Universidade do Algarve

Development of non-viral vectors for gene therapy for pathologies of the retina

Ana Vanessa Vieira Oliveira

PhD in Biomedical Sciences

Thesis supervised by Prof. Gabriela Silva, PhD and Daniel Chung, DO

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Resumo

A terapia génica é uma abordagem terapêutica que tem mostrado grande potencial para o tratamento de doenças genéticas hereditárias ou adquiridas. Geralmente envolve a substituição ou inibição do gene mutado ou ainda a inserção de um novo gene. O sucesso de uma estratégia de terapia génica depende da eficiência da transferência genética e da expressão estável do gene transferido. Atualmente existem duas abordagens que são utilizadas no desenvolvimento de terapias génicas: a viral e a não viral. A abordagem viral, embora seja bastante eficiente, levanta preocupações com a segurança a nível imunológico e mutagénico. Por outro lado a abordagem não-viral caracteriza-se pela sua fácil utilização, capacidade de empacotamento de genes ilimitada e ausência de resposta imunitária. Apesar destas vantagens a aplicabilidade da abordagem não-viral encontra-se limitada pela sua baixa eficiência de transfeção quando comparada com a abordagem viral; deste modo, a optimização dos vetores não-virais visa sobretudo a melhoria da sua eficiência de transfeção.

O nosso objectivo era desenvolver vetores não-virais para terapia génica ocular, em particular da retina. Considerando o órgão alvo, o olho apresenta características únicas para terapia génica como por exemplo o seu reduzido tamanho, relativo isolamento em relação à circulação sistémica e facilidade de acesso a diferentes tipos de tecidos por vias de administração diferentes.

Grande parte das estratégias de terapia génica não-viral focam-se na utilização de lípidos ou polímeros catiónicos, mas até à data poucos estudos foram feitos sobre a sua utilização no olho e em particular na retina. Os polímeros, quitosano e ácido hialurónico ou os polímeros modificados (quitosano tiolado e ácido hialurónico aminado) foram escolhidos considerando a sua biocompatibilidade e biodegradabilidade para a preparação de diversas formulações de vectores. Estes polímeros já foram utilizados com bastante sucesso em aplicações como a entrega de fármacos, o que, aliado às propriedades referidas anteriormente, os torna bastante atrativos para aplicações de terapia génica, embora seja necessária a sua otimização. Esta otimização engloba a modificação química dos polímeros supra-referidos, tal como a modificação com cistamina que permite introduzir uma ponte dissulfeto, que pode ser clivada no meio intracelular pela glutationa, permitindo assim uma libertação do ADN mais facilitada. No caso do ácido hialurónico permite

ainda adicionar grupos amina, que poderiam interagir electrostaticamente com o ADN.

Experimentalmente podemos dividir este trabalho em 3 secções: *i*) a preparação de diversas formulações de vetores com quitosano e ácido hialurónico, que foram caracterizadas em relação às suas propriedades físicas, nomeadamente tamanho, polidispersão, carga superficial, eficiência da complexação de ADN, capacidade de proteção contra nucleases e estabilidade dos vectores em diferentes condições; *ii*) a caraterização dos vectores *in vitro* em relação à sua citotoxicidade e eficiência de transfeção em células epiteliais pigmentadas de retina e células HEK293; *iii*) a administração *in vivo* dos vectores com melhores resultados *in vitro* e a sua caracterização em relação à sua expressão génica na retina de ratinhos através de injeção subretiniana.

Os nossos resultados mostram que os vetores têm tamanho e carga superficial adequados à entrega de genes, embora variem consoante a formulação. Apresentam também estabilidade a longo prazo tanto em condições de armazenamento como em condições fisiológicas de temperatura e pH. Os vetores mantêm-se estáveis após vários ciclos de congelação e descongelação e são capazes de proteger eficazmente o ADN da degradação por nucleases. Observou-se também um efeito do peso molecular do quitosano nas propriedades dos vetores: a utilização de um polímero com maior peso molecular resultou em vetores de maiores dimensões com tendência para uma carga superficial superior, mas com uma estabilidade mais reduzida comparativamente aos vetores preparados com o polímero de menor peso molecular. Este efeito do peso molecular do polímero estendeu-se à eficiência de transfeção e vectores preparados com o polímero de menor peso molecular obtiveram melhores resultados.

Os ensaios de transfeção mostram também que a eficiência de transfeção e a expressão do transgene é afectada pelo tipo de células e modo de entrega da integrase com os poliplexos. A eficiência de transfeção foi superior em células HEK293 do que em células pigmentadas da retina. A elevada estabilidade dos poliplexos tem sido associada com uma baixa eficiência de transfeção e a utilização de polímeros aniónicos tem sido usada como uma das opções para solucionar essa questão. A incorporação de ácido hialurónico nas formulações afectou a estabilidade das formulações, como era esperado, mas não afectou a complexação nem a proteção do ADN no poliplexo. A combinação de quitosano e ácido

hialurónico nos poliplexos mostrou uma melhoria significativa na eficiência de transfeção comparada com vectores baseados apenas em quitosano.

Os ensaios de transfeção usando vectores preparados com os polímeros modificados (quitosano tiolado e ácido hialurónico aminado) não revelaram melhorias significativas em relação aos polímeros não modificados. Pensa-se que isto poderá estar associado a interferências do contra-ião de grandes dimensões – tosilato no caso do quitosano tiolado - e a uma baixa percentagem de modificação no caso do ácido hialurónico aminado. Seria necessário repetir as reações de modificação de modo a substituir o contra-ião por cloreto e conseguir uma percentagem de modificação mais elevada do ácido hialurónico, respetivamente.

De modo a obter uma expressão génica continuada os vectores foram combinados com a integrase do fago phiC31 para promover a integração do transgene no genoma da célula de forma segura e eficaz. A estratégia combinada de entrega baseada em vectores de quitosano e integrase demonstrou expressão génica prolongada tanto de genes de pequena dimensão, como o gene que codifica para a proteína verde fluorescente (com aproximadamente 1 kb) como de genes de maiores dimensões como o gene da proteína centrossomal CEP290 (com aproximadamente 8 kb) várias semanas após a transfeção.

A administração subretiniana *in vivo* dos nossos vetores revelou transfeção eficiente e expressão do transgene continuada em células epiteliais pigmentadas de retina pelo menos 6 meses após a transfeção. Os nossos resultados indicam que esta abordagem baseada em vetores de quitosano pode ultrapassar as limitações de empacotamento encontradas nas técnicas de transferência de genes mediadas por vírus adeno-associados, mantendo um elevado perfil de segurança e expressão génica continuada, constituindo assim uma alternativa para a terapia génica na retina.

Palavras chave: Quitosano, Ácido hialurónico, Terapia génica, Retina, Vetores não-virais.

Abstract

The success of gene therapy relies on efficient gene transfer and stable transgene expression. Our goal was to develop non-viral vectors optimized for retinal gene therapy with continued gene expression. Polymers, chitosan and hyaluronic acid or the modified polymers (thiolated chitosan and aminated hyaluronic acid) were chosen considering their biocompatibility and biodegradability to prepare several formulations. Vectors were formulated and characterized regarding their physical properties, biocompatibility and gene transfer efficiency *in vitro* on both retinal pigment epithelial and HEK293 cells and gene expression in the mouse retina.

Our results show that our vectors exhibit size and surface charge consistent with gene delivery. They also present long-term stability in both storage and physiological conditions, remain stable after several freeze-thaw cycles and are capable of efficiently protecting DNA from nuclease degradation.

Transfection studies show that transfection efficiency and transgene expression is affected by cell type, polymer molecular weight and mode of integrase delivery with the polyplexes. The incorporation of hyaluronic acid affected formulation stability, as expected, but it did not affect DNA loading and protection. The combination of chitosan and hyaluronic acid in polyplexes showed a significant improvement of transfection efficiency compared to chitosan-based vectors. In order to achieve sustained gene transfer vectors were combined with phiC31-integrase to promote transgene integration. The combined strategy of chitosan-based delivery and integrase demonstrate prolonged gene expression of both small (GFP, 1 Kb) and large genes (CEP290, 8Kb) several weeks post-transfection.

In vivo sub-retinal administration of our vectors showed efficient transfection and sustained transgene expression in RPE cells at least 6 months post- injection. Our results indicate this chitosan-based approach may overcome size limitations found in commonly used adeno-associated viruses mediated gene transfer, while maintaining a high safety profile and prolonged, sustained gene expression, thus constituting an alternative for retinal gene delivery.

KEYWORDS: Chitosan, Hyaluronic acid, Gene therapy, Retina, Non-viral vectors.

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List of abbreviations, acronyms and symbols

ºC - Degree Celsius

x *g* - Times gravity

A

AAV - Adeno-associated virus

ABCA4 - ATP-binding cassette sub-family A 4

ADH - Adipic acid dihydrazide

AND - Ácido desoxirribonucleico

AMD - Age-related macular degeneration

ARVO - Association for Research in Vision and Ophthalmology

B

BCA - Bicinchoninic acid

BDNF - Brain-derived neurotrophic factor

BOP - Benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate,

C

C1 - Chitosan CL 113

C2 - Chitosan CL 213

CD44 - Cluster determinant 44

CEP290 - Centrosomal protein of 290 kDa

CHM - Rab escort protein 1

CS - Chitosan

CMV - Cytomegalovirus

CNGA3 - Cone-specific cyclic nucleotide gated channel alpha (A3)

CNGB3 - Cone-specific cyclic nucleotide gated channel beta (B3)

CNTF - Ciliary neurotrophic factor

CS-(AEDTP) - Chitosan-3-(2-aminoethyldithio) propionyl

CS-(PDP) - Chitosan-3-(2-pyridyldithio) propionyl

CRALBP - Cellular retinaldehyde-binding protein

CRB1 - Crumbs homolog 1

D

DAPI - 4′,6-diamidino-2- phenylindole

DCC - Dicyclohexyl carbodiimide)

DD - Degree of de-acetylation

DIPEA - N,N-diisopropylethylamine

DLS - Dynamic light scattering

DNA - Deoxyribonucleic acid

dsDNA - Double stranded deoxyribonucleic acid

DMEM - Dulbecco’s modified eagle medium

DMSO - Dimethyl sulfoxide

DTT - Dithiothreitol

E

ECM - Extracellular matrix

EDAC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA - Ethylenediaminetetraacetic acid

ERGs - Electroretinograms

F

FBS - Fetal bovine serum

FELASA - Federation of Laboratory Animal Science Associations

FITC - Fluorescein isothiocyanate

FRET - Fluorescent resonant energy transfer

G

GCL - Ganglion cell layer

GDEP - Gene differentially expressed in prostate cancer

GDNF - Glial-cell derived neurotrophic factor

GFP - Green fluorescent protein

GR - Glutathione reductase

GUCY2D - Retina-specific guanylate cyclase

H

h - Hour

HA - Hyaluronic acid

HA132 - Hyaluronic acid with 132 kDa

HA214 - Hyaluronic acid with 214 kDa

$HASSNH_2$ - Hyaluronic acid-*co*-*N*-cystaminyl-hyaluronamide,

HASH- Hyaluronic acid-*co*-*N*-cysteaminyl-hyaluronamide

HARE - Hyaluronic acid receptor for endocytosis

Hd-Ad - Helper-dependent Adenovirus

hIRBP - Human interphotoreceptor retinoid binding protein

HMDA - Hexamethylenediamine

I

IFN-α - Interferon alfa

IL-10 - Interleukin-10

K

K5 - Plasminogen kringle 5

L

LCA - Leber's congenital amaurosis

LRAT - Lecithin retinol acyl-transferase

LYVE-1 - Lymphatic vessel endothelial hyaluronan receptor-1

M

MERTK - c-mer proto-oncogene tyrosine kinase

min - Minutes

mRNA - Messenger ribonucleic acid

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW - Molecular weight

MYO7A - Myosin VIIA

N

NADPH - Nicotinamide adenine dinucleotide phosphate

NHS - N-hydroxysulfosuccinimide

NLS - Nuclear localization signals

N/P ratio - NH_3^+/PO_4^- ratio

NPs - Nanoparticles

NMR - ^{1}H nuclear magnetic resonance

O

OCT - Optimal cutting temperature

ONL - Outer nuclear layer

OTs - *p*-Toluenesulfonate

OTs^- - Tosylate ion

P

PAMAM - Poly(amido amine

PB - Potassium phosphate buffer with ethylenediaminetetraacetic acid

PBS - Phosphate buffered saline

PDI - Polydispersion index

pDNA - Plasmid deoxyribonucleic acid

PEI - Polyethylenimine

PEG - Poly (ethylene glycol)

pDMAEMA - Poly (2-(dimethylamino)ethyl methacrylate)

PDGF - Platelet-derived growth factor

PEDF - Pigment epithelial derived factor

PLGA - Poly (lactic-co-glycolic acid)

PLL - Polylysine

PVP - Poly(vinylpyrrolidone)

PPI - Poly(propylenimine)

R

RAFT - Reversible addition-fragmentation chain transfer

RDH5 - Retinol dehydrogenase 5

RGR - Retinal G protein coupled receptor

RNA - Ribonucleic acid

RP - *Retinitis pigmentosa*

RPE - Retinal pigmented epithelium

RPE65 - Retinal pigment epithelium-specific 65 kDa protein

rpm - Revolutions per minute

RHAMM - Receptor for hyaluronate-mediated motility

RHO - Rhodopsin

S

SDS - Sodium dodecyl sulfate

siRNA - Small interfering ribonucleic acid

S/MARs - Scaffold- or matrix-attachment regions

ssDNA - Single stranded deoxyribonucleic acid

SPDP - N-succinimidyl-3-(2-pyridyldithio)-propionate

T

TAE - Tris-acetate-ethylenediaminetetraacetic acid

TBE - Tris-borate-ethylenediaminetetraacetic acid

TGFb - Transforming growth factor-b

TE - Tris- ethylenediaminetetraacetic acid

TEM - Transmission electron microscopy

TETA - Triethylenetetramine

TLC - Thin layer chromatography

TPP - Tripolyphosphate

U

UV - Ultraviolet

USH2A - Usherin

V

VEGF - Vascular endothelial growth factor

VEGFR1 - Vascular endothelial growth factor receptor 1

VEGFR2 - Vascular endothelial growth factor receptor 2

V/V - Volume per volume

X

X-SCID - X-linked combined severe immunodeficiency

W

W/V - Weight per volume

Z

ZP - Zeta potential

Chapter I

General Introduction

Chapter I – General Introduction

1. Gene therapy

Gene therapy can be defined as a medical treatment by the transfer of therapeutic genetic material, DNA or RNA, into a group of cells (tissue or organ) in order to correct or modify expression of the gene influencing a disease process. [1] Gene therapy can be achieved in three ways:

i) Substitution of altered genes, where genetic mutations causing loss of function can be corrected by replacement of the defective gene and repair of the mutated sequence; [2, 3]

ii) Inhibition or counteraction of deleterious effects, which can be carried out by targeted inhibition of gene expression. This process occurs by blocking promoters, interfering with the mechanisms of gene expression using antisense RNAs, that are complementary to mRNA, bind and block them, or more recently, using siRNA (small interfering RNA), which block specific RNA sequences, neutralizing the mutated mRNA transcript and preventing it from being translated into a protein; [2, 3] and

iii) Insertion of new genes. This can be achieved by targeted deletion of specific cells through the insertion of suicide genes that destroy their host cell or stimulators of the immune response genes. A copy of the normal gene can also be inserted to replace the function of the mutant gene that does not produce the correct protein. [2]

Gene therapy can be used in the treatment of genetic or acquired diseases and is currently used to treat diseases such as cancer, peripheral vascular diseases, arthritis, neurodegenerative disorders and AIDS (Fig. 1.1). For example, gene therapy can be used to treat cancer by delivering to the tumor a gene encoding for an enzyme that will activate a pro-drug with no adverse effects for healthy cells. [4]

An ideal targeted gene therapy should be *i*) a gene delivery technique that is efficient and non-toxic; *ii*) well characterized in terms of the genetic basis of the disease, or its biochemical basis, so that the appropriate therapeutic approach can be selected; *iii*) controlled in a way that cells and tissues can support levels of gene product expression; *iv*) able to demonstrate proof of principle in an experimental animal model of the disease for preclinical testing of the therapy. [5]

