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Research paper

Nucleic acid delivery to differentiated retinal pigment epithelial cells using cell-penetrating peptide as a carrier

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ABSTRACT

Nucleic acid delivery to the eye is a promising treatment strategy for many retinal disorders. In this manuscript, retinal gene delivery with non-coated and chondroitin sulphate (CS) coated amphipathic and cationic peptides was tested. The transfection and gene knockdown efficiencies were evaluated in different retinal pigment epithelial (RPE) cell models including both dividing and differentiated cells. In addition, the mobility of peptide-based gene delivery systems was examined in porcine vitreous by particle tracking analysis. The results indicate that amphipathic and cationic peptides are safe *in vitro* and are capable of high transgene expression and gene knockdown in dividing cells. We further demonstrate that incorporation of CS improves the efficiency of gene delivery of peptide-based systems. Most importantly, the transgene expression mediated by both non-coated and CS coated peptides was high in differentiated as well as in human primary RPE cells which are typically difficult to transfect. Coating of peptide-based gene delivery systems with CS improved diffusion in the vitreous and enhanced the stability of the polyplexes. The results indicate that a peptide-based system can be fine-tuned as a promising approach for retinal gene delivery.

1. Introduction

Retinal diseases such as age-related macular degeneration, diabetic retinopathy, glaucoma, and retinitis pigmentosa are the leading causes of blindness in the world [1]. The number of patients suffering from retinal diseases is increasing due to the aging population. Retinal diseases are mostly incurable and there is an urgent need for new mode of therapies.

Gene therapy represents one promising approach for the treatment of retinal diseases especially rare, inherited, retinal diseases. The eye can be considered as a prime target for gene therapy since it is a relatively isolated and immune privileged organ. Gene therapy has great medical potential. However, the efficiency and safety of the cargo (DNA or RNA) and its delivery to the back of the eye remain a limiting factor [2–4]. Although viral vectors can deliver genes efficiently, there are problems related to their safety and large-scale production [5]. In contrast, non-viral vectors, such as cationic polymers and lipids, are safer than viral vectors but exhibit low transfection efficiency [6]. Therefore, safer and more efficient gene delivery systems are needed.

Cell penetrating peptides are one potential class of non-viral gene delivery systems; these can be either cationic or amphipathic in nature. These peptides have cell penetrating segments and they have been used to deliver small molecules, antibodies and nucleic acids into cells [6–9]. Furthermore, these peptides are biocompatible, have a large cargo carrying capacity, low toxicity and immunogenicity and can be modified to target specific cells [11,12]. Amphipathic peptides contain arginines to allow for proper DNA condensation, histidines for triggering endosomal escape, cysteines for improved stability and hydrophobic residues which improve their interactions with cell membranes [13]. Cysteine modified amphipathic peptides have been demonstrated to be non-toxic and have a high gene delivery efficiency in multiple cell lines such as in the Chinese hamster ovary cell line (CHO-K1), a breast cancer cell line (MCF-7), a pulmonary epithelial cell line (A549) and a melanoma cell line (WM-2661) [14]. Cationic arginine homopeptides and arginine containing peptides modified with histidine and cysteine residues have been found to possess minimal toxicity as well as an

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efficient gene delivery capability in a wide range of cell lines e.g. CHO-K1, MCF-7 and others [10,15]. There have been only a limited number of studies describing the application of peptide-mediated delivery to the retina [16–17]. Intravitreal injection is the most important mode of administration used for the treatment of retinal diseases. In intravitreal injection, the therapeutic agent is administered into vitreous inside the eye, from where it needs to diffuse through vitreous towards the retina. The vitreous is a transparent, viscous meshwork consisting mostly of hyaluronic acid and collagen polymers [18]. The vitreous retards the mobility of nanoparticles depending on their size, shape and surface properties [19,21]. The mobility of nanoparticles can be improved by masking the positive surface with hydrophilic molecules such as polyethylene glycol [22,23]. A hyaluronic acid (HA) coating has also been shown to enhance the mobility of nanoparticles across the vitreous [24,25] and this approach has been investigated as a means for retinal gene delivery [24,26,27].

Chondroitin sulphate (CS) is a negatively charged natural glycosaminoglycan present in the extracellular matrix. Similar to HA, CS is also known to bind to the cell surface CD44 receptor and to internalize cargo through receptor-mediated endocytosis [28]. The CS coating has also been reported to increase the cellular uptake and transfection efficiency in multiple cell lines [4,29]. Thus, CS may be a good candidate for improving the mobility of positively charged gene delivery systems in vitreous. However, it has not been clarified how a CS coating can alter the movement of particles in vitreous and if this offer advantages in ocular delivery.

In the present study, CS coated and non-coated polyplexes composed of the cationic and amphipathic peptides were characterized and their properties tested for retinal gene delivery. The delivery efficiency of plasmid DNA (pDNA) and siRNA of the peptide-based systems was also evaluated in dividing and differentiated human retinal pigment epithelial (RPE) cells i.e. both secondary and primary cells. Furthermore, their cell toxicity was also evaluated. The intravitreal mobility of the uncoated and CS coated polyplexes was also compared by exploiting a particle tracking based model to evaluate the efficacy of this peptide-based system *ex vivo*.

2. Materials and methods

All chemicals and cell culture media were purchased from Sigma, unless mentioned otherwise.

2.1. Preparation of polyplexes

2.1.1. Peptides

Peptides used in the study were custom synthesized (> 95% purity) from GL Biochem (Shanghai) Ltd. The peptides were dissolved in de-ionized water at a concentration of 10 mg/ml and stored in small aliquots at −80 °C to avoid repeated freeze-thawing. Information about the peptides' characteristics and sequences is given below (Table 1).

2.1.2. Plasmid DNA (pDNA)

Cytomegalovirus (CMV) promoter driven Gaussia luciferase (pFB-Gluc) and green fluorescent protein (pEGFP-C2, Clontech) reporter plasmids were used to assess the efficiency of the peptide carriers. The pFB-Gluc plasmid was a kind gift from Dr. Robert Kotin (University of Massachusetts Medical School, USA). The plasmids were amplified in *Escherichia coli*. Plasmid isolation and purification were done with Qiagen Plasmid Mega kit according to the instructions of the manufacturer. For the evaluations of DNA condensation, release and cytotoxicity we used the pMIR plasmid. pMIR (6.4 kb) (Clontech Laboratories) was amplified in *Escherichia coli* DH5α strain and isolated using GenElute HP Endotoxin-Free Plasmid MaxiPrep kit (Sigma). Plasmid integrity was confirmed by agarose gel electrophoresis. The concentration of pDNA was determined by its absorbance at 260 nm. The purity of plasmid was checked by the ratio of 260/280.

