DNA into target cells, enhanced delivery to the nucleus and elevated rates of transgene expression. Gene Ther 7:1337–1343.

Fisher KD, Stallwood Y, et al. 2001. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 8:341–348.

Godbout R, Ingram R, et al. 1986. Multiple regulatory elements in the intergenic region between the alpha-fetoprotein and albumin genes. Mol Cell Biol 6:477–487.

Green NK, Herbert CW, et al. 2004. Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. Gene Ther 11:1256–1263.

Nemerow GR. 2000. Cell receptors involved in adenovirus entry. Virology 274:1–4.

Nicklin SA, Seggern DJV, et al. 2002. Selective transduction of certain human tumour cells using genetically retargeted adenoviruses. Mol Ther 5:S209.

Nicklin SA, White SJ, et al. 2000. Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display. Circulation 102:231–237.

Nishi Y. 2003. Enzyme/abzyme prodrug activation systems: Potential use in clinical oncology. Curr Pharm Design 9:2113–2130.

Oupicky D, Ogris M, et al. 2002. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. Mol Ther 5:463–472.

Parker AL, Oupicky D, et al. 2002. Methodologies for monitoring nanoparticle formation by self-assembly of DNA with poly(L-Lysine). Anal Biochem 302:75–80.

Schrewe H, Thompson J, et al. 1990. Cloning of the complete gene for carcinoembryonic antigen: analysis of its promoter indicates a region conveying cell type-specific expression. Mol Cell Biol 10:2738–2748.

Stallwood Y, Fisher KD, et al. 2000. Neutralisation of adenovirus infectivity by ascitic fluid from ovarian cancer patients. Gene Ther 7:637–643.

Trotman LC, Mosberger N, et al. 2001. Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. Nat Cell Biol 3:1092–1100.

Zarovni N, Monaco L, et al. 2004. Inhibition of tumor growth by intramuscular injection of cDNA encoding tumor necrosis factor alpha coupled to NGR and RGD tumor-homing peptides. Human Gene Ther 15:373–382.