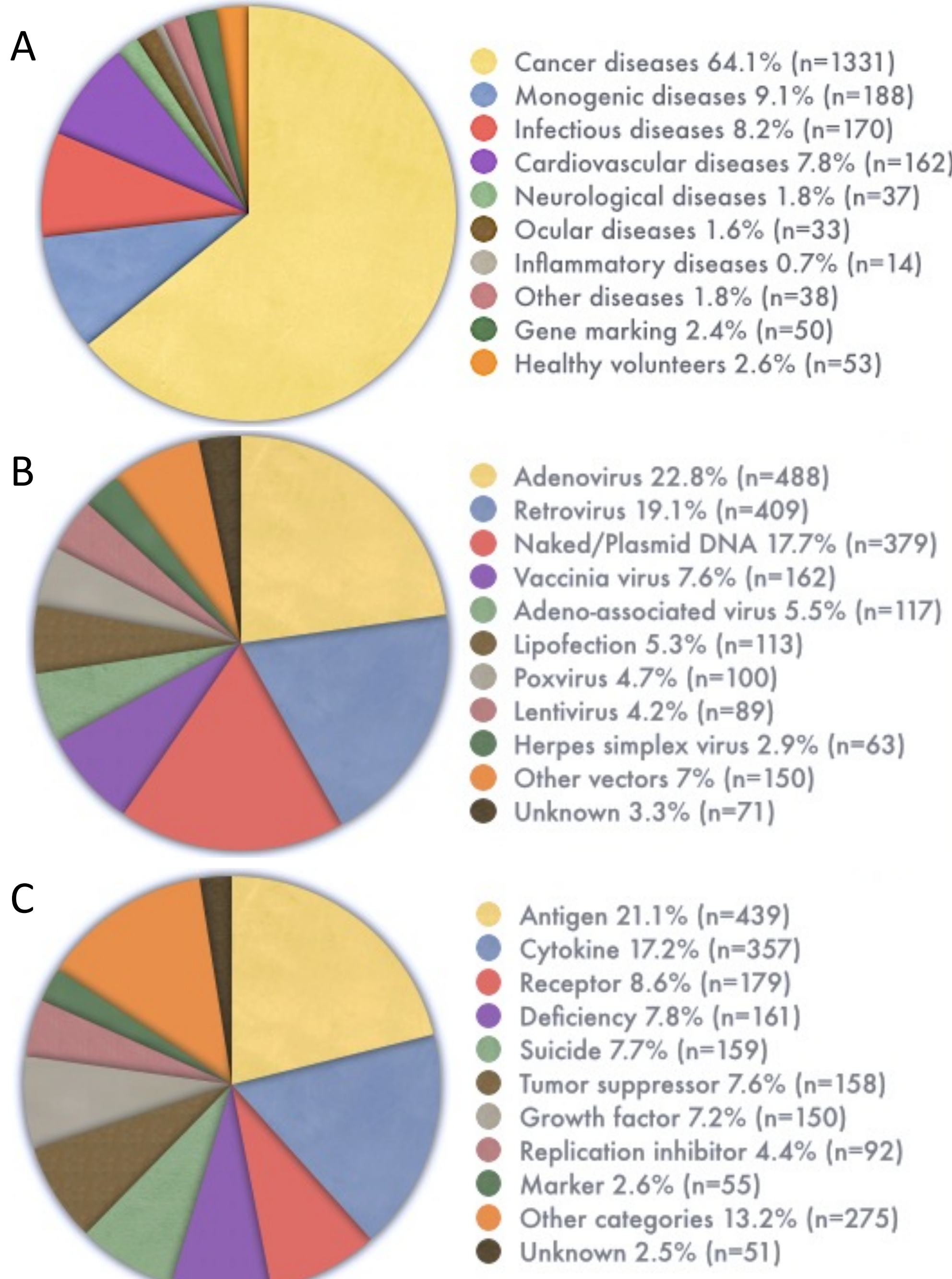

Figure 1.1 – Diversity in gene therapy clinical trials: A) focused diseases, B) strategies used and C) types of genes delivered. Adapted from Wiley clinical trial database (http://www.abedia.com/wiley/).

The efficiency of *in vivo* gene therapy is determined by the choice of method or vector used to deliver the gene, the route of administration and the selected gene and target cells. [6, 7] While some requirements are common to several strategies, some depend on the targeted disease and transgene product. For example, characteristics of the target cells (location, life span, and blood flow rate) are

determinant for the efficacy of gene therapy. Consequently, it is unlikely that a single system will be suitable for all applications. [7]

Other factors governing the success of gene therapy are cell-specific gene transfer, levels and duration of transgene expression. Regarding cell specificity, besides the use of different delivery routes, the use of a tissue or cell specific promoter is one of the strategies for achieving targeted gene transfer. Since some promoters are only active in a specific type of cell, high specificity of transgene expression can be achieved. For example, the rhodopsin promoter drives expression in rod photoreceptors, and the human red opsin promoter drives expression in cone photoreceptors. [8] Still, cell specific promoters tend to have a weak transduction efficiency that limits their usefulness. [7]

Considering levels of transgene expression in target cells, these can be directly correlated with the efficacy of the gene transfer approach, which in turn depends on the strength of the promoter and the amount of DNA that reaches the nucleus of target cells. The number of transformed cells and number of DNA copies taken up by each cell determine the levels of transgene expression. [7] Various ways to enhance the levels of expression have been examined to increase the amount of DNA in the cytoplasm, such as the use of fusogenic lipids or peptides to disrupt the endosomal membrane and the use of vectors with a high buffering capacity and the ability to swell when protonated. Other strategies aim at increasing the amount of DNA that reaches the nucleus using nuclear localization signals (NLS) that actively target DNA to the nucleus. [7]

Since most gene delivery methods do not undergo chromosomal integration, the degradation of DNA and the transcriptional inactivation of the promoter are the major causes for the loss of transgene expression. Approaches to extend transgene expression involve the continuous supply of DNA to target cells by controlled release systems; improved DNA stability with the use of optimized promoters; and/or reduce the CpG content, known to have an immunostimulatory effect. [7] The obstacles to gene therapy will be discussed in more detail in section 3.

2. Gene delivery systems

A successful gene therapy strategy requires a safe and effective gene delivery system as well as the appropriate gene delivery method. Since naked DNA

molecules cannot effectively enter to the cell due to their hydrophilic nature, large size and negative charge derived from the phosphate groups in its backbone, several methods have been developed to ensure gene transfer to target cells. [4]

Current delivery methods can be divided into two major classes: viral and non-viral. Non-viral delivery methods can further be classified as physical and chemical methods.

2. 1. Viral gene delivery

Viral-mediated gene delivery systems use recombinant, replication-deficient viruses to deliver and express genes carried in their modified viral genome using the cell's own machinery. [4, 9] Recombination is achieved by deleting all, or some, of the coding regions from the viral genome, but leaving intact sequences that are required for packaging the vector genome into the viral capsid or integration of vector DNA into the host chromatin. [10] Viral gene transfer is currently further advanced in terms of development due to its high efficiency, specificity in cell entry and gene expression. [9]

Viral vectors are derived from five main classes of clinically applicable viruses: adenoviruses, retroviruses, lentiviruses, adeno-associated and herpes simplex-1 viruses. These can be categorized into integrating and non-integrating vectors. Integrating vectors (*e.g.* adeno-associated viruses, retroviruses and lentiviruses) have the ability to integrate into the chromosomal DNA of the host cell, which will possibly allow lifelong gene expression. Non-integrating viruses (*e.g*. adenoviruses and herpes simplex-1 viruses) deliver their genomes into the nucleus of the target cell, where they remain episomal. [10, 11]

However, these systems have some limitations, including the use of viruses during the production stage (helper viruses), immunogenicity, oncogenicity, toxicity and lack of optimization in large-scale production. [4] Some of the most important characteristics of viral vectors are summarized in Table 1.1.

Table 1.1 – Main properties of viral gene therapy systems.

Transfection vector	Viral genome	Packaging capacity	Tropism	Integration	Advantages	Limitations	Refs
Adenoviruses vectors	dsDNA	8 – 36 kb	Broad (dividing and non-dividing cells)	No	High efficacy transduction in most tissues	Immune and inflammatory responses, short-term gene expression due to episome loss	[4, 9-11]
Adeno-associated vectors	ssDNA	<5 kb	Broad (dividing and non-dividing cells)	90% episome 10% integrated*	Site specific integration by non-homologous integration, non-pathogenic	Limited transgene capacity, pre-existing immunity due to natural infection	[9-11]
Retroviral vectors	RNA	8 kb	Dividing cells only	Yes	Transgene persistence in dividing cells	Low efficiency *in vivo*, risk of insertion mutagenesis	[4, 9, 11]
Lentivirus vectors	RNA	8 – 18 kb	Broad (dividing and non-dividing cells)	Yes	Transgene persistence in dividing cells, low immunogenicity	Difficult design and construction, risk of insertion mutagenesis	[9, 11, 12]
Herpes simplex virus vectors	dsDNA	40 – 150 kb	Broad (dividing and non-dividing cells)	No	Large transgene capacity, strong tropism for neurons	Short-term gene expression due to episome loss	[9-11]

* AAV genome integration is *rep* protein dependant. dsDNA – double stranded DNA, ssDNA – single stranded DNA

2. 2. Non-Viral gene delivery

Non-viral delivery systems can be classified according to preparation, as physical or chemical. In broad terms, physical delivery methods employ a physical force to increase permeability of the cell membrane and achieve gene delivery, while chemical methods utilize natural or synthetic carriers to perform a similar task.

Compared to viral delivery systems, non-viral vectors are less toxic, less immunogenic, easily produced and have the potential for repeated administration. However, they are still less effective and can rarely accomplish transgene expression at therapeutic levels. [4, 13, 14]

2. 2. 1. Physical methods

Initially, gene therapy was done using the simplest gene transfer system that is the injection of naked plasmid DNA. However, naked DNA once inserted into the cell is rapidly degraded by nucleases in the serum and clearance by the mononuclear phagocyte system resulting in generally low transfection efficiency and transient expression. [6, 15] Gene transfer with naked DNA is highly ineffective unless the DNA is associated with other molecules and/or physical energy is applied to help cell entry. [16]

Physical delivery methods may circumvent some of the limitations associated with other approaches, such as limited packaging of large genes. However, most require labor-intensive protocols and/or the use of costly instruments. [9]

Several methodologies have been developed to enhance the transfection efficiency using physical methods that act by bringing DNA into the vicinity of the cell membrane and/or cause temporary disruption of the cell membrane, thus increasing DNA cell entry. [16] Among others, examples of such methods are electroporation, jet injection, gene gun, ultrasound, gene transfer by laser beam, which are described next.

Electroporation

Electroporation, also known as electropermeabilization, is a delivery method that introduces foreign DNA into cells through an electric pulse that creates temporary pores in the cellular membrane. [6, 17] The pore formation is a very rapid event, occurring within 10 nanoseconds after electroporation and the size of the pore is estimated to be <10 nm in diameter. It is believed that small molecules, such as nucleic acids, can enter cells through these pores into the cytoplasm, by diffusion or a local electrophoretic effect. [6, 16]

Electroporation can be done *in vivo* by local insertion of electrodes into the tissue or by holding the tissue with plate-type electrodes. It has been successfully applied to a range of tissues such as skin, kidney, lung, liver, muscle, joints, spinal cord, brain, retina, cornea, vasculature and solid tumors. [6, 16] *In vivo* electroporation has shown increases in transgene expression up to 1000-fold, compared with injection of naked pDNA without electroporation. [6, 7] However, electroporation has some limitations such as tissue damage associated with the procedure and limited transfection efficiency due to low tissue penetration. [6]

Jet injection

Jet injection is a needle-free delivery method that uses high-speed pressure forcing DNA to penetrate the target tissue. Although jet injection is well tolerated by the target tissues its transfection efficiency is generally low. [17]

Gene gun

Gene gun delivery, also known as bioballistics or DNA-coated particle bombardment, uses a high-voltage electric discharge device that accelerates the DNA-coated particles to high velocity, enabling efficient penetration of target organs, tissues, or cells. DNA can be coated with microparticles of gold, tungsten or silver in order to increase density. [17] This technique can target the cell nucleus directly and has been used successfully in skin, liver, and muscle cells after surgical exposure of the tissue. [6]

The main advantages of this delivery system are the absence of toxic chemicals, no need for specific receptors, ability to transport DNA fragments of various sizes, high reproducibility and easy production of metal particles. Nevertheless, gene expression is generally low and short-termed. [4]

Ultrasounds

Ultrasounds can be used not only as a diagnostic tool but also as a therapeutic device. Gene transfer by ultrasounds, also known as sonoporation, uses acoustic cavitation that induces cell membrane permeabilization. [6, 17] In diagnostic applications low-intensity ultrasounds are used to prevent biological effects, whereas in therapeutic applications the intensity of the ultrasound energy is increased. [4]

The major advantages of this technique are its safety and noninvasiveness. However, the success of sonoporation depends largely on the tissue type since ultrasounds suffer loss of intensity due to absorption, deflection and refraction phenomena within the tissue. [17]

Gene transfer by laser beam

Laser beam gene transfer is similar to electroporation: naked DNA is injected into the target site followed by direct application of a laser beam instead of an electrical pulse. This method is considered safe, effective and reproducible for intradermal gene delivery. Compared with electroporation it causes substantially less damage. [17]

Magnetofection

Gene transfer by magnetic force, also known as magnetofection, uses magnetic fields to achieve transfection of DNA bound to magnetic particles. The major advantages of this technique are rapid and efficient transfection at low vector doses. [17]

2. 2. 2. Chemical delivery methods

In order to protect the therapeutic genetic material and enable a more efficient delivery, different chemical methods have been developed. Among these, cationic lipid- and polymer-based systems have been the most extensively studied. Lipids and polymers can interact with plasmid DNA and form nano-sized complexes, lipoplexes and polyplexes, respectively, that can pass through the cell membrane. Other chemical delivery methods include the use of inorganic compounds and synthetic peptides.

General mechanisms of transfection with lipidic and polymeric carriers involve particle cell surface bind by non-specific, electrostatic interactions between the positively charged carriers and the negatively charged cell surface, and cell entry by endocytosis or endocytosis-like mechanisms. Once inside the endosomes, the pH decreases from 7 to 5.5 and part of the bound nucleic acids escape into the cytoplasm, by mechanisms that differ for lipid- and polymer-based systems. Then, the carriers dissociate and the released nucleic acids can enter the nucleus by two hypothesized pathways, either passive DNA entry into the nucleus during mitosis or active import of the DNA through nuclear pores. [18]

2. 2. 2. 1. Inorganic compounds

Inorganic nanoparticles can be prepared from metals (iron, gold, silver), inorganic salts, or ceramics (phosphate or carbonate salts of calcium, magnesium, or silicon). [13] One of the first inorganic particles to be used for gene therapy was a calcium phosphate particle. Calcium phosphate gene transfer is mediated by DNA-hydroxyapatite particles. DNA-calcium phosphate co-precipitates spontaneously in supersaturated solutions, however, to achieve high transfection, precipitates have to be generated under strict conditions, because the formation of DNA-hydroxyapatite particles is affected by calcium, phosphate and DNA concentrations, temperature and reaction time. This technique has been widely used and is considered easy-to-use, cost-effective and highly safe. [9]

2. 2. 2. 2. Lipid-based systems

Lipid-based systems are generally comprised of cationic lipids, amphiphilic molecules containing a positively charged hydrophilic head and hydrophobic tail. [18] Most common hydrophilic head groups are primary, secondary, or tertiary amines, or quaternary amine salts that are positively charged and can interact with negatively charged phosphate groups in nucleic acids. Structural properties of the lipid and charge ratio used are very important for the formation of lipid/DNA complexes and for the transfection effectiveness. [4] Depending on the ratio of positive charges on the lipids and negative charges in the DNA phosphodiester bonds, lipid complexes can be anionic, neutral or cationic. It is general belief that a slight excess of positive charges promotes higher transfection efficiencies due to electrostatic interactions between complexes and the cell membrane as well as negatively charged proteoglycans. [18]

Cationic lipid/DNA complexes (lipoplexes) have been used in several clinical trials, which largely validate the concept of human gene therapy. Lipoplexes are formed in a self-assembly process triggered by DNA-mediated fusion of liposomes and involves a large-scale lipid rearrangement. [19] Plain lipoplexes can form two types of structures: a multilamellar structure with DNA monolayers sandwiched between cationic membranes or an inverted hexagonal structure with DNA molecules encapsulated within cationic lipid monolayer tubes. [18] Cationic lipids also have detergent and/or buffering properties that are thought to facilitate DNA release enhancing transfection efficiency. [19] One of the factors limiting the use of lipoplexes is its instability in biological fluids. In the presence of serum lipoplexes aggregate and their interaction causes its disintegration, which leads to DNA release and degradation. The rate of aggregation and disintegration is dependent on the structure of cationic and helper lipids. [19] Some examples are given in Table 1. 2.