Table 1
Peptide sequences.

Peptide	Nature	Sequence	References
CRHC	Cationic	CRRRRRHHHHHHHHRRRRC	[13]
MGPE-9	Amphipathic	CRRLRHLRHHYRRRWHRFRC	[10]

2.1.3. pDNA-peptide polyplexes

Peptide-pDNA complexes (subsequently will be termed simply as polyplexes) were prepared at different charge ratios (0.1, 0.5, 1, 2, 5 and 10) expressed as peptide nitrogen per nucleic acid phosphate (N/P). In most studies an N/P charge ratio of 10 was used since this ratio had shown the highest transfection efficiency in an earlier study [13]. Briefly, the pDNA stock was diluted to a concentration of 20–40 ng/μl in de-ionized water and added drop-wise to an equal volume of the appropriate peptide dilution while vortexing. The polyplexes were incubated for 30 min or 1 h at room temperature. For coating with CS, CS at 0.25 w/w of peptides was added to the polyplexes and incubated for 30 mins before performing any experiments.

Lipofectamine 2000™ transfection reagent (Thermo Fisher Scientific) was used as a positive control and lipoplexes with pDNA were prepared according to the manufacturer's instructions. Briefly, lipofectamine 2000 was gently mixed before use and 8 μl of lipofectamine solution was diluted with 92 μl of OPTI-MEM serum free medium and incubated for 5 min. Subsequently, pDNA (2 μg pDNA/well in 100 μl) was mixed gently with the lipofectamine solution and incubated for 20 mins before performing the experiment.

2.1.4. siRNA-peptide polyplexes

GAPDH siRNA (20 μM working solution) was diluted in nuclease-free water (AM9937, Thermo Fisher Scientific) to obtain a final 50 nM siRNA concentration for each well. DharmaFECT4 transfection reagent (T-2004–01, Dharmacon) was used as a positive control. The peptides diluted in nuclease-free water were mixed with siRNA at N/P ratios of 10 and 30. The complexes were incubated at room temperature for 30 mins. The coating was achieved by adding, CS at a concentration of 0.25 (w/w of peptide) to the complexes and which were then incubated for a further 30 mins.

2.2. Gel electrophoresis

pDNA-peptide polyplexes were prepared at different charge ratios (N/P) ranging from 0.1 to 5.0. Polyplexes (20 μl) containing 20 ng of pDNA were loaded onto 1% agarose gel containing ethidium bromide (0.5 μg/ml). Electrophoresis was carried out at 100 V in Tris acetate-EDTA buffer for 30 mins and the binding efficiency of peptides with the pDNA was checked by agarose gel electrophoresis through a Gel Doc-imaging system.

In the DNA release assay the polyplexes formed at N/P ratio of 5.0 were treated for 30 min with increasing amounts of an anionic agent, heparin (H3149-100KU), at heparin/peptide ratio of (wt/wt) 1:1, 5:1, 10:1, 20:1 and 50:1 and analyzed on 1% agarose gel as described above. The pDNA released from the polyplexes was analyzed and the stability of the complexes was checked as a measure of the pDNA released in agarose gel electrophoresis by the Gel Doc imaging system.

2.3. Dynamic light scattering (DLS)

The mean hydrodynamic diameter and zeta potential of the polyplexes were measured at +25 °C by using a dynamic light scattering device (Malvern, Zeta sizer Nano ZS90) at a fixed angle of 90°. Uncoated and CS coated polyplexes were prepared at an N/P ratio of 10 at a pDNA concentration of 40 ng/μl in deionized water. The mean hydrodynamic diameter and zeta potential were measured after 1 h

incubation of the polyplexes. A minimum of three readings was recorded for each sample.

2.4. Atomic force microscopy (AFM)

The polyplexes at a charge ratio of +10 with or without CS coating were imaged by depositing 2 µl of the polyplex solution on freshly cleaved mica and drying it in air. Imaging was done with a 5500 scanning probe microscope (Agilent Technologies, Inc., AZ) using PicoView software. Images were obtained in the AAC mode in air with 225 µm long silicon cantilevers (Agilent Technologies, Inc.) that have a resonance frequency of around 75 kHz and a force constant of 2.8 N/m. The scan speed used was 1 line/s. The minimum image processing (first order flattening and brightness contrast) was employed. Image analysis was performed using PicoImage software.

2.5. Cell culture

2.5.1. ARPE-19 cells

ARPE-19 cells are human RPE cells; they were purchased from ATCC (Manassas VA, USA). Cells were cultured in a growth medium composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin (100 U/ml) and streptomycin (100 mg/ml) as antibiotics. Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

The experiments were performed either with dividing (=undifferentiated) or differentiated (=post-mitotic) cells. The dividing cells were seeded on 24 well plates at the desired cell density (21,000 cells/ cm^2) and cultured in growth medium as described above for 24 h before the experiment. To achieve differentiation ARPE-19 cells were seeded on a 12-well Transwell permeable support (Costar 3460) at 160 000 cells/cm^2 cell density. Cells were cultured for one month and in growth medium containing only 1% FBS; this was otherwise the same as described above, but the medium changed twice a week.

2.5.2. hfRPE cells

hfRPE cells are human fetal primary RPE cells (HRPEpiC cell, #6540, lot 12612) which were purchased from ScienCell Research Laboratory (San Diego, CA, USA). The cells were cultured in EpiCM medium (ScienCell Research Laboratory, San Diego, CA, USA) supplemented with 2% FBS, 1% epithelial cell growth supplements and 1% penicillin/streptomycin solution. The cells were expanded and at passage 3 they were seeded on 12 well tissue culture plate at a cell density of 200 000 cells/cm^2 [30] hfRPE cells were differentiated for one month at 37 °C in a 5% CO_2 atmosphere before performing any experiments. Cell growth medium was changed three times a week.

2.6. Transfection assay

Transfection studies were performed in dividing and differentiated ARPE-19 and differentiated hfRPE cells. Gaussia and GFP pDNAs were used to assess the reporter gene activity. Polyplexes at charge ratio +10 and lipofectamine containing lipoplexes (2 µg DNA/well in 100 µl) were added to the ARPE-19 cells in serum free culture media. After 4 h incubation at 37 °C, the cells were replenished with 500 µl complete growth medium.