Liposomes, the most common lipid based gene delivery systems, are colloidal systems with membrane-like spherical structures composed by fatty acids or phospholipids with one or more lipid layers surrounding an aqueous core. [4, 9] Typically liposomes contain at least two components, a cationic lipid and a neutral lipid also referred to as helper lipid. [18] Liposome formation is influenced by several factors such as preparation procedure, mixing ratio, pDNA concentration, size of the cationic lipid and ionic strength of the buffer used in the preparation. [9] Parameters

like size, number of layers, and surface charge are determinant for the performance of liposomes *in vitro* and *in vivo.* [4] Several cationic lipids, such as quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, and lipid derivatives of polyamines have been investigated for liposome-based gene delivery. [20]

Table 1. 2 – List of commonly used lipids in gene therapy. Adapted from [13]

Name	Common abbreviation	Feature
1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine	DOPC	Helper lipid
1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine	DOPE	Helper lipid
Cholesterol	Chol	Helper lipid
N-[1-(2,3-Dioleyloxy)propyl]N,N,N-trimethylammonium chloride	DOTMA	Cationic lipid
1,2-Dioleoyloxy-3-trimethylammonium-propane	DOTAP	Cationic lipid
Dioctadecylamidoglycylspermine	DOGS	Cationic lipid
1,2-Dioleyl-3-trimethylammonium-propane	DOPA	Cationic lipid
6-Lauroxyhexyl ornithinate	LHON	Cationic lipid
Ethyldimyristoylphosphatidylcholine	EDMPC	Cationic lipid
N-Palmitoyl D-erythro-sphingosyl carbamoyl-spermine	CCS	Cationic lipid
N1-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine	CDAN	Cationic lipid
3β-[N-(N′,N′-Dimethylaminoethane)-carbamoyl]cholesterol	CD-Chol	Cationic lipid
1,2-Distearoyl-sn-glycero-3-ethylphosphocholine	DSEPC	Cationic lipid

In general, liposomes are considered non-toxic and biodegradable. However, their cationic nature may cause non-specific interactions with negatively charged cellular components resulting in reduction of cellular adhesion, hemolysis, and low

transfection efficiency compared to viral vectors. [9, 21] There is also the issue of potential cytotoxicity mainly caused by their cationic nature and the linker group. [9]

Recent developments have focused on optimizations to improve transfection efficiency and reduce cytotoxicity by modifications of the positively charged headgroup or the linker functionalization group. Other strategies have conjugated cationic liposomes with polyethylene glycol (PEG) and/or other molecules such as ligands and peptides to improve stability. [9]

2. 2. 2. 3. Polymer-based systems

A successful gene delivery system should protect the negatively charged phosphate DNA backbone from anionic cell surface repulsion, should provide nano- or micro-sized structures in order to be compatible with endocytosis or phagocytosis and should protect DNA from extracellular and intracellular nuclease degradation. In order to achieve this, three strategies are currently used to produce polymer-based vectors: electrostatic interactions, encapsulation and adsorption, as shown in Fig. 1.2. [4]

Polymers are composed of small repeating molecules called monomers that form long-chained structures. Polymers can be homopolymers if they are composed of just one repeating monomer or copolymers if they are composed of two or more monomers. [4]

Polymer DNA binding generally occurs by both hydrophobic and electrostatic interactions; the latter develop between DNA phosphate groups and polymer cationic groups, usually amine groups. Polymer DNA biding affinity is influenced by their intrinsic properties such as number of charged groups per polymer molecule, type of charged groups (primary, secondary, terciary amino groups, quaternary ammonium groups, amidine groups), spacing of charged groups within the polymer, degree of branching in the polymer backbone and hydrophobicity. [22] Interactions with DNA are pH-dependent in which higher charge-density polymers show stronger DNA binding. However, stronger biding can have an adverse effect on transfection efficiency since it can hamper DNA release to the cytoplasm. [23]

Cationic polymers, unlike cationic lipids, do not contain a hydrophobic moiety and are water-soluble. Also, compared to cationic liposomes they form relatively smaller complexes with DNA, which can facilitate transfection. [18, 21] Polyplexes

can condensate into spherical, globular, rod-like or toroid structures. [18] Another approach to polymer-based delivery is the use of dendrimers. Dendrimers are repeating, branched, large spherical molecules, and have functional groups on their surface that can be used for modification and optimization. [4] The degree of polymerization of dendrimers is given by the number of generations of repeated branching cycles during its synthesis. [4]

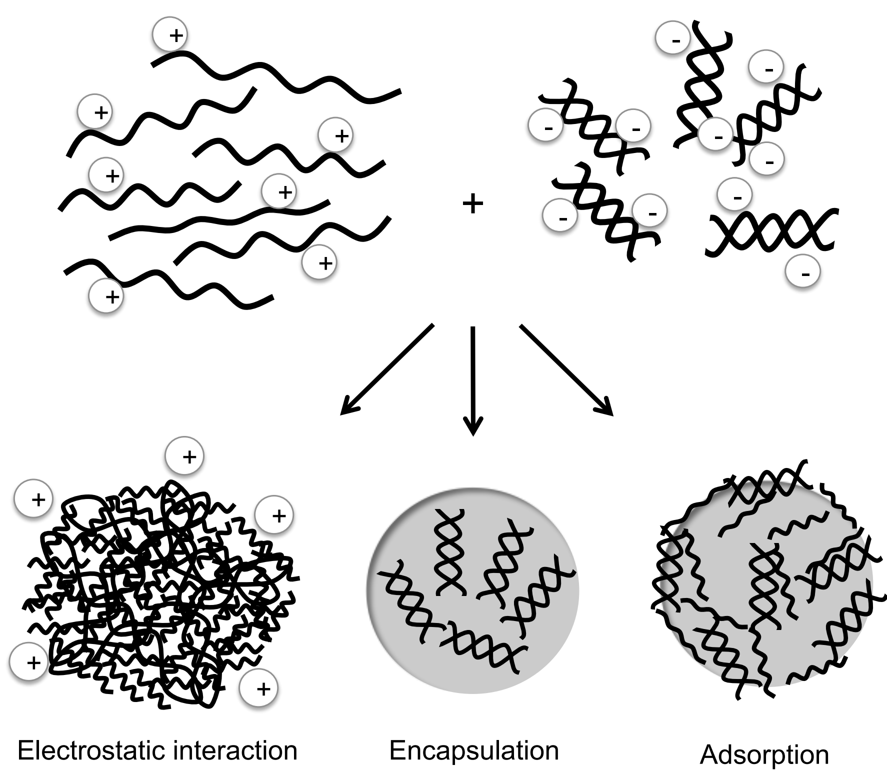

Figure 1.2 – DNA packaging strategies. Three strategies can be used to package DNA in polyplexes: electrostatic interactions, encapsulation within or adsorption onto biodegradable nano- or microspheres. Adapted from [24].

Cationic polymer/DNA complexes (polyplexes) have been actively studied as gene delivery systems and are a safe, biodegradable and non-toxic promising alternative to viral therapy. [9] Polyplex formation is influenced by several conditions, such as: ionic strength of the polyplex solution, concentration and positive/negative charge ratios of polymer and DNA and process of polyplex formation (kinetic vs. thermodynamic process). [13] These also affect the stability of the carrier and consequently its transfection efficiency. [18]

Polyplexes rely on endocytosis of polymer-based carriers for gene delivery and have showed triggered nucleic acid release, structural diversity and relatively higher transfection efficiency and stability than liposomes as well as more efficient DNA condensation. [9, 19, 21] A positive surface charge is required for efficient cellular uptake since the cell entry process encompasses interactions with cell-surface proteoglycans. [18]

Selecting the most appropriate polymer for gene delivery is difficult due to structural differences and requires the characterization of chemical, mechanical, and biological features of the polymer. [4] Numerous kinds of polymers have been examined for gene delivery and the majority of these cationic polymers are derived from polyamines such as polylysine, polyarginine, chitosan, polyethylenimine and polyamidoamine dendrimers. [9]

Several different types of polymers, both synthetic and natural have been evaluated as gene delivery vehicles. The most commonly used polymers are listed in Table 1.3. In many cases, polymers are chosen to address one of the particular barriers gene delivery faces, for example, DNA packaging and *in vivo* stability, biocompatibility or endosomal escape. [22]

2. 2. 2. 3. 1. Synthetic polymers

Among synthetic polymers, polyethylenimines (PEIs) are the most promising and the ones of most use in gene therapy. PEIs have strong DNA condensation capacity and intrinsic endosome-buffering properties that enable them to mediate efficient gene transfer. [9] They have been used with different molecular weights, branched or in a linear form resulting in variable levels of transfection efficiency and toxicity. [19]

One of the factors influencing PEI-mediated transfection efficiency is PEI molecular weight (MW). High MW PEIs have been associated with higher transfection efficiencies along with acute cytotoxicity due to cell membrane disruption, followed by induction of apoptosis, while low MW PEI has been associated to low cytotoxicity and low transfection efficiencies. [9, 13] Besides toxicity, PEIs have other drawbacks like tendency to aggregate red blood cells, bind complement components and poor degradability. [9]

Table 1. 3 – List of polymers commonly used in gene therapy. Adapted from [13, 24]

Name	Common abbreviation	Feature	Structure
Polyethylenimine	PEI	Cationic synthetic polymer	
Chitosan	CS	Natural polysaccharide	
Poly(ethylene)glycol	PEG	Inert synthetic polymer	
Poly(L-lysine)	PLL	Cationic synthetic polymer	
Poly(N-vinylpyrrolidone)	PVP	Neutral synthetic polymer	
Triethylenetetramine	TETA	Cationic synthetic	
Poly(D,L-lactic-*co*-glycolic acid)	PLGA	Biodegrabable synthetic polymer	

Poly(propylenimine)	PPI	Synthetic dendrimer	
Poly(amidoamine)	PAMAM	Synthetic dendrimer	
Poly(2-(dimethylamino)ethyl methacrylate)	pDMAEMA	Cationic synthetic polymer	

One of the strategies used to modify surface charge and overcome the issue of non-specific binding and toxicity is co-formulation with anionic polymers. [13]

Polyamidoamine (PAMAM) dendrimers are highly branched spherical polymers that form mono-dispersive molecules with primary amino groups in their surface. PAMAM dendrimers can be produced in large quantities and synthesized over a range of molecular weights. Their structure begins with a core molecule (ammonia or ethylenediamine) and a stepwise polymerization process adds layers or generations by successive addition of methyl acrylate and ethylenediamine. [9, 22] The number of surface amine groups increases with each new layer and can be used to attach antibodies or contrast agents. [9]

Dendrimers produce very stable and highly soluble DNA complexes due to ionic interactions with DNA. However, PAMAM dendrimer-based gene delivery is limited by their production costs and their dependence in generation number. Also, high transfection efficiency is associated with cytotoxicity, which further limits their use. [9]

Poly(lactide-*co*-glycolide) (PLGA) is composed of lactic and glycolic acids linked together by ester bonds. PLGA can incorporate DNA into particles protecting DNA from degradation and allowing a controlled DNA release. Some of the limitations it presents are slow DNA release rate, reduced DNA encapsulation due to negative charges that hinder the process, and structural changes to DNA due to the acidic microenvironment of the PLGA particles. [9]

Poly-L-lysine (PLL) is a biodegradable synthetic polypeptide capable of pDNA condensation and one of the first cationic polymers used for gene delivery. Nonetheless, its application in gene therapy is limited by cytotoxicity and low transfection efficiency. [6, 9] Another example of a synthetic polymer is poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), a derivative of PLL, in which the ester link was substituted with amide. This water-soluble polymer can condensate DNA and then release it upon hydrolysis of the polymer with a much lower cytotoxicity that PLL. [20]

Another class of polymers used in gene therapy application are poly(amino-esters). Poly(amino-esters) can be readily synthesized via the conjugate addition of primary or secondary amines to diacrylates. The use of poly(amino-esters) has various advantages since diamine and diacrylate monomers are inexpensive and commercially available materials, polymerization is done in a single synthetic step

reaction with no byproducts generated during polymerization. Compared to PEI, poly(amino-esters) have a more favorable profile due to their biodegradability via hydrolytically degradable ester groups, reduced cytotoxicity, ability for triggered DNA release within the cell, and potential for structural diversity. [22]

Acrylate based vectors such as poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA) have also been evaluated for gene therapy. High MW forms of pDMAEMA (>300 kDa) were able to condense DNA effectively into positively charged polyplexes. [22] This polymer can be produced with defined sizes by reversible addition-fragmentation chain transfer (RAFT). Recent studies have shown that pDMAEMA polyplexes were able to achieve efficient gene delivery expression in a polymer:DNA ratio dependent manner without induction of inflammation *in vivo*. [25]

Modification of polyplexes with PEG, a process commonly known as PEGylation, can stabilize polyplexes against salt, protein, and complement-induced inactivation. This increase in stability is presumably due to steric effects that lead to a decrease in polyplex–polyplex and polyplex–protein interactions. The effect varies with PEG molecular weight, the grafting density, and the method of attachment of PEG to the polymer. Also, PEG has other desirable features such as the lack of immunogenicity, antigenicity, and toxicity, and a high solubility in water and in many organic solvents. [22] Studies have also shown a reduction in hemolytic potential and cytotoxicity after PEG coating of polyplexes. [22] A study compared the effects of PEG coating versus a natural polymer (chitosan) coating on a drug delivery application showing that penetration rate was dependent on the coating composition. Furthermore, PEG coating accelerated transport across the whole epithelium while the chitosan coating privileged retention of the carriers in the superficial layers of the epithelium. [26]

2. 2. 2. 3. 2. Natural polymers

While synthetic polymers constitute the majority of non-viral gene delivery systems, natural polymers have distinct advantages such as environmental responsiveness via degradation and remodeling by cell-secreted enzymes, as well as the potential for *in vivo* applications due to their non-toxic and cytocompatible profile at high concentrations. [14, 21] Natural biopolymers such as gelatin and

chitosan have been previously used in drug delivery applications. Studies have shown that these polymers can also form stable polyplexes when mixed with DNA. [19] Some examples of natural polymers used in gene therapy are listed below. Chitosan and hyaluronic acid, as the polymers of interest in this thesis, will be described in more detail in the following sections.

Gelatin is obtained by thermal denaturation of collagen, obtained from animal skin and bones. In its structural unit, it contains many glycine, proline and 4-hydroxyproline residues. It shows excellent controlled biodegradation and it can also protect plasmid DNA from rapid degradation by nucleases. [21] Cationic gelatin can be easily obtained by modification and introduction of amine residues in the carboxyl groups. Several techniques can be used to produce gelatin polyplexes, such as self-assembly, desolvation, coacervation-phase separation, emulsification-solvent evaporation, and nanoprecipitation, among others. [27] Gelatin derivatives, obtained by chemical introduction of different amine compounds, such as ethylenediamine, spermidine, and spermine are also used for gene delivery. Among these, the latter is the most effective gene carrier due to its superior buffering ability. [21]

Alginate is a naturally occurring linear polysaccharide that can be obtained from brown algae and bacterial species of *Pseudomonas* and *Azotobacter*. It is composed by D-mannuronic acid and variable amounts of L-guluronic acid, and has the ability to form hydrogels in the presence of divalent cations like Ca^{2+}. [21] Alginate can be used to encapsulate genetic material, other vectors or cells. For example, it has been used to encapsulate PEI/DNA complexes in order to reduce their toxicity and improve their serum stability. [28]

Peptides containing highly basic amino acids (arginine and lysine) have been widely used as delivery vectors. Arginine is a *α*-amino acid and its side chain consists of a 3-carbon aliphatic straight chain with a guanidinium group at the end. Arginine/DNA complexes of various sizes have been tested and no relationship between the complex size and the cellular distribution of the complex was found. Also, studies have shown that arginine/DNA complexes were transferred into the cell nucleus without the dissociation of the arginine peptide. [21]

Collagen is composed of three helical polypeptide chains with a rod-like structure and is the main constituent of the connective tissue. Collagen has two very advantageous properties for gene delivery: it can stay in liquid form at low temperatures and it becomes fibrous and then solid when implanted in the body due

to body temperature. Therefore, it can form a matrix structure that protects DNA from immunological reaction and enzymatic attack. [21] This way collagen can be used to produce matrices with naked DNA or other vectors and achieve sustained gene delivery. [29]

Chitosan

Among the natural polymers, chitosan (CS) and their derivatives are the most reported non-viral vectors due to their reduced cytotoxicity and immunogenicity, high biodegradability and excellent biocompatibility. [14, 21] Chitosan is a naturally occurring linear cationic polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine units linked by a β (1→4) glycoside bond at varying ratios and of varying chain lengths. It is obtained by the deacetylation of chitin, which can be obtained from crab and shrimp shells by chemical processing. [21] Other sources of chitin besides shellfish include insects and mushrooms. [30] Chitosan is a weak base, insoluble at neutral and alkaline pH values. However, in acidic conditions it is soluble as its amine groups become positively charged leading to a high charge density of the polymer. [21]

Over the years CS has been used in several biomedical applications such as drug delivery and tissue engineering due to its biocompatibility, biodegradability, ecological safety, low toxicity, low immunogenicity and antimicrobial activity. [9] Biodegradability is assured by a variety of enzymes also present in humans such as lysozyme, di-N-acetylchitobiase, N-acetyl-beta-D-glucosaminidase and chitiotriosidase. [30] The mucoadhesive properties of CS allow a prolonged interaction between polyplexes and membrane epithelia and therefore a more efficient uptake. It also has the ability to open intercellular tight junctions that facilitate transport into the cells. [21] Due to this characteristic, CS has been applied to oral and nasal gene therapy delivery systems quite successfully. [31, 32]

Chitosan can effectively complex the negatively charged DNA due to its positive character and can protect it from nuclease degradation. Chitosan has the added advantage that there is no need for sonication and organic solvents, during the complex formation with DNA, therefore lowering possible DNA damage during complexation. Moreover, DNA-loaded CS polyplexes remain stable during storage. [21] Chitosan polyplexes are easy to prepare and their size and stability varies with

CS MW and CS/DNA ratios. [21] Besides MW and N/P ratio, several parameters can influence polyplex size but concentration of desolvating agent (*e.g.* sodium sulphate) and plasmid size (ranging from 5.1–11.9 kb) does not have an effect. [33] Several polyplex preparation methods can be used including complex coacervation, ionic gelation, matrix inclusion and the use of self-aggregating modified chitosans. [30] Also, various methods can be used to determine the extent of DNA complexation or encapsulation into CS polyplexes, such as gel electrophoresis, Pico Green assay and photoelectric methods. [21]

Transfection efficiency is influenced by several factors: degree of acetylation, CS molecular weight, CS/DNA ratios, serum concentration and pH of the transfection medium, and physicochemical properties of polyplexes (Fig. 1.3). [9, 21] The degree of acetylation determines the amount of amines available to interact with nucleic acids and influences degradability, inflammation and immune responses. It also has been shown to influence polyplex size and as the degree of deacetylation decreases there is a slight increase in size. This effect has been related to a weaker binding of chitosan to nucleic acids and to stiffening of the polymer chain. [30]

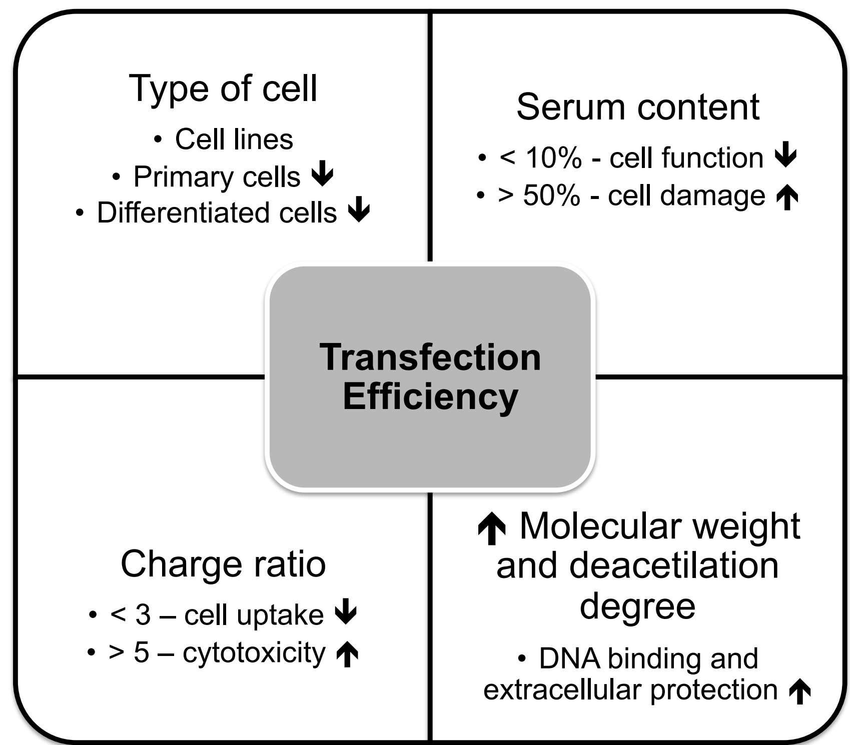

Figure 1.3 – Schematic representation of factors affection chitosan polyplex transfection. Adapted from [34].

Considering CS MW, it is necessary to balance several factors in order to choose an optimal MW. Chitosan with high MW has shown better DNA complexation and stability although it also showed aggregation and low solubility at neutral pH. [35, 36] Whereas CS with low MW has a more efficient intracellular release associated with low complexation. [37]

One of the key advantages of CS polyplexes compared to other non-viral systems is their relatively high transfection efficiency in serum that allow *in vitro* studies to be conducted in physiologically relevant conditions. [30, 38, 39] Several groups have shown a cell type dependency in transfection efficiency with significant differences in transfection levels between different cell types given comparable transfection conditions. [14, 40, 41] One of the explanations to this cell type dependency is that it is related to the preferred endocytic pathways used to internalize polyplexes and not the level of uptake. In a study by Douglas *et al*. cell lines preferring clathrin mediated pathways, such as HEK293 and COS7, showed increased transfection while cell lines preferring caveolin-mediated endocytosis did not. [42] This cell type dependency is not unique to CS and it should be considered when selecting and designing a gene therapy strategy.

The mechanism of transfection with CS polyplexes is still not fully elucidated. A major drawback to the understanding of this mechanism is that most transfection studies rely entirely on end-point quantification of reporter genes to determine efficacy and potential formulation improvements. [30] According to Ishiii *et al*. CS/DNA polyplexes condense and form large aggregates, which are then adsorbed at the cell surface. Polyplexes are endocytosed and released from endosomes due to swelling of endosomes along with the swelling of the polyplexes, causing the rupture of the endosome. Finally, polyplex accumulation in the nucleus is observed. [43]

Several studies have showed, using different cell lines, that cellular uptake is not problematic and that variations in cellular uptake do not necessarily lead to variations in transfection. [30, 37, 44] Other studies indicate that lysosomal sequestration is one of the bottlenecks CS transfection has to overcome. This rate-limiting step is not affected by MW or degree of acetylation and is responsible for the delayed gene expression observed, generally 48 h after transfection. [30, 45] Excess free polymers can help transfection by promoting polyplex release from lysosomes. [30] Polyplex dissociation is also a rate-limiting step and has been evaluated through

the use of techniques such as fluorescent resonant energy transfer (FRET). Both quantum dots and organic dyes can be used to monitor dissociation. Using the latter, Thibault *et al.* have shown that polyplex dissociation kinetics was the most critical formulation-dependent intracellular process and could correlate transfection efficiency with CS structure, confirming the effect of MW and degree of acetylation on optimal binding and consequent fine-balance. [46]

Some of the limitations CS presents are its insolubility at physiological pH, insufficient charge and low transfection efficiency. Chitosan has different chemical groups (amine and hydroxyl) that can be easily modified to enhance gene delivery. [9] Efforts to achieve higher transfection efficiencies and overcome the aforementioned limitations include various chemical modifications that are described in the next section of this chapter.

Chitosan derivatives: modifications and optimizations

Approaches to design and optimize polymer chemical structures can include either the rational design of the chemical structure taking into account identified biological barriers to gene transfer or the systematic modification of synthetic polymers to establish structure–activity relationships. [18] For example, a polymer library of over 2000 biodegradable poly(β-amino esters) with different MWs revealed that amine alcohol formulations performed better in terms of cell viability and transfection efficiency. [47]

As mentioned above, polymer modifications are done to address specific issues. In the case of CS numerous modifications have been investigated that aimed at the enhancement of solubility at physiological pH, enhancement of colloidal stability, and increase of proton sponge capacity. [30] Chitosan modification can be achieved by chemical grafting in the C2 amine groups or C6 hydroxyl groups. Modifications in chitosan's amine functional group can reduce electrostatic interactions with nucleic acids and create steric hindrances while modifications in the hydroxyl group are generally more difficult to achieve due to the high reactivity of the amine groups. [30] Examples of the most common CS modifications are given next and are summarized in Table 1.4.

In order to modulate CS solubility, hydrophobic and hydrophilic modifications have been tested to obtain CS derivatives with more favorable characteristics. On one hand, hydrophobic modifications with deoxycholic acid and thiolation have showed improved cell binding, reduced serum inhibition, DNA protection from degradation, and enabled pDNA internalization. On the other hand, hydrophilic modifications using PEGylation and quaternization also enhanced transfection efficiency due to increased solubility at physiological pH and improved intracellular pDNA release. Both hydrophobic and hydrophilic modifications present different merits that have promoted the evaluation of amphiphilic CS derivatives. [9]

Several investigators reported that the incorporation of hydrophobic moieties by alkylation into the chain backbone of CS increased transfection efficiency. [21] In a study by Liu *et al.* the incorporation of hydrophobic alkyl groups into CS chain backbone with alkyl bromide not only increased the transfection efficiency but also facilitated cellular entry and increased the unpacking of DNA in the nucleus. [48]

As mentioned above, quaternization has been used to improve CS solubility at physiological pH. Quaternization can be achieved by *N*-trialkylation using halogenoalkanes (typically CH_3I) or the reaction with a quaternized epoxide. [30] For example, *N*-trimethylated CS oligomers synthetized by reductive methylation showed excellent solubility in water at different pH values. The improvement in solubility of the quaternized amine is related to the permanent positive charge, independent of pH. However, increasing degrees of trimethylation lead to an increase in toxicity of both oligomers and polymer. [49]

Amidation can be done through the use of carboxylic compounds with carbodiimides such as EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) or DCC (dicyclohexyl carbodiimide) coupled with NHS (*N*-hydroxysulfosuccinimide) to accelerate the reaction. This method has been used to incorporate several different species, such as amino acids, imidazole derived acids and PEG. [30] For example, incorporation of amino acids has shown improvements in terms of solubility, cytotoxicity, cellular uptake, nuclear localization, and transfection efficiency. [50]

Reductive *N*-alkylation allows the engraftment of aldehyde derivates to CS and has been used to incorporate several different species, such as fluorescent probes, phosphorylcholine and dextran. [30]

Table 1. 4 – Modifications on the amine functional group of chitosan. Adapted from [30]

Type of modification	Purpose	Reagents
Methylation	Improve solubility	CH_3I
Amidation	Improve phamacokinetics	PEG-succinimidyl ester
	Improve uptake	Arginine
	Enhance proton sponge effect	Histidine
	Target liver cells	Galactosylated lactobionic acid
	Improve solubility	PEG-succinimidyl ester
	Fluorescent chitosan	Texas Red Succinimidyl Ester
Reductive *N*-alkylation	Improve polyplex stability	Dextran-aldehyde
	Fluorescent chitosan	9-Anthraldehyde
	Improve solubility	Phosphorylcholine-glyceraldehyde
Thiourethane	Fluorescent chitosan	Rhodamine isothiocyanate
Reductive *N*-alkylation to oxidized chitosan	Decrease cytotoxicity and improve transfection efficiency	PEI
Dissulfide bond	Improve endosomal escape	2-Iminothiolane and then thiolated histidine dendron

Examples from the literature have also shown improved transfection by modification of CS with deoxycholic acid. In one approach, polyplexes were prepared as self-aggregates by sonication and their performance was compared at different serum concentrations. It was concluded that in the presence of serum, serum proteins inhibit complex formation by weakening the electrostatic interactions

between self-aggregates and DNA, resulting in increased transfection. In another approach, conjugates were synthesized by a coupling reaction with the *N*-hydroxysuccinimide ester of deoxycholic acid to achieve improved CS solubility, reduced toxicity and increased DNA unpacking after cellular uptake of polyplexes. It was also found that transfection efficiency decreased with an increasing degree of substitution. [21]

Reduction sensitive cationic polymers can suffer rapid degradation via thiol-disulfide exchange due to the high levels of glutathione in the cytosol. This can contribute to a reduction in toxicity by preventing the buildup of high molecular weight polymers inside the cell and can improve intracellular nucleic acid release. [51] Studies have shown that thiolated CS has improved mucoadhesiveness and cell penetration and can form intra and intermolecular disulfide bonds that allows crosslinking. [52, 53] Thiolation can be achieved by reaction with thioglycolic acid and carbodiimides, as described above for the amidation process. Lee *et al.* demonstrated that thiolated CS enhanced gene expression and that by crosslink a slow, sustained release of pDNA could be achieved. [52]

Hyaluronic acid and its derivatives

Hyaluronic acid (HA), also referred to as hyaluronan, is a biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory natural polymer. HA unique viscoelastic, biocompatible and non-immunogenic properties have led to its use in numerous biomedical applications, including drug delivery, ocular surgery, tissue engineering, joint fluid supplementation in arthritis and regeneration of surgical wounds. [54, 55]

HA is an anionic linear polysaccharide composed of alternating units of D-glucuronic acid and *N*-acetyl-D- glucosamine linked by a $\beta(1\rightarrow4)$ glycosidic bond. [54] HA is abundant in the extracellular matrix (ECM) of almost all animal tissues and it can be found in significant amounts in synovial fluid, skin, lung, intestine, brain, vitreous of the eye, umbilical cord and blood. [54, 56] It can be obtained commercially from animal tissues and microbial fermentations. [54]

HA can regulate tissue hydration and osmotic balance and its viscoelasticity can promote cushioning and lubrication to cartilaginous joints, aqueous humor and synovial fluid. [56] HA has been associated with cell migration and proliferation with

important roles in wound healing, cell motility, angiogenesis and composition of ECM. It has also been shown that HA function can be dependent on MW, with high MW HA maintaining cell integrity and water content in ECM while smaller fragments can induce receptor-mediated intracellular signaling. [54] HA degradation, in mammals, is carried out by hyaluronidase (hyase), *β*-D-glucuronidase, and *β*-*N*-acetyl-hexosaminidase that can be found in the intracellular milieu or in serum. [55]

HA can interact with several specific receptors such as cluster determinant 44 (CD44), receptor for hyaluronate-mediated motility (RHAMM), HA receptor for endocytosis (HARE) and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) that are associated with various biological functions such as endocytosis, degradation and signal transduction. [54, 56] Cell surface RHAMM has been involved in the mediation of cell adhesion and migration in both normal and tumor cells in response to oligo-HA following activation of the platelet-derived growth factor receptor (PDGF) and subsequent activation of c-src and ras-ERK pathways. [56] CD44 is a glycoprotein expressed in most cell types that mediates cell adhesion and homotypic cell aggregation and can non-covalently bind HA to the surface of endothelial cells hence facilitating its interaction with cellular surface. [55, 56] CD44 and RHAMM overexpression in cancer cell is well documented and has been associated with cancer progression. [56]

Appropriate modifications of the original polymers can make them targeted gene delivery systems to specific cell types, and can also improve their transfection efficiency, as well as extend their residence time once delivered *in vivo*. [21] HA can be easily degraded and its physiological turnover is quite fast. However, this can hamper long-term applications and so, several modifications have been tested in order to extend HA half-life time. Modifications are mainly on hydroxyl and carboxyl groups and include esterification, and activation of HA with carbodiimide followed by crosslinking with divinyl sulfone, glycidyl ether or dialdehyde. [54] Since carboxyl groups are necessary for HA receptor recognition the chemical modification of these groups can lead to changes in the biological properties of HA. Studies indicate that modifications up to 25mol % did not show a significant effect in receptor-mediated endocytosis. [54]

Various functional groups can be added to HA using the aforementioned methods. Of particular interest to gene therapy is the introduction of amine groups

that can be achieved by conjugation with adipic acid dihydrazide (ADH), hexamethylenediamine (HMDA), or cystamine. [54, 57, 58] Introduction of thiol functional groups can be achieved by conjugation with cystamine followed by reduction with dithiothreitol (DTT). [58] Other examples of HA modification are listed in Table 1. 5.

HA can also be used to prepare hydrogels either by direct crosslink of HA or HA derivatives. Crosslinking can occur with the addition of a crosslinker to the reaction mixture, such as bis(sulfosuccinimidyl)suberate or via Michael addition with dithiols or peptide linkers with cysteine groups. [54]

Table 1.5 – Examples of hyaluronic acid derivatives. Adapted from [54]

Modified HA	**Added compound**	**Conjugation reagent***
HA-NHS	*N*-Hydroxy succinimide	EDC, NHS
HA-ALD	Aldehyde	Sodium periodate
HA-DOPA	Dopamine	EDC, sulfo-NHS
HA-Tyramine	Tyramine	EDC, sulfo-NHS
HA-AEMA	Aminoethyl methacrylate	BOP, DIPEA
HA-VS	Vinyl sulfone	BOP, DIPEA
HA-I	NaI	Bromoacetic anhydride

*EDAC – 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide, NHS – *N*-hydroxysuccinimide, BOP – benzotriazol-1-yloxy-tris(dimethylamino) phosphonium hexafluorophosphate, DIPEA - *N,N*-diisopropylethylamine.

HA applications in nucleic acid delivery can be divided in two categories: *i*) HA or HA derivatives can form hydrogels that incorporate nucleic acids or ii) HA or HA derivatives can coat cationic carriers.