2.6.1. Gaussia luciferase

The amount of secreted luciferase reporter protein was determined from the culture medium which had been bathing the transfected cells. The samples of 200 µl were collected at various time points after complex removal and stored at −20 °C. In the experiments with dividing ARPE-19 cells and differentiated hfRPE cells the selected time points were 24, 48 and 72 h and in the experiments with differentiated ARPE-19 cells the samples were collected from apical and basolateral sites at a 1, 2, 3, 6, 8, 10 days after exposure to the complex. At the end of the experiment, the cells were lysed in 50 µl of lysis buffer and luciferase activity and protein amount (Bio-Rad Protein Assay) were determined from 20 µl and 2 µl of lysate, respectively. The amount of expressed transgene was quantified with a Victor^2 1420 multilabel counter (Wallac, Finland). The recorded luminescence activity was normalized to the amount of protein in each sample by using the Bradford protein assay (Bio-Rad) with bovine serum albumin (Sigma-Aldrich, Germany) as a standard.

2.6.2. GFP transgene expression

To assess GFP reporter expression, the cells were seeded and grown on eight well micro chamber slides (Ibidi, USA) as described above. After pre-determined time intervals ARPE-19 cells were incubated with polyplexes (50 µl) in serum free medium (150 µl) for 4 h. After medium replacement, the cells were grown for another 48 h. Subsequently, cells were fixed with 4% formaldehyde for 10 mins. The samples were washed with PBS and then treated with 0.1% Triton-X (5 mins) for permeabilization of the membrane. Then, the whole construct was washed with PBS and treated with 1% bovine serum albumin (BSA) for 20 mins. Finally, the cell nuclei were stained with DAPI (5 µg/ml) for 15 mins, GFP reporter gene expression was observed in a confocal microscope (Zeiss LSM 800 Airyscan, Carl Zeiss).

2.7. Gene silencing

The efficiency of peptides for siRNA delivery was also evaluated in dividing and differentiated ARPE-19 cells by measuring the knockdown of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with qPCR. GAPDH siRNA and scrambled siRNA sequences are shown in table 2. SiRNA-polyplexes were added to the wells and to the apical side of permeable supports (50 nM siRNA/well) in serum-free medium. After 24 h transfection, the cells were washed with PBS (with Ca and Mg, Sigma-Aldrich, D8662) and fresh complete growth medium was added. Forty-eighth hours after transfection, the cell culture medium was removed and TRIzol reagent (ThermoFisher, 15596018) was added to the cells, and total RNA was isolated. The total RNA concentration was determined with a spectrophotometer (DeNovix Inc., Wilmington, USA). cDNA was synthesized using RevertAid First strand cDNA synthesis kit (ThermoFisher, K1621). Quantification of GAPDH mRNA was performed with LightCycler 480 SYBR Green I Master (Roche, 04 887 352 001). The primers were used at a concentration of 0.5 µM with 4 ng of cDNA used in the final 10 µl reaction volume. Quantitative PCR was performed on a Light Cycler® 480 Instrument (Roche Molecular Systems Inc., Pleasanton, USA). Sequence-specific amplification of cDNAs was verified by performing melting-point analysis. GAPDH mRNA expression was normalized to endogenous β-actin.

2.8. Mobility study of polyplexes in vitreous

Fresh porcine eyes were obtained from a slaughterhouse (HKScan, Forssa, Finland) and covered with ice during transportation. After transportation, the extraocular tissues were removed and the eyes were quickly dipped in 70% ethanol and placed in 10x PBS and stored at 4 °C

Table 2
Used siRNA primer sequences.

siRNA/primer	Sequence
siRNA GAPDH	SS: 5′-GGUCAUCCAUGACAACUUU[dT][dT]-3′ AS: 5′-AAAGUUGUCAUGGAUGACC[dT][dT]-3′
siRNA scramble	SS: 5′-UUCUCCGAACGUGUCACGUTT-3′ AS: 5′-ACGUGACACGUUCGGAGAATT-3′
Human GAPDH primers	F: 5′-GTCAGCCGCATCTTCTTTTG-3′ R: 5′-GCGCCCAATACGACCAAATC-3′
Human b-actin primers	F: 5′-AGAGCTACGAGCTGCCTGAC-3′ R: 5′-AGCACTGTGTTGGCGTACAG-3′

prior to the diffusion experiments. The eyes were used within 30 h after sacrificing the animal. For the intravitreal injections the anterior part of the eye was cut and removed. Briefly, a short incision was made into the sclera with a scalpel blade 2 mm away from iris. The anterior section was removed by cutting with the scissor circumferentially around the eye. The anterior part of the eye ball was carefully lifted off with tweezers making sure that the lens was removed. The eyeball was then placed on a petri-dish well with the anterior part facing up. The intravitreal injection was done with a 30 G BD Micro-Fine TM + insulin syringe (BD, Franklin Lakes, NJ, USA). In each eyeball 50 µl of FITC tagged peptide-untagged pDNA complexes coated and uncoated with CS were injected in to the vitreous body at a depth of 0.5 cm. The injected eyes were then carefully placed vitreous facing down into 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA, USA) making sure that no air bubbles were trapped between the vitreous gel and the glass. Super glue was used to fix the edge of the eyecup to the dish. The prepared vitreous eye sample with the injected polyplexes was incubated at 25 °C for 15 min prior to microscopy.

Movement and distribution of CS coated and uncoated polyplexes composed of FITC tagged peptide-untagged DNA (GL Biochem Ltd. Shanghai) were followed and recorded by multiple particle tracking with an Andor Neo sCMOS camera mounted on a spinning disk confocal microscope (Marianas, Intelligent Imaging Innovation (3i), Colorado, USA) at 488 nm -excitation wavelength. High-resolution 15 sec movies at 150 ms temporal resolution were taken. Movies were taken using Slidebook®Software V.6 (Intelligent Imaging Innovation (3i), Colorado, USA). Visualization was done at a depth of 0.15–0.45 cm from the cut surface. Microscopy was performed at 37 °C and the samples were kept at 37 °C for 20 mins prior to visualization. Particles were visualized with a 63X objective. Movies were analyzed with Imaris software (Bitplane AG, Zurich, Switzerland) to gather time-dependent positional data. The mean square displacement ($< MSD >$) and effective diffusivity (D_{eff}) were calculated as previously described [20].

3. Results

3.1. Physicochemical characterization and stability of coated and un-coated peptide-pDNA complexes

Gel electrophoresis assay was carried out to determine the binding and release of pDNA as a function of the charge ratio (Fig. 1). The cationic peptide showed efficient retardation of the electrophoretic mobility of pDNA from the charge ratio +0.5 (Fig. 1a). The amphipathic peptide showed complete binding and retardation of pDNA at the charge ratio of +2 (Fig. 1c). Coating of polyplexes with exogenous CS did not change the electrophoretic mobility profile of pDNA (Fig. 1 b and d).