The use of hydrogels has been mostly in the tissue engineering and regeneration field. Kim *et al.*, were one of the first to use a HA matrix to deliver plasmid DNA. HA was derived and crosslinked with adipic dihydrazide to prepare hydrogels for the delivery of DNA encoding a model cytokine, PDGF. DNA protection from enzymatic degradation as well as sustained DNA release was observed. Matrix degradation and DNA release kinetics could be modulated varying incubation time with crosslinkers or varying DNA loading. [59] Using a similar approach, Yun *et al.*, were able to produce microspheres with extended DNA release profiles (up to 2

months) and adaptable for targeting specific cellular receptors distinguishing between E- and P-selectin expressing cells. [60]

Some of the problems that can arise with the use of cationic carriers are cytotoxicity and low serum stability due to their positively charged surface. Anionic glycosaminoglycans, like HA, have been used to modify the surface of these carriers. Examples include the use of HA with CS [61, 62], PEI [63], PLL and liposomes. [54] The use of a ternary complex consisting of DNA, linear PEI and HA showed increased transfection efficiency associated with improved DNA stability in the extracellular environment and at the early stages of intracellular trafficking as well as timely dissociation of the polyplex. [63] Also, HA can enhance transcription of PEI polyplexes by loosening DNA-PEI electrostatic interactions and allowing easier access to transcription factors. [64] On another study two polysaccharides, HA and CS, were used to produce polyplexes for corneal gene delivery. The rational for the incorporation of HA in polyplexes was to increase cellular targeting using the specific HA receptor CD44 that is present in ocular tissues and is involved in receptor-mediated internalization as well as HA degradation. Polyplexes containing HA were able to achieve relatively high transfection levels and were internalized by endocytosis in a process mediated by the CD44 receptor. [62]

The HA receptor-mediated endocytosis has been exploited in several other studies. Lee *et al*. used a thiolated HA derivative and crosslinked it via disulfide bond formation to produce nanogels and deliver siRNA. They compared cellular uptake and gene-silencing efficiency in a cell line over-expressing CD44, HCT-116 cells, and in a CD44 deficient cell line, NIH-3T3 cells. CD44 receptor positive cells on the surface readily took up HA nanogels. Also, siRNA release rates could be modulated by the concentration of glutathione indicating that an intracellular reductive agent controlled the release pattern of siRNA. [58]

3. General obstacles to gene therapy

A successful gene therapy strategy has to overcome extracellular and intracellular obstacles as well as achieve persistent gene expression. It is important to understand how these obstacles hinder gene transfer in order to allow a more effective polymer modification and better vector design.

Extracellular barriers: Undesirable interactions and targeting

In order to reach the target cells, polyplexes have to overcome several extracellular physical barriers as well as remain stable in blood and other biological fluids. Strategies must take into account the physicochemical properties such as size, shape and flexibility, overall charge, charge density, and non-electrostatic interactions at the surface of the polyplex. [65]

Whereas *in vitro* gene delivery is mostly concerned with intracellular barriers, *in vivo* gene delivery must also consider extracellular barriers, where the choice of administration route has great importance. General delivery routes as wells as specific ocular delivery routes are described in section 4.1.

When administered *in vivo,* polyplexes interact with different factors (salts, lipids, carbohydrates, proteins, and enzymes) that can affect their stability and bioavailabity due to aggregation, dissociation, or degradation. Interactions with non-target cells or tissues can also be problematic. The use of receptor-specific ligands can be used to answer this problem and target specific cells. However, this does not prevent non-specific interactions and internalization via other methods. The administration of polyplexes can also elicit an immune response including phagocytosis, inflammation, and other non-specific mechanisms such as the complement system that will lead to the destruction of the carriers. One of the strategies to overcome the above-mentioned issues is to resort to *ex vivo* gene transfer where cells are transfected in a cell culture environment and then transplanted to the host. [65]

Intracellular barriers: Cell entry, endosomal escape and nuclear entry

Once vectors reach the target cells they need to be internalized in order to fulfill their role (Fig. 1.4). Cellular uptake is achieved by endocytosis in a process that involves the invagination of the cellular membrane and the formation of vesicles. Endocytosis can be classified as macropinocytosis, phagocytosis and receptor-mediated endocytosis (clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis). [24] Phagocytosis is usually only carried out by specialized cells, such as monocytes, macrophages and neutrophils, and is not ideal for polyplex uptake. Macropinocytosis

is a more prevalent form of endocytosis and involves charge-mediated interactions with proteoglycans that are present on the cell surface and the formation of large vesicles. [23, 24] Although polyplexes can enter the cell through macropinocytosis the biggest contributor to polyplex uptake is receptor mediated or dependent endocytosis. Among these caveolae-mediated endocytosis has been the most studied because it bypasses the lysosome. [23]

Polyplex uptake pathways vary with their structure, composition, size and also with the type of cell, since different cell lines utilize uptake pathways differently. Larger particles are internalized via micropinocytosis, whereas smaller polyplexes (≤ 200 nm) are generally taken up through the clathrin-mediated pathway. [23]

Upon cell entry the endocytic vesicles containing the polyplexes can have 3 different destinations: *i*) recycling back to the cell surface, *ii*) turning into acidic degradative vesicles (*e.g.* lysosome or phagosome), or *iii*) delivery to an intracellular organelle (*e.g.* Golgi apparatus or endoplasmic reticulum) depending on the internalization pathway. Polyplexes can be trapped within endosomal vesicles and carried through the endo-lysosomal pathway. In order to avoid enzymatic degradation within the lysosomal compartment polyplexes need to escape from theses vesicles. [24]

Cationic protonable polymers can induce endosomal escape through the proton sponge effect. The proton sponge hypothesis states that at physiological pH only a few nitrogen atoms in the amine groups of the polymer are protonated. In the endosomal compartment, the pH will decrease and the amount of protonated nitrogen increases generating a charge gradient that leads to a Cl^{-} influx. This influx will increase Cl^{-} concentration inducing a water influx that will eventually lead to endosome swelling and rupture. [18] Although widely accepted the proton sponge hypothesis may not be valid for polymers with a buffer capacity at pH around 5. [18] Further studies are required to understand the molecular mechanisms of polyplex behavior in the endosomal compartment and interaction with endosomal membranes.

Endosomal escape can also be promoted by the use of vesicle-disturbing peptides conjugated to polyplexes or the use of chemicals agents, often referred to as lysosomotropic agents, such as weak bases (*e.g.* chloroquine, procaine, and spermidine) that can promote pH buffering in endosomal vesicles. [23, 65]

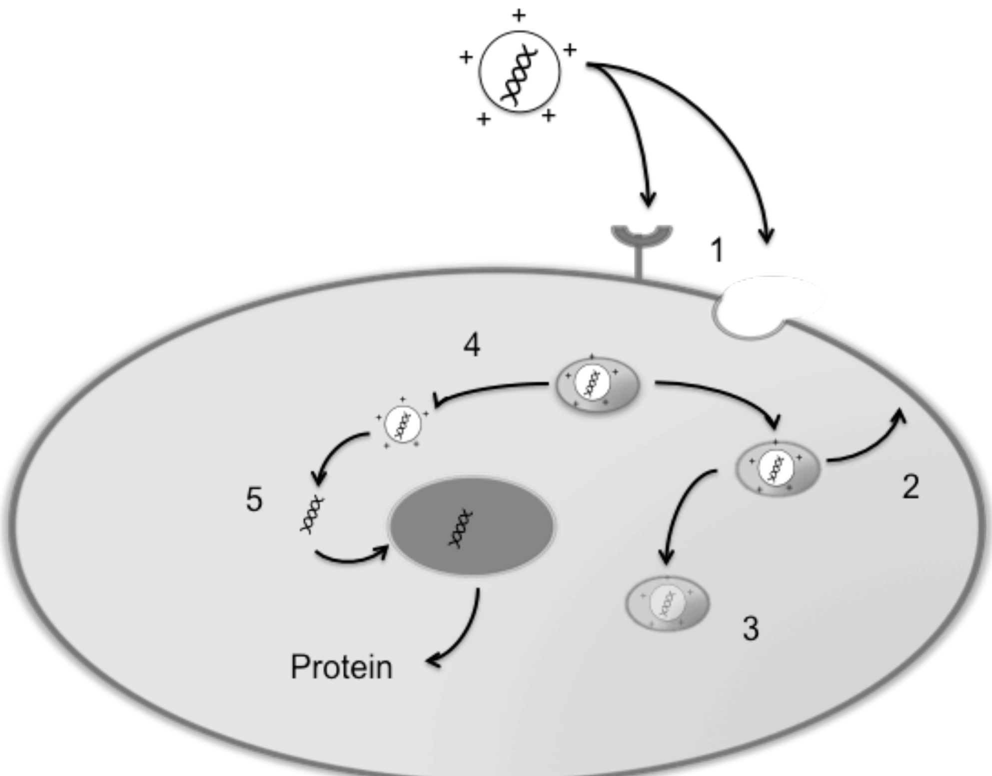

Figure 1.4 – Cellular trafficking and intracellular barriers of non-viral vectors. 1) Cellular uptake can occur by receptor-mediated endocytosis or non-specific endocytosis. 2) After uptake endocytic vesicles can be removed by exocytosis. 3) Vectors in endocytic vesicles can also be degraded in late endosomes. 4) Vectors that can escape the endosomes will need to navigate the cytoplasm and dissociate. 5) Upon dissociation the therapeutic DNA must reach the nucleus and recruit the necessary transcriptional machinery to yield a therapeutic protein. Adapted from [1].

Upon endosomal escape polyplexes need to reach the nucleus, as well as dissociate. A limiting step in this process is the polymer's high affinity for DNA that will difficult the separation of the DNA and polyplex dissociation. Chain length and polymer molecular weight are key factors that dictate the efficiency of DNA release and should be optimized for each polymer used for transfection. [23] Dissociation is much faster when short polymers are used and this can significantly increase short-term gene expression both *in vitro* and *in vivo*. [66] Strategies to improve DNA release include polymer modification to create reducible polymers that can be degraded in the intracellular milieu. [18, 67]

Plasmid DNA can achieve nuclear entry by two different mechanisms: either by passive diffusion when there is nuclear membrane disruption during cell division, or by active transport through receptors in the nuclear membrane. [18] Large molecules can be actively transported to the nucleus via nuclear pore complexes

embedded in the nuclear membrane by recognition of NLS. These have been previously employed to improve nuclear import and the most widely used are NLS from the SV40 virus large T-antigen (PKKKRKV). [23]

Transient gene expression

A successful gene therapy strategy requires long-term and sustained transgene expression levels both *in vitro* as well as *in vivo*. Persistence of the transferred DNA within the nucleus and gene expression can be compromised by several factors: *i*) degradation by intranuclear nucleases; *ii*) DNA loss during cell division; *iii*) transfected cell loss due to apoptotic, inflammatory, or immune responses; *iv*) gene silencing by transcriptional shut-off; and *v*) inefficient intranuclear trafficking. [65]

There are different ways to surpass transient gene expression and the most promising include genomic integration, minicircle DNA and episomal replication. The Sleeping Beauty transposon is the most extensively studied integration system for gene therapy applications. Transposons are naturally occurring DNA sequences capable of enzymatically excising themselves, through the activity of a self-encoded transposase component, out of one chromosomal locus and re-inserting itself into another locus. However, transposon-mediated integration is limited by random integration that can cause serious side effects. [24]

One of the most promising strategies to promote integration and long-term expression exploits a site-specific recombinase, the phage phiC31 integrase. PhiC31 integrase catalyzes recombination between attachment sites on phage and bacterial genomes, *attP* and *attB*, respectively. [68, 69] PhiC31 integrase has also been shown to promote site-specific integration in mammalian cells using pseudo *attP* sites endogenous to the mammalian genome, typically confined to a few chromosomal hotspots. [69, 70] This sequence specificity makes this system superior to viral systems that rely on integration for persistence of expression due to the low risk of insertional mutagenesis. This system has been tested in rat retina and transgene expression was observed for up to 4.5 months. [68]

Another promising strategy involves vector modifications to overcome transgene silencing. Minicircle technology is based on the use of a site-specific intramolecular recombination site in the plasmid. Upon induction the plasmid can

recombine originating two circular fragments, one containing the expression cassette and one containing the prokaryotic sequence. The minicircle containing the expression cassette can be purified and used for gene therapy with persistent expression. [71]

The ideal gene therapy vector should rely on chromosomal elements and act as an independent functional unit after integration into the genome or retention as an episome. Mammalian gene expression and replication can be regulated by numerous elements such as enhancers, locus control regions, boundary elements, insulators, scaffold- or matrix-attachment regions (S/MARs) and CpG depletion. [71, 72] These elements can be used in vector design to create replicating episomal vectors as expression systems in mammalian cells without interfering with the host genome. [71] For example, enhancers are DNA sequences that sequester transcription factors to increase gene transcription. The combination as well as the location of the enhancer and promoter within a plasmid vector is very important for expression augmentation and several combinations have been studied to maximize gene expression. Some of the enhancers tested for ocular tissues are the human inter-photoreceptor retinoid binding protein (hIRBP) promoter and GDEP (gene differentially expressed in prostate cancer) enhancer. [72]

4. The eye as a therapeutic target

In this section, the characteristics of the eye as a therapeutic target will be addressed as well as specific delivery routes and obstacles that ocular gene delivery faces.

The eye can be divided in two anatomical regions: the anterior segment, where the cornea, lens and conjunctiva are the main prominent structures, and the posterior segment, where the retina plays important functions. [73] The anterior segment is responsible for focusing light onto the photoreceptor cells in the retina in the posterior segment while photoreceptors are responsible for converting the light image to electrical signals and transmitting it to the brain via the optic nerve to enable vision. [74]

The cornea is a transparent and avascular tissue composed of five to seven layers characterized by high resistance to passive diffusion and withstanding the intraocular pressure. The cornea has a small surface area compared to the

conjunctiva, which is composed of more permeable epithelium. Previously it was though that the conjunctiva had a mainly protective role and functioned as a passive physical barrier. Currently it is known that there are several transporters that perform a significant role in the influx and efflux transport in the conjunctiva. The mucus layer covers the cornea and conjunctiva and forms part of the tear film, which is secreted by the goblet cells of the conjunctiva. The lachrymal film has multifunctional properties, such as hydration, cleanness, lubrication and defense against pathogens. Furthermore, the lachrymal film is a dynamic fluid that is in constant renewal, which limits the exposure time of pharmaceuticals on the eye surface. [73]

The posterior segment of the eye is comprised of three layers, the sclera, choroid and retina that surround the vitreous cavity, which is filled by the vitreous, a transparent gel composed of water, collagen, HA and proteoglycans. The vitreous acts as a shock absorber and supports the shape of the eye by keeping the retina flat against Bruch's membrane. The sclera is a tough outer layer constituted largely of connective tissue with protective functions and it helps maintaining the eye shape. The choroid is a vascular layer, which in conjunction with the retinal blood supply feeds the blood supply supporting retinal cells. The retina is the sensory inner layer of the posterior segment of the eye and is separated from the choroid by Bruch's membrane. [74]

The retina is comprised by five major neuronal cell classes: *i*) the Müller glial cells that provide metabolic and homeostatic support; *ii*) photoreceptors that convert light energy to membrane potential changes leading to neurotransmitter release and that can be classified as rods and cones; *iii*) horizontal cells that modulate synaptic transmission between photoreceptors and bipolar cells; *iv*) bipolar cells that are involved in the synaptic transmission from the photoreceptors and contact retinal ganglion cells and amacrine cells; *v*) ganglion cells that function as output neurons of the retina. [75] The retinal pigment epithelium (RPE) is a monolayer of pigmented epithelial cells that line the back of the retina and is responsible for multiple functions important for the maintenance of the neural retina and vision, including nourishment and protection of photoreceptor cells, absorption of excess light entering the retina, secretion of growth factors, and recycling retinoids as part of the visual cycle. The apical membrane of the RPE faces the photoreceptor outer segments and the basolateral membrane faces Bruch’s membrane, which separates the RPE from fenestrated endothelium of the choriocapillaris. [76] Additionally, tight junctions

between RPE cells form the blood retina barrier that supports the eye's immune privileged status. [72, 76]

The eye as a therapeutic target for gene therapy has several advantages: *i*) easy access to various ocular tissues that can be targeted by different delivery options, *ii*) immune privileged situation that arises from existence of the blood-ocular barrier, which limits systemic exposure, and *iii*) existence of animal models for retinal diseases and easy assessment of structural and functional rescue after treatment that allow the eye to be a useful model for both proof-of-principle and clinical studies. [71]

The phagocytotic nature of the RPE makes it a great target for gene delivery since the exogenous genetic material can be efficiently internalized, probably through interactions with RGD binding domains. The vast majority of gene delivery vectors have transduced the RPE with a high degree of efficacy. [72, 77] Comparatively to the RPE, gene delivery to photoreceptors is much more difficult probably due to their non-phagocytotic nature as well as the continuous movement of outer segment discs away from the nucleus. This tightly packed lipid rich discs can also act as an additional physical barrier between the subretinal space and the cell nucleus located in the outer nuclear layer. [77]

Clinical endpoints in many of the proof of principle studies use electrophysiological data as an indicator of retinal function improvement assessed by electroretinograms (ERGs), which can be recorded noninvasively. Most ocular diseases are bilaterally symmetrical so the contralateral eye can serve as control for a better interpretation of results. In terminal studies this data can also be supplemented with histological/ immunohistochemical data. [65]

Several ophthalmological diseases are chronic and progressive, such as macular degeneration and glaucoma while others are due to genetic mutations, like retinal degeneration. [78] The genetic basis of a wide range of inherited retinal diseases has been identified and over 261 retinal disease genes and loci have been mapped (http://www.sph.uth.tmc.edu/RetNet/). [79] Several animal models of these mutations have been generated to help develop therapeutic strategies and design treatments.