Release of pDNA: The extracellular and intracellular spaces are enriched in variety of negatively charged macromolecules such as glycosaminoglycans that may affect the stability of the positively charged polyplexes [10,31] In order to examine the stability of the peptide based polyplexes *in vitro*, we have challenged them with a model anionic agent, heparin (Fig. 2). Exposure to the lowest concentration of heparin resulted in complete release of pDNA from non-coated peptide polyplexes (Fig. 2a and c). The addition of CS provided slightly better protection to the compacted pDNA from heparin since only partial release of pDNA was detected (Fig. 2b). The observations from the gel condensation and release are similar to those observed earlier, although different plasmids were used in this study [10,13,44].

3.1.1. Morphology of the peptide-pDNA polyplexes

The morphology of the polyplexes with and without exogenous CS was studied by AFM (Fig. 3) and the hydrodynamic diameters and surface charge were evaluated DLS (Table 3). The native non-coated pDNA containing polyplexes exhibited a monodisperse population of particles (Fig. 3a and c) with a mean diameter of about 50 to 65 nm (Table 3). Addition of a low amount of CS, resulted in the formation of more polydisperse polyplexes (Fig. 3b and d) which were slightly larger in size 161 nm and 133 nm for cationic and amphipathic complexes, respectively (Table 3). It is notable that peptides formed approximately three times smaller complexes with pDNA than with siRNA. The zeta potential of the non-coated pDNA containing polyplexes was highly positive and decreased slightly upon addition of small amounts of CS, although it remained positive (Table 3).

3.2. Gene and siRNA delivery efficacy of coated and non-coated peptide based systems

3.2.1. pDNA delivery

The transfection efficiency of the polyplexes was determined in dividing and differentiated ARPE-19 and differentiated hfRPE cells by using secreted Gaussia luciferase (Fig. 4) and GFP (Fig. 5) as reporter genes. Experiments with Gaussia luciferase reporter gene clearly showed that cationic peptides achieved 10–100 fold greater transgene

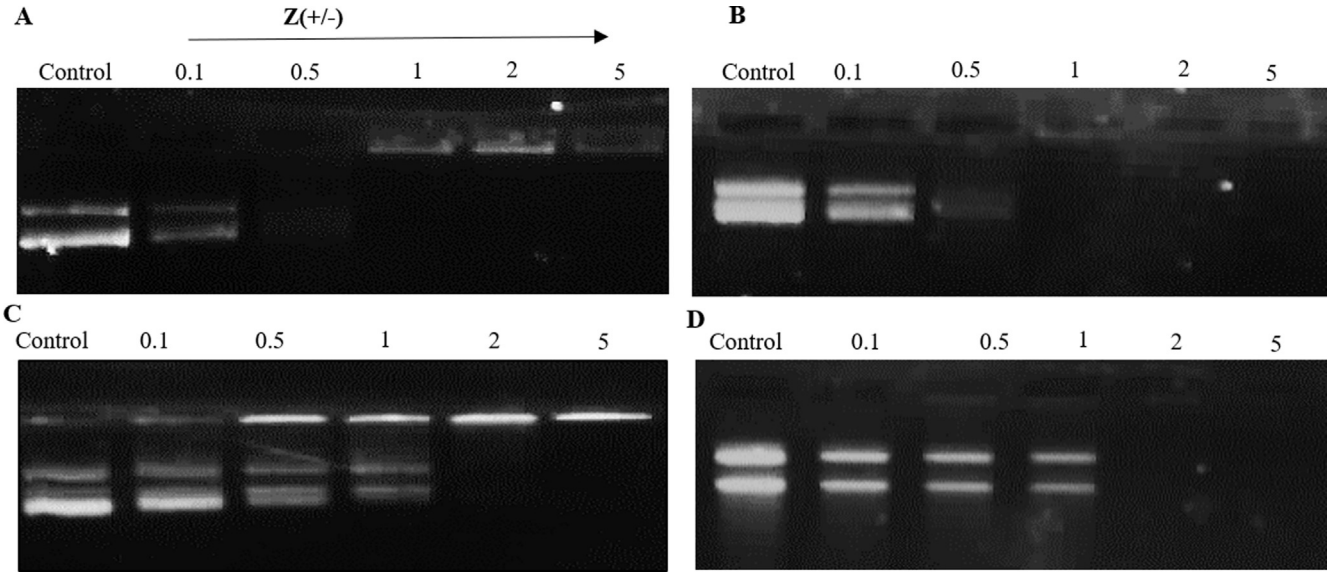

Fig. 1. Gel electrophoresis of the uncoated cationic (A), CS coated cationic (B), uncoated amphipathic (C), and CS coated amphipathic polyplexes (D). Lane 1: only DNA (control), Lanes 2–6 showing polyplexes formed at different charge ratio (0.1 0.5, 1.0, 2.0, 5.0).

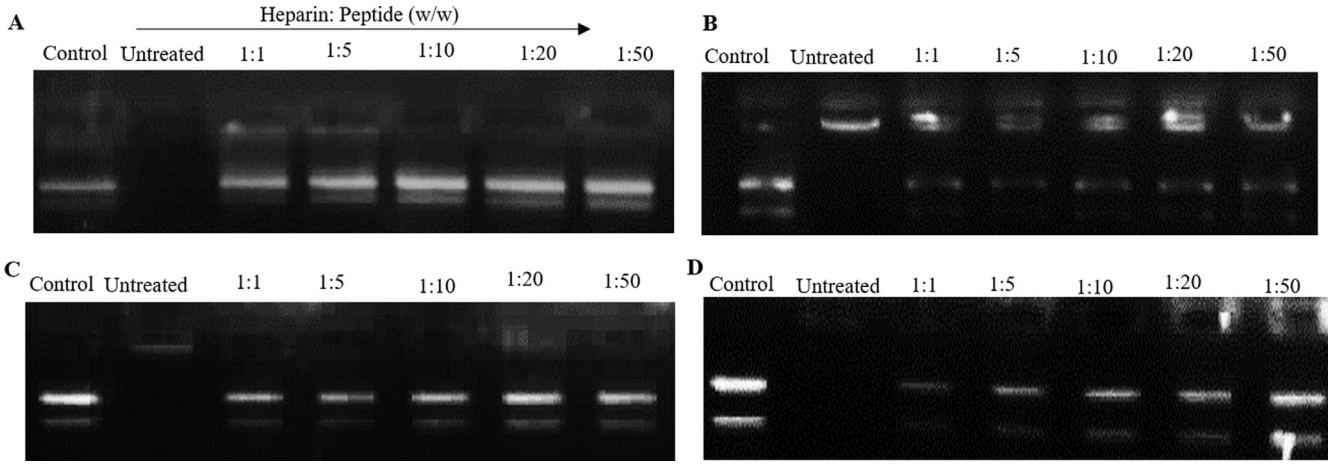

Fig. 2. Gel based DNA release assay showing relative stability of uncoated cationic (A), CS coated cationic (B) , uncoated amphipathic (C) and CS coated amphipathic (D). Polyplexes, at Z(±) 5.0, are resistant to anionic challenge (heparin) after coating with low amounts of CS (0.25 w/w). In panels A-D, lane 1 (control) is uncomplexed pDNA, lane 2 is the respective untreated polyplex at the charge ratio of 5.0 and lanes 3 to 7 are the polyplexes treated with heparin with different wt/wt ratios as indicated in the figure.

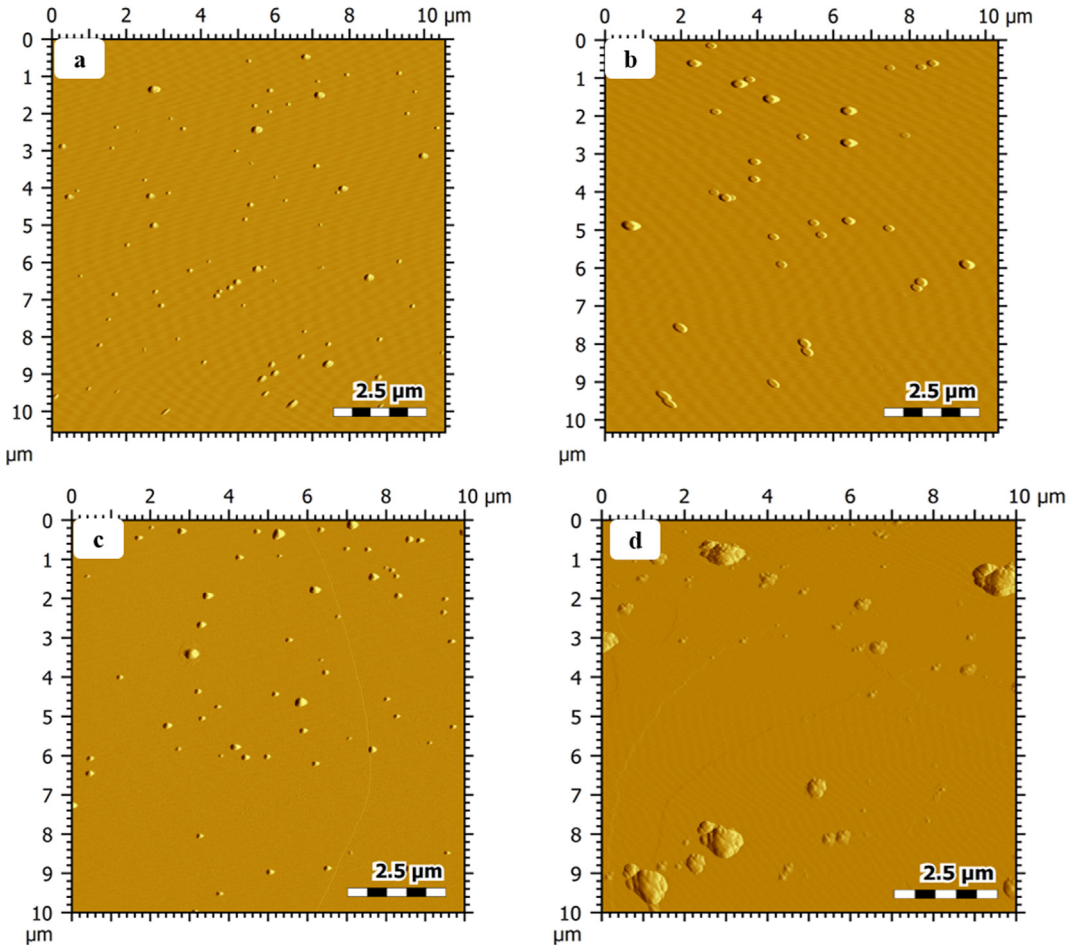

Fig. 3. Effect of soluble CS on morphology of peptide-DNA polyplexes at Z (+/)10.0, Polyplexes were coated with CS (0.25 w/w, CS: peptide). Uncoated amphipathic peptide-DNA complexes (a) and CS-coated amphipathic peptide-DNA complexes (b). Uncoated cationic peptide-DNA complexes (c) and CS-coated cationic peptide-DNA complexes (d). 2 µl of the resulting polyplex was deposited on mica, air-dried and imaged using an Atomic Force Microscope. Each image has dimensions of 4x4 microns. Scale bar represents 2.5 µm.

expression than amphipathic peptides in all studied cell types. CS coating of the peptide based polyplexes improved transfection efficiency in both dividing ARPE-19 cells by 10 times and by five times in the corresponding differentiated cells (Fig. 4A and C). The same trend in transgene expression was also confirmed in experiments with the GFP reporter gene (Fig. 5). All polyplexes were well tolerated *in vitro* and showed over 70% cell viability in dividing ARPE-19 cells (Supplementary Fig. 2).

Table 3
Size and zeta potential of CS coated and uncoated peptide-DNA and size of peptide-siRNA complexes.

Carrier	Size (nm), + pDNA	Zeta-Potential (mV), + pDNA	Size (nm), + siRNA
Cationic peptide	57.30 ± 4	24 ± 7	184,0 ± 68
Cationic peptide + CS	161.5 ± 3	19 ± 0.8	151,0 ± 40
Amphipathic peptide	50.63 ± 0.4	24 ± 2	173.9 ± 67
Amphipathic peptide + CS	133 ± 2.0	20 ± 4	156.1 ± 29

The effect of cell differentiation and cell type (primary vs. secondary) on transgene expression was also evaluated. Upon differentiation both ARPE-19 and hfRPE cells expressed the tight junction protein, occludin, this was localized on the plasma membrane (supplementary Fig. 1). As expected occludin expression was more prominent in primary hfRPE cells than in secondary ARPE-19 cells (supplementary Fig. 1). The commercially available transfection agent, lipofectamine, was used as a control in our studies. In dividing ARPE-19 cells lipofectamine resulted in equal or lower (3–40 fold) transgene expression than obtained with the peptide based systems (Fig. 4A). In fact, in both differentiated RPE cell models the transfection efficiency of lipofectamine was reduced by 1000 fold and was close to the limit of detection (Fig. 4B-D). Interestingly, peptide-based gene delivery systems showed relatively high transgene expression even in differentiated ARPE-19 cells. The transgene expression of non-coated and coated amphipathic peptides was 10 fold lower in differentiated than in dividing ARPE-19 cells (Fig. 4A and B) and was further decreased by 10 fold in differentiated hfRPE cells (Fig. 4B) while CS coated cationic peptide polyplexes, retained their delivery efficiency and showed almost equal transgene expression in all cell types. The above results were further supported by studies with the GFP reporter gene (Fig. 5).