4.1. Ocular delivery routes

In systemic administration carriers are generally administered intravenously, where the circulatory system as well as various non-target cells and organs are major obstacles. On the other hand, in local administration, *e.g.* direct injection into the target area, carriers do not face circulatory issues but still face barriers such as extracellular matrix or inflammatory and immune responses. [65] Also, in local administration DNA characteristics limit its diffusion and absorption upon injection since both processes are affected by molecular weight. DNA delivery with polyplexes is also affected by size and net charge that limits diffusion and generally results in transfection restricted to the injection site. [7]

One of the major drawbacks of systemic administration for ocular purposes is that only 1–2% of the administered drugs reaches the vitreous. [80] This is due to the existence of the blood–retinal barrier that is selectively permeable to more lipophilic molecules and determines the entry of drug molecules into the posterior segment of the eye. [73]

The eye is a small and complex organ separated from the rest of the body by multiple layers of biological barriers. Also, intraocular structures and tissue are protected from the external environment by tight junctions of the corneal epithelium and the mucosal surface. Therefore, it is necessary to bypass these protective barriers in order to achieve therapeutic effectiveness. [80] The eye's multiple tissues can be easily accessed and targeted by therapeutic agents using different delivery options.

Surface or topical instillation

Topical administration as a non-invasive technique is clinically favored and would be the most convenient gene delivery route. [73] Surface instillation can be used to target corneal epithelium, but clearance mechanisms of the corneal surface, which include lacrimation, tear dilution, and tear turnover results in limited bioavailability. [71, 73, 80] Also, due to DNA's size and charge it is unable to cross the cornea and transfection is generally limited to the superficial epithelial layer. [73]

Intracameral injection

Injections into the anterior chamber of the eye can be used to target corneal endothelium, iris and aqueous outflow pathway, which have especially relevant application to research on aqueous outflow and glaucoma. However, this delivery method has the same drawback as surface instillation, and it is difficult to maintain therapeutical vector concentrations. [71]

Subconjunctival injection

Subconjunctival injection is a less invasive delivery method compared to any of the intraorbital injections and allows delivery of large volumes as well as repeated injections without significant adverse effects from the procedure. However, the blood-ocular barrier limits its utility and only extraocular cells are accessible this way. Moreover, this delivery method has also been associated with systemic exposure due to the presence of lymphatic and blood vessels and extraocular side effects may be a problem. [71, 73]

Intravitreal injection

Intravitreal injection is a common delivery method in both human and animal models and it can be used to target the optic nerve, lens, inner retina and occasionally the outer retina or the anterior chamber. However, it faces the same problems as intracameral injection and repeated application may be necessary which might lead to lens damage, retinal detachment, endophthalmitis, intravitreal hemorrhage and increased risk of cataract development. [71, 73, 80] The vitreous consists mainly of collagen, HA, and proteoglycans containing chondroitin sulfate and heparan sulfate so diffusion of the therapeutic material can be challenging. [73]

Subretinal injection

Subretinal injection is a very precise way to target posterior retinal layers like the photoreceptors and the RPE, but uptake can be limited to the site of injection. Additionally, subretinal injections can cause lesions of RPE cells, hemorrhages,

retinal tears, sub- or pre-retinal fibrosis and uncontrolled retinal detachment. [78] Although technically challenging, subretinal injection is a clinically viable procedure. [71]

5. Diseases and current treatments

There are several blinding ocular diseases requiring therapeutic treatment. Most of these diseases are associated with intraocular structures located in the posterior part of the eye as the retina, macula, RPE, or choroid. Local infections or blinding disorders such as chronic uveitis, glaucoma, and choroidal neovascularization are usually treated with anti-inflammatory drugs, anti-bacterial agents, or even angiogenesis inhibitors; however, the efficiency is limited by the variety of aforementioned ocular barriers. [80] So far, biomaterials have been used mainly to provide positional support or sustained release drug therapy. [74]

Ideally an ocular delivery system should ensure *i*) controlled and sustained release in order to maintain therapeutic concentration and reduce frequency of administration; *ii*) specific targeting and prolonged retention in the affected tissues to improve efficiency and reduce side effects; and *iii*) patient-friendly delivery routes that minimize side effects due to the administration method. [80] One of the major difficulties in ocular therapeutics is to maintain an effective concentration at the site of action for an appropriate period of time, in order to achieve the expected therapeutic response. [73]

Current treatment strategies for retina-associated diseases include oral/intravitreal administration of retinoids, delivery of agents designed to alleviate endoplasmatic reticulum stress, and cell transplantation. Replacement of dying photoreceptors and RPE with cells derived from human embryonic stem cells or retinal progenitor cells is a very promising strategy for ocular diseases. However, it is still limited by problems with cell integration and survival after transplantations. [72] Intravitreal injection of bevacizumab (Avastin) or ranibizumab (Lucentis) is being tested as treatment to vascular diseases associated with the posterior segment of the eye, including retinopathy of prematurity or choroidal neovascularization, respectively. [80]

Several non-viral carriers (microsphere, micelles, liposomes, hydrogels) have been considered for ocular drug delivery. Treatment for a variety of illnesses has

been tested (diabetic macular edema, ocular hypertension, dry eye, glaucoma, ocular neovascular activity) using different materials like CS-coated liposomes, PAMAM and PLGA. [74, 80]

Numerous retinal disorders including Leber's congenital amaurosis (LCA) and *retinitis pigmentosa* (RP) involve mutations in genes specific to the RPE like RPE65, bestrophin, and lecithin retinol acyl-transferase (LRAT). [72] Some of the identified mutations in genes associated with the visual cycle causing retinal degeneration are listed in Table 1.6. Other retinal diseases, such as choroidal neovascularization and age-related macular degeneration (AMD), are also related to defects in RPE structures, gene products, and metabolism. Blindness frequently develops due to photoreceptor loss or malfunction secondary to RPE degeneration or functional defect. [72] Some examples are given next.

Neovascularization

Ocular neovascularization is a common cause of blindness and visual impairment, and is characterized by the abnormal growth and proliferation of new blood vessels. [74] There are 3 types of ocular neovascularization: *i*) choroidal neovascularization that can occur in diseases of the Bruch's membrane/RPE cell layer, particularly neovascular age-related macular degeneration, the most prevalent cause of severe vision loss in persons over the age of 60 in developed countries; *ii*) retinal neovascularization can occur in ischemic retinopathies, such as diabetic retinopathy; and *iii*) corneal neovascularization that can occur as a consequence of corneal infection and/or inflammation, such as herpes simplex keratitis. [81] Retinal detachment is another important cause of blindness that is characterized by the separation of the neural retina from the underlying retinal pigment epithelium and it may be spontaneous or derive as a complication of other pathologies. Neovascularization and retinal detachment, as most posterior segment disorders, require treatment either by laser photocoagulation or surgery. [74]

Proliferative vitreoretinopathy

Proliferative vitreoretinopathy is a process characterized by the formation of retinal scar tissue and fibrosis, associated with the exertion of traction that can

ultimately cause retinal detachment. This can affect about 10% of retinal reattachment surgery patients and requires additional surgery and substantially reduces visual prognosis. [81]

Table 1.6 – Examples of mutations causing retinal disorders. [76, 77]

Mutated gene	**Associated diseases**	**Particularities**
ABCA4	Stargardt disease or *fundus flavimaculatus*	Maculopathy, cone-rod dystrophy and early loss of vision
CRALBP	*Retinitis punctata albescens*	Early-onset retinal dystrophy with the appearance of uniform white dots in the fundus picture
RDH5	*Fundus albipunctatus* night blindness	Strong bleaches and white dots in the fundus picture
RGR	*Retinitis pigmentosa*	Severe form of the disease
RPE65	Retinal dystrophies including Leber's congenital amaurosis	Early-onset retinal dystrophy
LRAT	Severe retinal dystrophy	Restricted visual field
MERTK	Leber's congenital amaurosis	Photoreceptor degeneration, childhood-onset rod-cone dystrophy and macular atrophy
CNGB3 and CNGA3	Achromatopsia or rod-monochromatism	Severe cone dysfunction
RHO	*Retinitis pigmentosa*	Mild to severe forms of the disease
CRB1	Leber's congenital amaurosis	Retinal thickening, absence of lamination and formation of photoreceptor rosettes
GUCY2D	Leber's congenital amaurosis	Cone-rod dystrophy
CEP290	Leber's congenital amaurosis	Photoreceptor dysfunction
MYO7A	Usher syndrome	Abnormal accumulation of opsin in photoreceptor cilium
CHM	Choroideremia	Progressive chorioretinal degeneration, night blindness and loss of peripheral visual field

Leber congenital amaurosis

Leber congenital amaurosis (LCA) is a severely debilitating disease characterized by reduced visual function, abnormal ocular movements (nystagmus), and is typically diagnosed during childhood. Several mutations in RPE genes have been associated with the development of LCA. The most common mutation is in gene RPE65, which codes for a conserved RPE specific 65 kDa protein. Mutations in this gene account for approximately 7% to 16% of LCA cases and can also cause other severe and early onset blinding diseases, including RP. [65] RPE65 mutations result in accumulation of all-trans retinol that causes cellular toxicity and while the fundus of LCA patients appears normal at birth, degeneration occurs within the first decade of life. [77] Mutations on photoreceptor-expressed genes are responsible for nearly half of LCA (~53%) and the most frequently mutated genes are CRB1 (10%), GUCY2D (12%) and CEP290 (15%). For example, CEP290 is a 290 kDa protein and one of the most abundant components of the centrosome. Within the retina, CEP290 is localized primarily to the basal body of the photoreceptor cilium, a structure that connects the photoreceptor inner and outer segments and is believed to participate in the intraflagellar transport of proteins between cellular compartments. CEP290 mutations result in inadequate protein transport across the cilium causing photoreceptor dysfunction. [77]

Achromatopsia

Achromatopsia, or rod-monochromatism results in severe cone dysfunction that can either be complete, with total dysfunction of all cone classes or incomplete, where one or more cone classes are partially functional. The resulting phenotype causes severe visual impairment but photoreceptor degeneration is generally mild or absent. This makes this disease an ideal candidate for retinal gene therapy since visual improvement could be achieved even in late stages of the disease. [77]

Retinitis pigmentosa

Retinitis pigmentosa (RP) is a genetic and phenotypic heterogeneous disorder characterized by progressive loss of photoreceptors. RP can be caused by an autosomal dominant (30-40%), autosomal recessive (50-60%) or X-linked (5-15%) mutation. Rhodopsin (RHO) mutations are involved in 25-30% of autosomal dominant RP, but only 1% of autosomal recessive RP, showing a tendency for rhodopsin mutants to abnormal gain-of-function. [77]

5.1. Retinal gene therapy

Regarding RPE-associated diseases, gene therapy is the most advanced treatment strategy. The most common viral vector used in pre-clinical and clinical gene transfer is adeno-associated virus (AAV) and there are extensive reports on AAV-based therapies. However, the usefulness of AAV vectors is limited by their small capacity that cannot accommodate large genes or promoter/ regulatory elements such as the RPE gene LRAT as well as photoreceptor genes such as ABCA4 (responsible for Stargardt's macular degeneration) and USH2A (responsible for Usher syndrome type 2). [72] An alternative solution to the limited cloning capacity of AAV, lentivirus and adenovirus is the use of helper-dependent adenovirus (Hd-Ad) vectors that have a cloning capacity of 36 Kb and are structurally equivalent to recombinant AAV vectors or the use of non-viral vectors. [82]

In the retinal gene therapy field AAVs are considered the least immunogenic of the viral vectors. However, there are reports of inflammatory responses, neutralizing antibodies and RPE atrophy following delivery of AAV into the subretinal space of non-human primates as well as T-cell responses to AAV capsid and local dropout of transgene expression. [82]

Some examples of current retinal gene delivery strategies and therapeutic approaches as well as examples of non-viral vectors applications as alternatives to the use of AAVs are described next.

Gene replacement and enhancement therapies

The choice of the appropriate therapeutic approach is very important, and usually autosomal recessive and X-linked disorders are treated through gene replacement therapy. This strategy can be very effective as long as there are no developmental abnormalities. [77] Treatment of LCA resulting from RPE gene mutations has several advantages, such as early diagnosis, preservation of photoreceptors until late stages of disease and the cooperation of the RPE to gene delivery, that make treatment with gene therapy quite promising. In fact several gene therapy trials are presently targeting RPE65 mutations. [77] One of the most successful gene replacement clinical trials was conducted in Briard dogs with a mutation in the RPE65. AAV serotype 2 were used to deliver RPE65 cDNA, which restored retinal function to mutant dogs after a single subretinal injection and with persistent results. [83] Currently, there are at least 7 active clinical trials using AAV-RPE65 for the development of treatment of RPE65-associated retinal diseases. [84]

Diseases that do not have a single genetic cause, like glaucoma and age-related macular degeneration can be treated by the expression of neurotrophic or anti-apoptotic factors or the suppression of angiogenic factors in a neuroprotective approach. [71, 77] With this strategy the main goal is to prolong the life span of the affected cells instead of correcting a specific gene function. This therapeutic approach is particularly promising in the case of neovascular age related macular degeneration, where the repeated administration of either bevacizumab (Avastin) or ranibizumab (Lucentis) is used to prevent angiogenesis. Gene therapy with long-term expression of an anti-angiogenic compound would greatly benefit patient care. [77] Researchers have used PEDF (pigment epithelial derived factor) to protect from ischemia reperfusion injury, delay the onset of RP and inhibit choroidal neovascularization. [71] Also, it has been evaluated in recent clinical trials for the treatment of age-related macular degeneration and proliferative diabetic retinopathy. [84] Other examples include the use of neurotrophic factors such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF) and glial-cell derived neurotrophic factor (GDNF) in the treatment of RP caused by rhodopsin mutations. [77]

Gene knockdown therapies

In the case of gain-of-function mutations strategies, the use of siRNA are currently being tested. The sequence specificity of siRNA allied to local administration in the relatively isolated confinement of the eye provides an ideal setting for eye-specific gene disruption. [71] One example of the utilization of siRNA is in neovascularization, namely in vascular endothelial growth factor (VEGF) targeted therapies, which has been associated with both corneal and choroidal neovascularization. Studies in a murine model of choroidal neovascularization induced by rupture of Bruch's membrane with laser photocoagulation showed that siRNA directed VEGF reduces rupture and leakage. The therapeutic potential is being explored in a phase I trial where the safety of intravitreous injection of siRNA directed against VEGF mRNA is being tested in patients with subfoveal choroidal neovascularization due to neovascular age-related macular degeneration. [81]

In a study by Kim *et al.*, administration of siRNAs targeting VEGF-A, VEGF receptor 1 (VEGFR1) or VEGFR2, or a mix of the three showed a significant inhibition of corneal neovascularization induced by herpes simplex infection or CpG oligodeoxy-nucleotides when given locally (subconjuntival injection) or systemically (systemic injection with commercial polymeric siRNA transfection reagent TargeTran®). [85]

Another example of this strategy targets ocular fibrosis by using siRNA directed at the transforming growth factor-b (TGFb) and its receptors. Reports show reduced TGFb receptor II expression, production of fibronectin, and migration in cultured human corneal fibroblasts. *In vivo* data, using a mouse model of induced subconjunctiva scarring, co-injection of siRNA significantly reduced the number of inflammatory cells and matrix deposition. [81]

Non-viral ocular gene delivery approaches

Few studies have focused the ocular distribution of DNA after topical ocular application. Hydrophilic molecules, like DNA, can enter both corneal and non-corneal epithelia via paracellular entry. However, corneal tight junctions act as a selective barrier and limit paracellular entry to very low molecular weight compounds. On the other hand, conjunctiva is a more permissive epithelium and has an important role in

the absorption of large hydrophilic molecules. [73] Still, it has been shown that topical administration of plasmids encoding for proteins with immunomodulating potential (IFN-α and IL-10) showed biological efficacy against viral infection and development of herpetic stromal keratitis. [78]

Studies have shown that DNA distribution in the retina upon intravitreal injection is dose and time dependent and that delivered DNA could be found in all neural layers and preferentially accumulated in RPE cells. An example of nucleotide delivery by intravitreal injection is the synthetic oligonucleotide, fomivirsen, which has been used to treat cytomegalovirus retinitis. [78] Electroporation has also been used to test DNA vectors in proof-of-principle studies in animal models, but side effects prevent it from being considered clinically viable. [71] However, recent reports have shown that electroporation can successfully deliver transgenes to choroidal and RPE cells with no ocular complications, namely no retinal detachment. [86, 87]

Liposomes have also been used for ocular delivery with surprising results considering the chosen delivery method: *i*) transgene expression in retinal ganglion cells was observed following topical instillation of cationic liposomes in rats, and *ii*) opsin promoter mediated gene expression was observed in the retina following intravenous administration without ectopic expression elsewhere in the rhesus monkey. However, liposomes also have some drawbacks and retinal toxicity has been observed upon liposome application as well as liposome aggregation that can interfere with vision. [71] Thus, liposome-based therapies for retinal disorders are not currently a very active research field. [72]

Biocompatibility and safety concerns have stimulated the development of biodegradable polymer-based gene therapies and some examples are given next.

Despite being one of the most studied polymers, there are few reports of PEI applications in the eye. Topical administration has showed positive results in the cornea but intravitreal injection has had limited success. [72] Poly(lactic) acid and poly(glycolic) acid polymers have effectively transfected RPE cells following intravitreal injection in rats with no significant toxicity. However, transfection only occurred in 10–35% of the exposed cells. [88]

Chitosan mediated gene delivery has shown positive results in the cornea and retina. Topical instillation of CS:HA polyplexes showed enhanced corneal transfection and extended expression compared to naked plasmid DNA. [62, 89] Stromal injection of CS polyplexes in rat corneas showed increased transgene

expression compared with PEI polyplexes. [90] Another study evaluated CS-mediated delivery of plasminogen kringle 5 (K5) on choroidal neovascularization and retinal inflammation. Administration was done by intravitreal injection and transfected mostly the inner retina. K5 expression was observed 2 weeks post injection and significant reductions in neovascularization and inflammation were observed. [91]

Compacted DNA polyethylene glycol/lysine polyplexes administered by intravitreal or subretinal injection in adult mice achieved reporter gene expression in retinal ganglion cells, cornea, lens, and trabecular meshwork and also in RPE and photoreceptor cells, respectively. More importantly, polyplex uptake was uniform throughout the retina after a single administration. [92] Koirala *et al.*, showed efficient photoreceptor targeting as well as visual function improvement in a RP model using CK30PEG10k-compacted DNA nanoparticles and using an RPE-specific reporter vector (VMD2-eGFP), they also achieved transgene expression in the retinal pigment epithelium. [93]

6. Specific aims

Despite the great potential of non-viral vectors, their application in ocular gene therapy is still in its infancy. In this work a multidisciplinary approach for the development of non-viral vectors for ocular gene therapy is presented.

In chapters III to VIII the development of several polymer-based vectors is described. The first aim was to formulate polymer-based vectors for retinal gene delivery using natural polymers, more specifically chitosan and hyaluronic acid. Optimizations to the polymer-based vectors included chemical modifications, namely chitosan thiolation and hyaluronic acid amidation, as well as mixtures of the aforementioned polymers. The produced vectors were extensively characterized regarding their physiochemical properties, namely size, zeta potential, morphology, DNA loading capacity and protection from nuclease degradation, and stability at physiological conditions and long-term.

The second aim was to functionally characterize the developed vectors *in vitro.* The *in vitro* characterization included evaluation of the cytotoxicity of polymers and produced vectors as well as the evaluation of transfection efficiency and gene expression in HEK293 and RPE cells (ARPE-19 and D407). Gene expression experiments included a commonly used reporter gene, GFP, and CEP290, considered a large gene (approximately 8kb) associated with the development of the retinal degenerative disease Leber's congenital amaurosis. In addition, long-term gene expression was also evaluated using phage C31 integrase to promote genome integration of the transgene.

The third aim was to evaluate the developed vectors in wild-type C57Bl6 mice for their functionality in transfecting mitotic (animals younger than 7 days, chapter VII) and post-mitotic cells (adult animals, chapter VIII) as well as sustained transgene expression.

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Chapter II

Materials and Methods

Chapter II – Materials and Methods

1. Plasmid constructs and cell lines

Four different plasmids (Fig. 2.1) were used:

i) pCMVeGFP-attB, expressing the reporter gene green fluorescent protein (GFP);

ii) pCMVCEP290attB, expressing the CEP290 gene (gene responsible for LCA 10), both driven by the cytomegalovirus (CMV) promoter and containing the attachment sites for integrase (attB);

iii) pCMVINT, an integrase expression plasmid and

iv) pCMVGFP, expressing enhanced green fluorescent protein (GFP) driven by the cytomegalovirus promoter (all kindly provided by Dr. Jean Bennett, University of Pennsylvania, USA) [1, 2]. Unless otherwise stated, the plasmid used in polyplex preparation was pCMVGFP. Plasmids were amplified in Top10 bacteria and purified using a Plasmid Maxi kit (Qiagen, USA) following manufacturer guidelines. Plasmid DNA (pDNA) was dissolved in TE buffer, and the concentration was determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) at 260 nm.

Four cell lines were used for cytotoxicity and/or transfection evaluation: human embryonic kidney HEK293T and HEK293 cells (kindly provided by Dr. Guilherme Ferreira, University of Algarve, Portugal and Dr. Jean Bennett, University of Pennsylvania, USA), and two human retinal pigment epithelial cells: D407 (kindly provided by Dr. Jean Bennett, University of Pennsylvania, USA) and ARPE-19 (kindly provided by Dr. Francisco Ambrósio, University of Coimbra).

2. Animals

C57BL/6 mice housed under controlled temperature and a 12 h light/dark cycle with food and water *ad libitum* were used for the *in vivo* experiments. All experimental procedures were carried out according to the Portuguese and European Union (FELASA) regulations for the use of animals and the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research. All procedures were performed under anesthesia (tribromoethanol, Sigma-Aldrich) induced by intraperitoneal injection (250 mg/kg).

Animals were humanely sacrificed by anesthesia and confirmed by cervical dislocation.

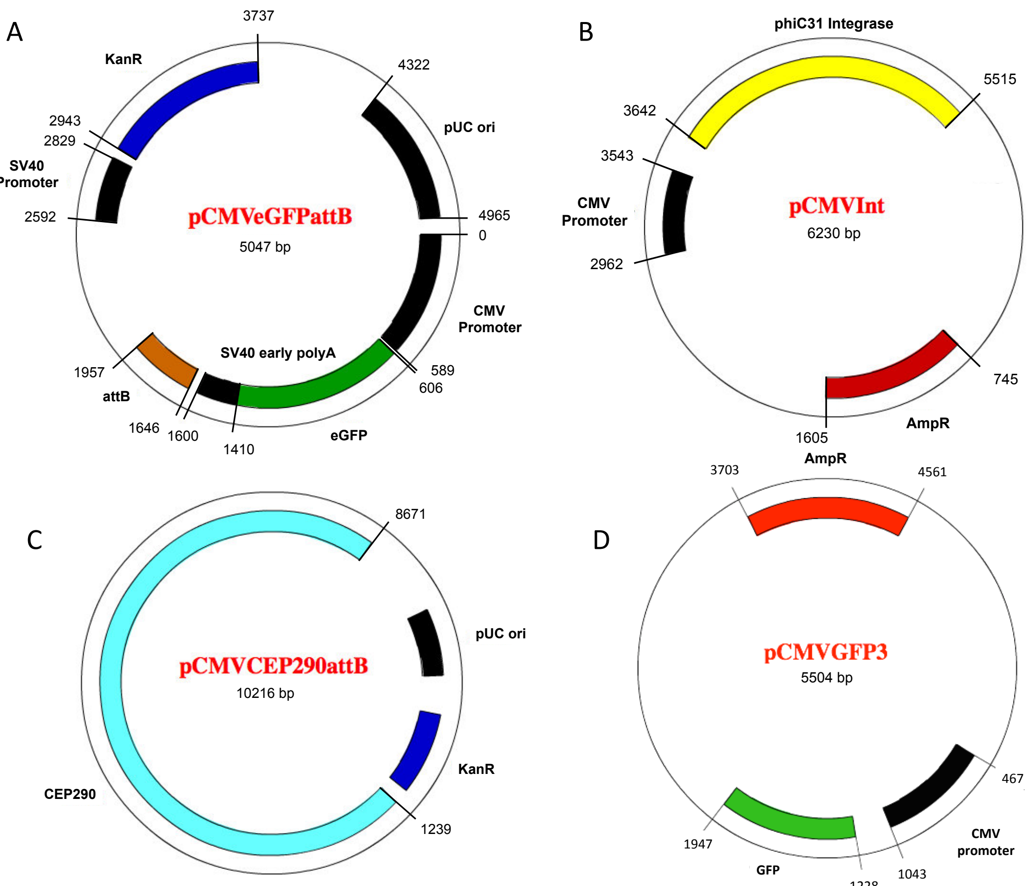

Figure 2.1- Schematic diagrams of the plasmids used. (A) pCMVeGFPattB and (D) pCMVGFP are plasmids for expression of GFP, (B) pCMVInt, a plasmid for expression of phiC31 integrase in mammalian cells, (C) pCMVCEP290attB, a plasmid for expression of the human CEP290 gene. Both pCMVeGFPattB and pCMVCEP290attB have the attB site for integration events. KanR and AmpR, are the genes for resistance to kanamycin and ampicillin, respectively; SV40, simian virus 40.

3. Polymers

Chitosan, with a MW of 15 kDa and deacetylation degree (DD) of 84% was purchased from Polysciences, Inc., USA (used in experiments described in Chapters III and IV). Ultrapure chitosan CL 113 and 213 (C1 and C2, respectively), with a molecular weight (MW) of 80 and 260 kDa and DD of 83% was purchased from Novamatrix (FMC BioPolymer AS, Norway). C1 was used in experiments described in Chapters V, VI, VII and VIII and C2 was used in Chapters V and VII.

Hyaluronic acid, with 132 or 214 kDa - from here on designated HA132 or HA214, respectively - was purchased from Lifecore Biomedical Inc., USA. All other reagents were analytical grade and used without further purification.

3. 1. Polymer modifications

3. 1. 1. Synthesis of chitosan-3-(2-aminoethyldithio) propionyl

Synthesis of chitosan-3-(2-aminoethyldithio) propionyl (CS-[AEDTP]) was performed by a two-step procedure, in an adaptation of what was previously described by Pichon *et al*. [3] The first step was the preparation of chitosan-3-(2-pyridyldithio) propionyl (CS-(PDP)). Briefly, CS (63 mg; 4.19 μmol; 352 μmol of NH_2 groups) was suspended in 5 mL of dimethyl sulfoxide (DMSO) along with 110 mg (352 μmol) of N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), and 49 μL (35.4 mg; 352 μmol) of triethylamine. The mixture was stirred under nitrogen atmosphere for 120 h, at room temperature. The reaction was monitored by thin layer chromatography (TLC), in silica plates (Merck), and through the decrease of SPDP. A mixture 6/6/1 (V/V/V) of chlorophorm/methanol/water was used as eluent; the plates were visualized under UV light and revealed with Dragendorff reagent. The reaction mixture was poured onto approximately 10x its volume of water and centrifuged at 4°C for 1 h at 3900xg. The supernatant was decanted and the residue was vacuum dried at 40°C for 48 h.

The second step was the preparation of CS-(AEDTP). The polymer obtained in the first step was re-suspended in 4 mL of DMSO and the solution kept under stirring, in a nitrogen atmosphere, at room temperature. p-toluenesulfonate mercaptoethylamine salt (a total of 61 mg; 244 μmol; TCI Europe) was added at a rate of 40 μmol/h (10 mg). The reaction was followed by UV spectrophotometry, by monitoring of the formation of the by-product pyridine-2-thione, which absorbs at 377 nm. Ultraviolet (UV) spectra of 30 μL aliquots diluted in 200 μL of DMSO and added to 2 mL of ethyl acetate were traced in the range 250-400 nm every 3 h for a period of 9 h. The suspension was left stirring overnight and, the following day, an extra 10 mg of reagent was added, until no further increase in the referred absorption band was detectable, in a total of 30 h. The reaction mixture was added to 10 volumes of ethanol and centrifuged at 4°C for 1 h at 3900xg. After decantation of the

supernatant, the residue was washed with ethanol, under sonication, centrifuged, and decanted. Afterwards, it was vacuum dried at 40°C for 72 h. ^{1}H nuclear magnetic resonance (NMR) (D_2O) δ 8.33 (d, aromatic H in *p*-toluenesulfonate (OTs), 8.33 (d, aromatic H in tosylate ion (OTs^-), 7.98 (d, aromatic H in OTs^-), 4.72 (s, H-1), 4.03 (m, H-3, 4 and 6), 3.70 (m, H-5 and 6'), 3.63 (s, S-CH_2-CH_2-NH_3^+), 3.47 (s, S-CH_2-CH_2-C=O), 3.33 (s, H-2), and 3.02 (s, CH_3 in OTs^-). Peak attribution was made according to Hiral *et al*. [4]

3. 1. 2. Modification of hyaluronic acid with cystamine ($HASSNH_2$)

The modification reaction was performed in an adaptation of what was previously described elsewhere and depicted in Fig. 2.2. [5-7] Briefly, 500 mg of HA was dissolved in 100 ml of distilled H_2O, a one and a half molar excess (relative to the carboxylic acid groups in HA) of EDAC (0.378 g, 1.974 mmol), and three molar excess of cystamine (0.889 g, 3.947 mmol) were added to the solution. The mixture was stirred at room temperature for 72 h. The reaction mixture was dialyzed against 4 g/L NaCl (MW cut off 2000) for 6 h and then against distilled H_2O for 24 h. The final product - $HASSNH_2$ - was lyophilized for 3 days and stored at room temperature until use.

Figure 2.2 – Schematic representation of the modification reaction and conditions for synthesis of aminated hyaluronic acid.

3. 2. Polymer characterization

3. 2. 1. Potentiometric titration of $HASSNH_2$

In order to characterize the protonation behavior of the modified polymer, a titration was performed [8]. Polymer solutions, HA and $HASSNH_2$, were prepared with a concentration of total titrable carboxyl group concentration of 10 mM in a volume of 10 ml. The initial pH was adjusted to 2 - 3 by adding 2.00 M HCl prior to the dropwise addition of the titrant 0.08 M NaOH to the polymer solutions under

constant stirring. Potentiometric measurements were made using a pH measurement electrode (Orion 9157BN) connected to an Orion pH meter (Thermo Orion 4 Star pH-ISE Benchtop; ThermoFisher Scientific (Waltham, MA USA).

3. 2. 2. Ellman's test

In order to determine the extent of the modification, the thiol groups were quantified by the Ellman's test. Firstly, 50 mg of $HASSNH_2$ were dissolved in distilled H_2O and treated with 30 molar excess of dithiothreitol (DTT, 0.418 g, 2.712 mmol) in order to expose the thiol groups. The mixture was stirred under N_2 atmosphere for 24 h, at room temperature. The reaction mixture was dialyzed against 4 g/L NaCl (MW cut off 2000) for 6 h and then against distilled H_2O for 24 h. The final product HASH was lyophilized for 2 days and stored at room temperature until use. Secondly, for the Ellman's test, 3.8 mg of HASH were dissolved in 1 ml of 0.1 M Tris buffer, pH 8.0. For each measurement, a tube containing 100 μl of Ellman's reagent (10 mM of 5,5'-dithio-bis-(2-nitrobenzoic acid) in 0.1 M Tris buffer), 1800 μl of 0.1 M Tris buffer and 100 μl of sample was prepared, incubated at room temperature for 15 min and then the absorbance at 412 nm was measured. [9]

3. 3. Polyplex preparation

3. 3. 1. CS, CS-(AEDPT) and CSHA polyplexes

Polyplexes used in Chapters III and IV were prepared as described in this section. CS and CS-(AEDPT) vectors were prepared as described previously. [10] Briefly, a CS or CS-(AEDTP) solution (0.02% (W/V) in 0.1 M acetic acid, pH 3) and a 5 mM of sodium sulphate solution were separately preheated to 55°C. An equal volume of both solutions was quickly mixed together, vortexed for 30 s, placed on ice and stored at 4°C. To prepare pDNA loaded vectors at a NH_3^+:PO_4^- ratio of 5:1, 50 µg (3.4 nmol; 26.52 nmol of NH_2 groups) of CS or CS-(AEDTP) and 16.1 µg (5.3 nmol; 4.8 pmol of PO_4^-) of pDNA, were used. pDNA was mixed with the sodium sulfate solution and this solution mixed with the CS or CS-(AEDTP) solution, as

described above, thus, producing CSpDNA 5:1, and CS-(AEDTP) pDNA 5:1 polyplexes.

CSHA polyplexes were prepared using a HA132 or HA214 solution (0.1% (W/V) in MilliQ water). The following CS:HA weight ratios: 3:1, 4:1, 5:1, 7:1 and 10:1 were used, keeping constant the CS amount (50 μg) and varying the HA amount. In order to use equal volumes of both solutions, HA was diluted in 5 mM sodium sulphate and then mixed with the CS solution, as described above. To prepare CS and CSHA polyplexes loaded with pDNA at a $NH_3^+:PO_4^-$ ratio of 5:1, 50 μg of CS and 16.1 μg of pDNA, were used as described above, thus producing CSpDNA 5:1 or CSHApDNA 5:1 polyplexes. The 5:1 ratio was chosen based on previous results for CS-pDNA, which showed this ratio to be the most appropriate for cellular transfection. [10, 11]

3. 3. 2. C1 and C2 polyplexes

CS polyplexes used in Chapters V and VIII were prepared as described in this section. Ultrapure CS polyplexes were prepared as described previously. [11] Briefly, a chitosan solution (1 mg/mL) in MilliQ water, pH 5.5) and a 25 mM of sodium sulphate solution were preheated separately to 55ºC. An equal volume of both solutions was quickly mixed together, spun for 30 s, placed on ice and then stored at 4ºC.