The kinetics of the transgene expression showed already relatively high transgene expression at 24 h after removal of polyplexes; this was retained at the same level for at least up to 72 h. There was no difference in the kinetic profile of transgene expression between the dividing and non-dividing ARPE-19 cells. In differentiated ARPE-19 cells the secretion of luciferase reporter protein occurred mainly from the apical site during the first 3 days after transfection. On day 6, luciferase was secreted as efficiently from the apical and basal compartments but on day 8, the secretion was at least 10 fold higher from basolateral site. After 10 days, the secretion of luciferase reporter protein was very low in both apical and basolateral compartments.

3.2.2. siRNA delivery

To investigate the suitability of peptide-based systems to deliver siRNA, we knocked down the expression of the GAPDH house-keeping gene with siRNA-peptide complexes in dividing and differentiated ARPE-19 cells. Fig. 6 shows extent of the knockdown in dividing cells. CS coated cationic complexes (CRHC-CS) at N/P ratio 30 achieved the best silencing effect i.e. 80% knockdown, followed by CS coated amphipathic peptide complexes (M9-CS, N/P 30) with 70% knockdown. The gene silencing ability of peptide complexes was, however, lost in differentiated cells, and we could not detect any knockdown in differentiated ARPE-19 cells (data not shown).

3.3. Mobility of pDNA containing polyplexes in vitreous

The mobility of CS coated and uncoated amphipathic and cationic peptide pDNA containing polyplexes was studied in porcine vitreous. The mobility studies in porcine vitreous indicated that CS coating slightly improved the mobility of polyplexes in vitreous (Table 4). CS coating improved the vitreal mobility of cationic polyplexes by 1.6 fold and amphipathic polyplexes by 1.3 fold. Interestingly, in water the uncoated cationic and amphipathic polyplexes moved about 2.7 times

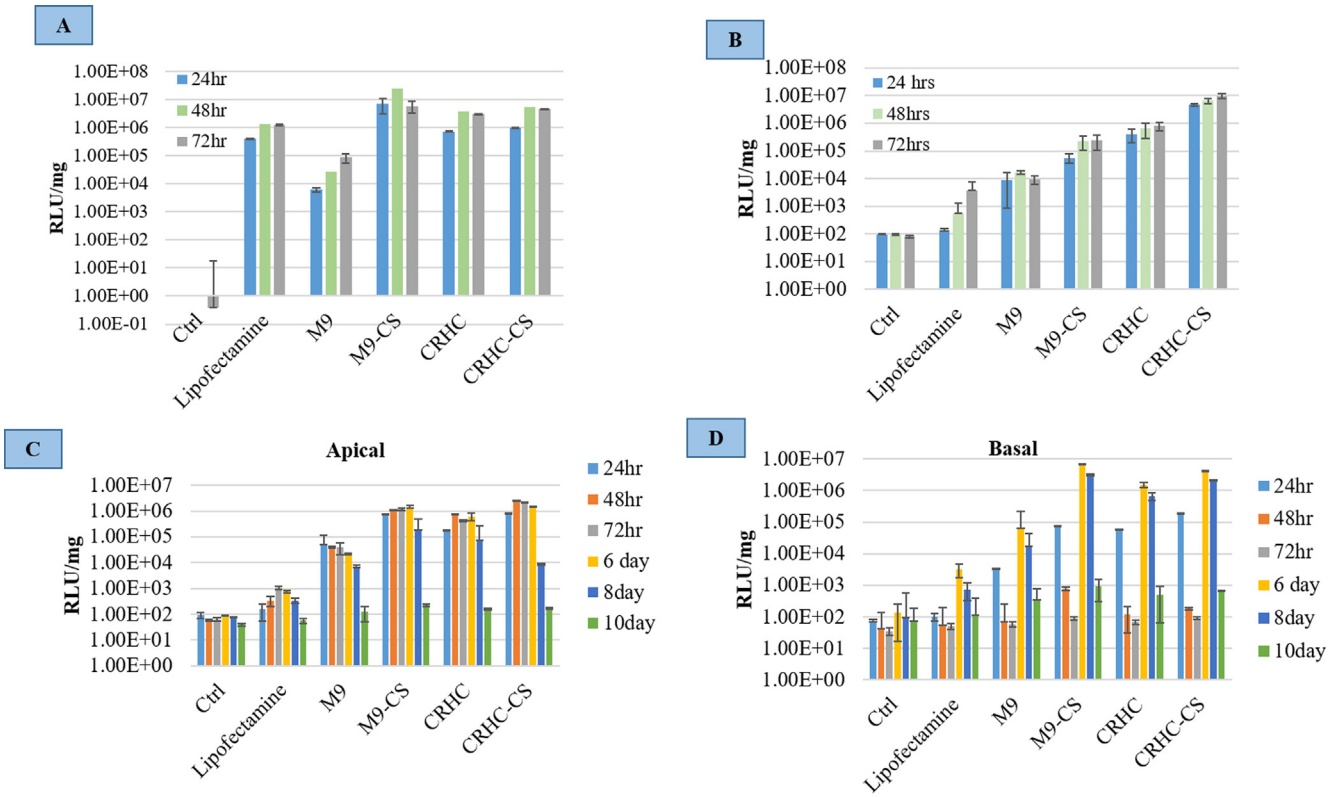

Fig. 4. Gaussia Luciferase expression in dividing ARPE cell line (A), primary hfRPE cell (B), differentiated ARPE cells in the apical (C), and basal compartments (D). M9 represent amphipathic peptide complexes, M9-CS represents CS coated amphipathic peptide complexes, CRHC represents cationic peptide complexes and CRHC-CS represent CS coated cationic complexes. Control (Ctrl) represents cells without any treatment. The data is shown as mean ± SD (n = 3).

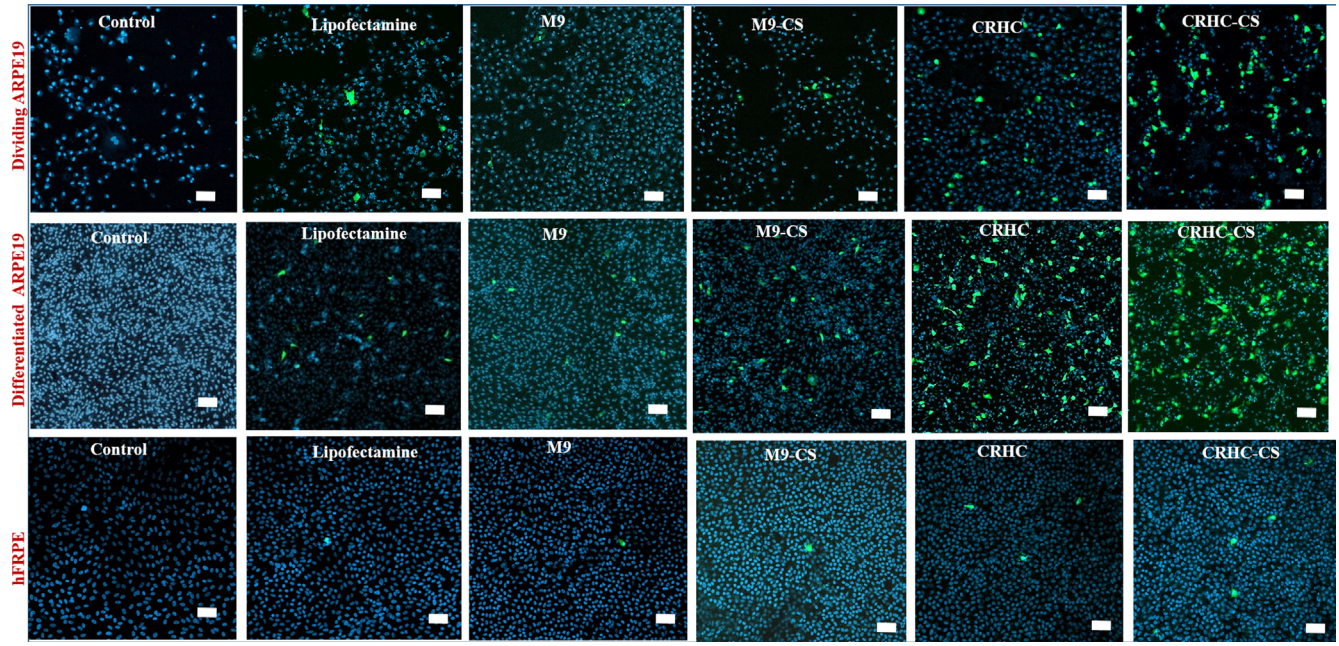

Fig. 5. Confocal microscopy image shows dividing ARPE cells (A), differentiated ARPE cells (B), primary hfRPE cells (C), transfected with polyplexes (pEGFP-amphipathic and pEGFP- cationic complex) at a charge ratio of 10 after 48 h. Scale bar represents 100 µm. Green color refers to pEGFP reporter protein expression and blue color is (DAPI) nuclear staining. M9 represent the amphipathic peptide complexes, M9-CS represents CS coated amphipathic peptide complexes, CRHC represents cationic peptide complexes and CS-CRHC represent CS coated cationic complexes.

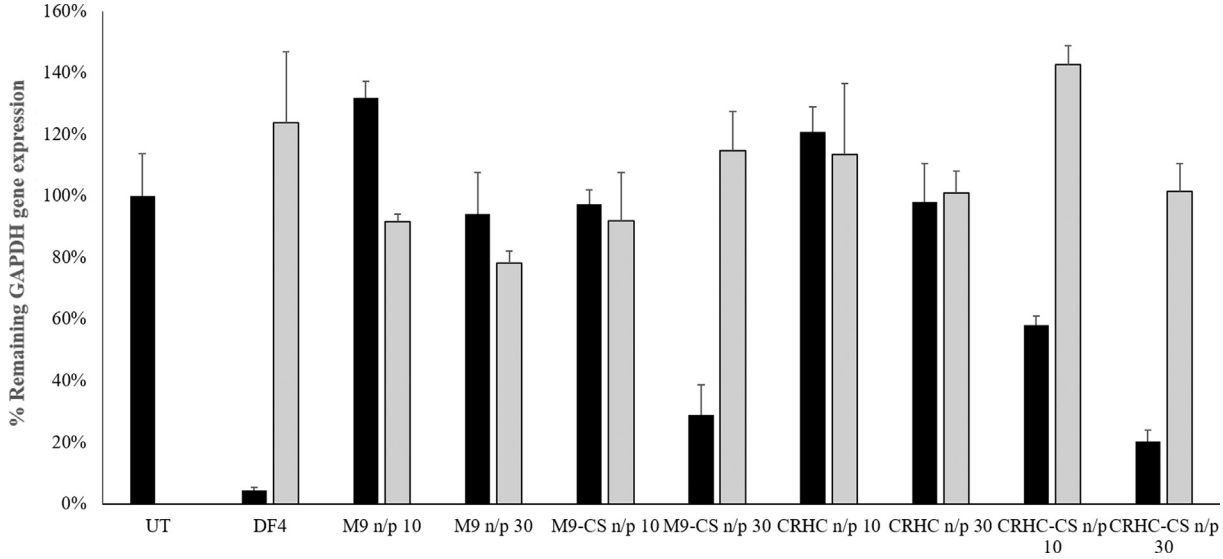

Fig. 6. GAPDH knockdown with siRNA-peptide complexes N/P-ratio 10 and 30) in dividing ARPE cells. Cells were transfected with 50 nM GAPDH siRNA (black bars). A scrambled siRNA (grey bars) served as control. GAPDH mRNA expression was evaluated with qPCR and normalized to endogenous beta-actin. M9 represents the amphipathic peptide complexes, M9-CS represents CS coated amphipathic peptide complexes, CRHC represents cationic peptide complexes and CRHC-CS represents CS coated cationic complexes. DharmaFECT4 (DF4) was used as positive control. The data is shown as mean ± SD (n = 3).

Table 4
The diffusion coefficient of CS coated and uncoated cationic and amphipathic peptide pDNA complexes. Dv refers to the diffusion coefficient in the vitreous calculated at a time scale of 1 sec. Dw is the theoretical diffusion coefficient in water calculated from the Stokes-Einstein equation (n = 3).

pDNA containing polyplexes	D_v ($\mu m^2/s$)	D_w ($\mu m^2/s$)	D_w/D_v
Cationic peptide	0.119 ± 0.09	8.6	72
Cationic peptide -CS	0.194 ± 0.06	3.0	15
Amphipathic peptide	0.166 ± 0.14	9.8	59
Amphipathic peptide -CS	0.22 ± 0.12	3.7	16

faster than their coated counter-parts. Non-coated amphipathic polyplexes moved slightly faster than cationic polyplexes both in vitreous and in water.

More obvious changes between coated and non-coated polyplexes were evident when comparing the theoretical diffusion coefficient in water and the measured diffusion coefficient in vitreous (Dw/Dv). The ratio of Dw/Dv reflects how many times more rapid a particlés diffusivity is in water than in the more viscous vitreous. This ratio for CS coated polyplexes was 4.8 and 3.7 times higher than the respective ratios of non-coated amphipathic and cationic peptides (Table 4).

4. Discussion

In this study, we have explored the effect of CS coating on an arginine rich cationic and amphipathic peptide-pDNA polyplexes for retinal gene delivery.

It is clear that peptides are good at condensing pDNA and a CS coating provides additional stability and protection of the compacted pDNA against anionic challenge. It is essential to achieve a balance between condensation and release of the polyplexes since pDNA needs to be stable before reaching the target cells but once inside the target cells, it has to be released in order to exhibit the appropriate biological activity.

The gene delivery efficiency of peptide based carrier system was also evaluated in dividing and differentiated ARPE-19 cells as well as in primary hfRPE cells. Both coated and non-coated amphipathic and cationic peptides displayed high transgene expressions in all cell types even in differentiated cells. This property is very important in retinal gene delivery because RPE cells are not dividing in the eye *in vivo*. The ability to transfect non-dividing cells efficiently is an interesting finding since dividing cells are known to be more easily transfected than their non-dividing counter-parts. This has been demonstrated in the literature by many groups [32,33] and was clearly shown here with lipofectamine which displayed a dramatic decrease in transgene expression in the differentiated ARPE-19 and hfRPE cells. This is attributable to the fact that during cell division the nuclear membrane is temporarily disrupted enabling the access of transgene to nuclear space [34]. The results obtained in differentiated cells suggest that peptide-based gene delivery systems are able to penetrate intact nuclear membrane or they can utilize nuclear pores to allow the transgene to gain access into the nuclear space. The exact mechanism is not known but the cell-penetrating properties possessed by both peptides may have role in this phenomenon.

Transfection data also showed that addition of small amounts of exogenous CS significantly increased the transfection efficiency of both reporter genes in all cell models. Similar trends with CS coated peptide-DNA complexes and DNA-chitosan complexes have also been observed by us [34] and others [35]. Some studies are showing that CS promotes CD44 mediated endocytosis of nanoparticles [36].CD44 receptor is also mediating the internalization of HA [37], which has been widely used for coating of gene delivery systems [24,26,27]. Only few studies have compared CS and HA coating in gene delivery [38,39]. These studies are indicating that CS coating displays equal or even higher transfection efficiency *in vitro* than HA-coating.

In addition to pDNA delivery, the efficiency of peptides for siRNA delivery was also evaluated by knocking down the expression of a housekeeping gene (GAPDH). While pDNA and siRNA are both nucleic acids their biological function and physicochemical properties are very different. pDNA is a very large molecule (size 4000–10 000 basepairs) with the target site in nucleus whereas siRNA is a much smaller molecule (size 21–23 basepairs) that needs to localize into the cytoplasm of the target cells. Our results showed that unlike in pDNA mediated gene transfer gene knockdown was not detected in differentiated ARPE cells. One possible explanation for this observation may be the differences in the physicochemical properties of the formed polyplexes ie. these peptides form larger complexes with siRNA than with pDNA. The optimal N/P ratio for siRNA containing polyplexes is higher than that for pDNA containing polyplexes. This may be due to the different packaging properties of siRNA over pDNA. As a large molecule even a single pDNA may provide a focus for aggregation and allows the formation of a stable complex whereas many copies of small siRNA molecules are required for the assembly of stable and a monodisperse complex when poly-L-lysine was used as a carrier [40]. The differences in the physicochemical properties may have an impact on the cellular uptake route and its efficacy followed by differences in the intracellular trafficking of polyplexes.

For the treatment of retinal diseases, intravitreal injection is a clinically accepted and widely used mode of drug administration. Efficient diffusion of the gene delivery systems in the viscous vitreous is a prerequisite for the drug to reach the retina after an intravitreal injection. Vitreous humor is composed mainly of negatively charged HA and collagen fibres forming a mesh-like structure in the vitreous [18]. The Dw/Dv of 60–70 achieved a clear retention of non-coated cationic and amphipathic peptide based systems (mean diameter 50–60 nm, zeta-potential 24 mV) inside the vitreous. This is understandable because cationic nanoparticles interact via electrostatic forces with anionic HA present in vitreous. There is evidence in the literature that vitreous represents a significant barrier for nanoparticles with positive surface properties [41–43]. The Dw/Dv ratio for cationic (zeta-potential +39 mV) nanoparticles of average size of 200 nm was 2200, being approximately 1000 times higher than for anionic/neutral nanoparticles of the same size [43]. The obtained Dw/Dv ratio for peptide based polyplexes was, however, significantly lower than that obtained by other investigators [43] and may be explained by smaller particle size as well as the lower zeta-potential. CS coating improved the mobility of peptide-based gene delivery systems in vitreous (Dw/Dv ratio 15) probably due to the decreased zeta-potential. Masking the surface charge of nanoparticles by hydrophilic coating such as with PEG chains has also been reported to improve the intravitreal mobility of cationic nanoparticles [24,45]. Further optimization of CS coated polyplexes in terms of size and zeta-potential may result in even greater improvements in vitreal mobility. CS coating also enhanced the stability of the polyplexes when exposed to exogenous heparin. This indicates that the presence of HA, CS coated polyplexes may retain their intactness better than their non-coated counter-parts in the vitreous.

5. Conclusion

The arginine-rich cationic and amphipathic peptides displayed a high transfection efficiency in retinal pigment epithelial cell lines with minimal cytotoxicity. More importantly, the transgene expression was high in differentiated non-dividing cells, which are known to be difficult to transfect. Coating of the peptide based polyplexes with exogenous CS improved the transfection and gene silencing efficacy of the polyplexes. Furthermore, CS coating increased the diffusion of the polyplexes in vitreous and improved the stability of the polyplexes against the exposure to anionic heparin sulphate. The *in vitro* and *ex vivo* studies offers the hope that it may be possible to deliver genetic material to the retina by a safe peptide-based delivery system.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.05.003.

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