Polyplexes were prepared with chitosan (of 80 or 260 kDa) and combined with 1) plasmid coding for GFP (pCMVeGFP-attB) or 2) plasmid coding for CEP290 (pCMVCEP290attB). Each condition was also prepared in combination with the plasmid coding for integrase (pCMVINT). Another formulation was prepared with the plasmid coding for integrase (pCMVINT) (all formations described in Fig. 2.3). To prepare pDNA loaded polyplexes at a $NH_3^+:PO_4^-$ ratio (N:P ratio) of 15:1, 250 μg of chitosan and 26.5 μg of pDNA were used. pDNA was mixed with the sodium sulphate solution and this solution mixed with the chitosan solution, as described above. When two plasmids were combined in the same polyplex the total pDNA amount was kept constant and a molecular ratio of 2:1 was used.

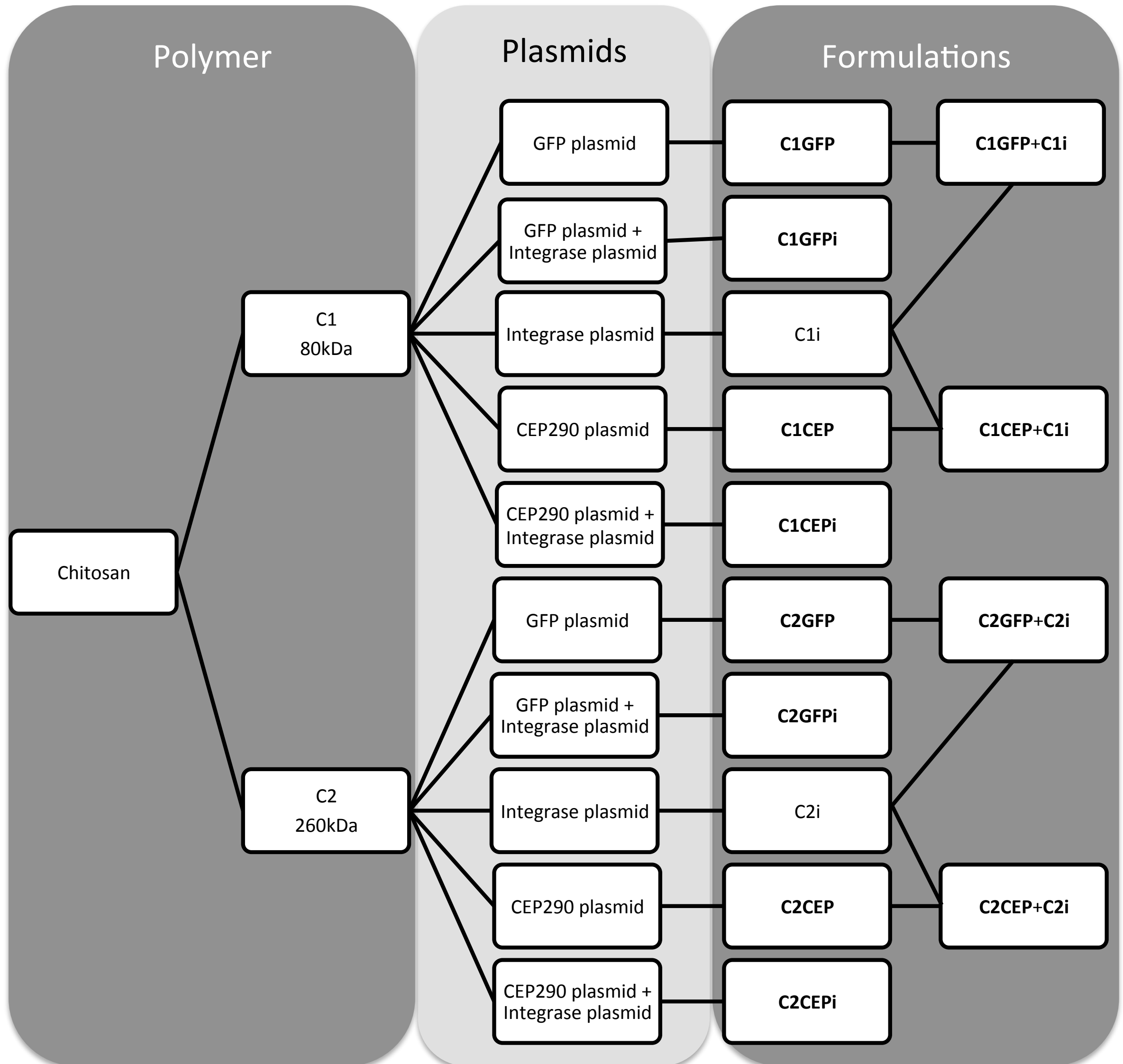

Figure 2.3 - Schematic representation of the different formulations and their composition. Formulations in bold represent conditions tested in transfection assays.

3. 3. 3. $HASSNH_2$ polyplexes

Polyplexes used in Chapter VI were prepared as described in this section. Polyplexes were prepared as previously described by our lab. [11, 12] Vector formulations were prepared at various ratios by adding anionic solutions to the $HASSNH_2$ solution, as indicated in Table 2.1. Briefly, polymer solutions (1 mg/ml in MilliQ H_2O, pH 5.5) and sodium sulphate solutions were separately heated to 55ºC. Equal volumes of both solutions were quickly mixed together, vortexed for 30 s, placed on ice and stored at 4ºC. Alternatively, HA and other anionic species (TPP

and κ-carrageenan) were tested by combining them to the sodium sulphate solutions.

Table 2.1- Vector formulations with different anionic agents.

Polymers		**Ratio (W/W)**	**Solvent**
$HASSNH_2$		-	5 - 150 mM Na_2SO_4
$HASSNH_2$	HA 132kDa	5:1 10:1 15:1 20:1	25mM Na_2SO_4
$HASSNH_2$	HA 214kDa	5:1 10:1 15:1 20:1	25mM Na_2SO_4
$HASSNH_2$	HA 214kDa	1:1 3:1 5:1	5 - 150 mM Na_2SO_4
$HASSNH_2$	TPP	1:1 3:1 5:1 10:1 15:1 20:1 30:1 40:1 50:1	25mM Na_2SO_4 or MilliQ H_2O
$HASSNH_2$	κ-carrageenan	5:1 10:1 15:1 20:1 30:1	25mM Na_2SO_4

Different weight ratios were tested keeping constant the $HASSNH_2$ amount (250 μg) and varying the HA amount. In order to use equal volumes of both solutions, HA was diluted in sodium sulphate and then mixed with the $HASSNH_2$ solution, as described above.

Vectors were prepared based on mixtures with chitosan (CS), originating CSHA or $CSHASSNH_2$ vectors. The CS:HA or CS:$HASSNH_2$ weight ratios 5:1 and 7:1 were chosen based on previous studies [12] keeping constant the CS amount

(250 μg). In order to use equal volumes of both solutions, HA or $HASSNH_2$ was diluted in 25mM sodium sulphate and then mixed with the CS solution, as described above.

To prepare polyplexes loaded with DNA at a $NH_3^+:PO_4^-$ ratio of 15:1, 250 μg of CS and 26.5 μg of DNA, were used. DNA was mixed with the sodium sulfate solution and this solution mixed with the CS solution, as described above. The resulting polyplex formulations are described in Table 2.2.

Table 2.2 – Polyplex formulations with chitosan and hyaluronic acid. All polyplexes containing DNA were prepared at a N:P ratio of 15:1.

Polymers		**Ratio CS: HA or $HANSSH_2$ (W/W)***	**Solvent**	**Name**
CS	HA 214kDa	5:1	25mM Na_2SO_4	CSHA5N
			MilliQ H_2O	CSHA5H
		7:1	25mM Na_2SO_4	CSHA7N
			MilliQ H_2O	CSHA7H
	$HASSNH_2$	5:1	25mM Na_2SO_4	$CSHASSNH_25N$
			MilliQ H_2O	$CSHASSNH_25H$
		7:1	25mM Na_2SO_4	$CSHASSNH_27N$
			MilliQ H_2O	$CSHASSNH_27H$

3. 3. 4. CS polyplexes

Polyplexes used in Chapter VII were prepared as described in this section. Chitosan polyplexes were prepared as described previously. [11] Briefly, a chitosan solution (0.2 mg/mL) in MilliQ water, pH 5.5) and a sodium sulphate solution (20 – 100 mM) were preheated separately to 55ºC. Equal volumes of both solutions were quickly mixed together, spun for 30 s, placed on ice and then stored at 4ºC. Polyplexes were prepared with chitosan (113 or 213) and combined with the plasmid coding for GFP. To prepare pDNA loaded polyplexes at a $NH3^+:PO4^-$ ratio (N:P ratio) of 5:1, 50 μg of chitosan and 15.92 μg of pDNA were used. pDNA was mixed with the sodium sulphate solution and this solution mixed with the chitosan solution, as described above.

3. 4. Polyplex characterization

3. 4. 1. Size, polydispersity, charge and morphology

Dynamic light scattering (DLS) and non-invasive backscatter technology were used to determine the size of vectors using a detection angle of 173°. Laser Doppler velocimetry and phase analysis light scattering technology were used to measure the zeta potential of the vectors (Zetasizer Nano ZS, Malvern instruments, UK). The polydispersity index was obtained by DLS using the Zetasizer Nano Series software v 6.20. All vectors were analyzed in ddH_2O at 25°C.

Transmission electron microscopy (TEM, JEOL JEM-1011 electron microscope, Tokyo, Japan) was used to evaluate the morphology of the polyplexes. Prior to analysis, samples were stained with 2% (W/V) phosphotungstic acid and placed on copper grids with Formvar® films.

3. 4. 2. pDNA complexation and protection from degradation

The pDNA complexation, retention, and integrity in the polyplexes were assessed by gel electrophoresis. The pDNA complexation capacity by the polymer was assessed by a retardation assay using an agarose gel electrophoresis with 1% (W/V) agarose in TAE or TBE buffer with ethidium bromide or GreenSafe® Premium (NZYtech, Portugal). Gels were run at 80 mV for 1 h and further visualized under UV light.

For the evaluation of pDNA protection capacity, free pDNA and polyplexes were separately incubated with 1 unit of DNAse I (Sigma-Aldrich®, USA) for 15 min at 37ºC. The reaction was stopped by 1µL of a 50 mM EDTA solution and heating at 70ºC for 10 min. The integrity of the pDNA was then analyzed by agarose gel electrophoresis in 1% (W/V) agarose in TAE buffer with ethidium bromide. Gels were subjected to a 70 mV voltage for 1.5 h and further visualized under UV light (AlphaImager®, Alpha Innotech, USA).

3. 4. 3. Effect of dithiothreitol and gluthatione in pDNA release

To evaluate the cleavage of disulfide bonds incorporated into the CS-(AEDTP), the complexes were incubated with glutathione and DTT. These assays were performed as described previously. [3] Briefly, a 0.4 M glutathione reduced-form (Sigma) stock solution was prepared in 0.1 M potassium phosphate buffer (pH 7.5), with 2 mM EDTA, pH 7.5 (potassium phosphate buffer with EDTA from here on referred to as PB). DNA loaded vectors prepared, as previously described, were incubated for 24 h at 37°C in PBS in the presence of 20 mM glutathione, 2 units/mL of glutathione reductase (GR; Sigma), and 0.5 mM of nicotinamide adenine dinucleotide phosphate (NADPH, Sigma). CS-(AEDTP) vectors were incubated for 24h at 37 ºC with increasing concentrations of DTT (10, 20, 40, 60, 80, 100 mM) in sodium acetate at 10 mM. Samples were then analyzed by agarose gel electrophoresis as in the previous section.

3. 4. 4. Polyplex stability

Polyplex stability was evaluated in different ways: stability at physiological temperature (37ºC) and pH (7.4) and also long-term stability (4ºC or 37ºC). Briefly, polyplexes were incubated in equal volumes of either PBS or Dulbecco's Modified Eagle's Medium [DMEM, with 10% FBS (fetal bovine serum)] at 37ºC for 1 to 3 days. Polyplex stability, evaluated by pDNA retention, was performed as described above. For long-term stability evaluation, polyplexes were incubated at 4 or 37ºC and their size, polydispersity and zeta potential monitored weekly for up to 3 months.

For freeze-thaw stability evaluation, polyplexes were centrifuged at 16000xg on a 10 μl glycerol layer for 30 min at 15°C (Himac CT15RE centrifuge, rotor T15A61-1205, VWR by Hitachi KokiCo, Lda.). The supernatants were discarded and polyplexes were ressuspended in a cryoprotectant (either glucose or sucrose 10%, W/V). [13] Polyplexes were subjected to freeze-thaw cycles (-20ºC for 1h followed by room temperature for 30 min to 1 h). After freeze-thaw cycles, the size and zeta potential of the polyplexes was evaluated as indicated above.

3. 5. Cell culture

All techniques requiring aseptic conditions were performed in a laminar flow hood (Hera safe Heraerus) after irradiation for 15 min with ultraviolet (UV) light. Cells were cultured in an humidified incubator (Hera cell 150 Heraerus) at 37ºC with 5% of CO_2 in 95% air and media and culture solutions were pre-warmed to 37ºC in a water bath prior to use. Microscopic examination of cell growth was preformed frequently by using an inverted light microscope (Leica DMIL).

Cells were cultured in the appropriate media: HEK293, HEK293T and D407 in DMEM and ARPE-19 in DMEM mixture F-12 HAM; supplemented with 5% to 10% of FBS, 1% penicillin/streptomycin and 1% Glutamine. All cell culture reagents were purchased from Sigma-Aldrich® (St. Louis, MO/USA).

3. 6. Polymer and polyplex cytotoxicity evaluation

An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the cytotoxicity of the polymers or polyplexes. Cells were plated, at a density of 1.5×10^4 cells/well in 48-well flat-bottom tissue culture plates. After 24 h, the culture medium was replaced by culture medium containing different amounts of polymer or polyplexes. Cells were incubated up to 72 h. As positive and negative controls of cell viability, cells cultured in standard cell culture conditions and cells incubated with a latex extract in culture medium (1.5 cm^2/mL) were used, respectively. [14] At the selected time point, 25 µL of MTT (5 mg/mL) were added to each well and cells were further incubated for 4 h. To dissolve the formed formazan crystals, the medium was replaced with 300 µL of 0.04N HCl in isopropanol.

Absorbance was measured using a microplate reader (Tecan Infinite 2000, USA), at 570 and 630 nm, for cell viability/formazan formation and background, respectively. After subtracting the background (OD = OD570nm – OD630nm), cell viability was calculated as follows: Cell viability (%) = (ODsample) / (ODcontrol) × 100, where ODcontrol and ODsample are cells not challenged and challenged by polyplexes, respectively. Each value was averaged from triplicates and each experiment was carried out thrice.

3. 7. *In vitro* transfection assays

For the transfection studies, cells were plated at 1 – 2×10^5 cells/well in 6-well tissue culture plates. FuGENE® HD (Promega, USA) was used as positive transfection control according to the manufacturer's instructions. Polyplexes were added to plated cells at a ratio of 1 µg of pDNA per well and further incubated for 72 h. Non-transfected cells were used as negative transfection control.

For long-term transfection studies, HEK293T cells were plated at 1.5 – 3×10^5 cells/well in clear 6-well tissue culture plates 24 h prior to transfection. FuGENE® HD (Promega, USA) was chosen as positive transfection control and used according to manufacturer instructions. Formulations (indicated in Fig. 2.3) were added to the cells to obtain a total of 1 µg of pDNA per well and further incubated for 5 h in serum free media. Untreated cells were used as negative control for transfection.

For selection studies, HEK293 cells were transfected as described above and 72 h post-transfection selection with Geneticin® (Gibco, USA) was initiated. Selection of transfected cells was carried out for 2 weeks using 300 – 500 µg/mL of Geneticin® in complete media. HEK293 cells were used instead of HEK293T because they are resistant to geneticin selection.

3. 7. 1. Transfection efficiency evaluation by flow cytometry

Transfection efficiency was evaluated quantitatively by flow cytometry by scoring GFP-positive cells (FACScalibur, BD Biosciences, USA) using FL-1H, green channel. A total of 1×10^5 events were counted for each sample. The percentage of positive events corresponds to the gated events minus the non-transfected cells.

3. 7. 2. Transfection efficiency evaluation of by western blot

Transgene expression was evaluated by western blot analysis. Samples of transfected cells were collected at different time points, lysates obtained and stored at -80ºC. Prior to electrophoresis the total protein concentration in cell lysates were determined by the Micro BCA assay (Thermo Scientific, USA). Cell lysates were electrophoresed on 4-12% Bis-Tris or 3-8% Tris-Acetate SDS-polyacrylamide gel

(NuPAGE, Invitrogen, USA); for GFP and CEP290 samples respectively, and transferred to a nitrocellulose membrane. Immunodetection was carried out using mouse anti-GFP (1:3000, Roche, USA) and rabbit anti-CEP290 (1:2000, Abcam, USA) antibodies followed by anti-mouse (1:10000, Amersham, USA) and anti-rabbit (1:10000, Amersham, USA) secondary antibodies, respectively. Alpha tubulin was used as a loading control (mouse anti-α-tubulin, 1:10000, Abcam, USA). Transgene over-expression was determined by gel lane analysis using ImageJ software, v. 1.46r.

3. 7. 3. Transfection efficiency evaluation of by fluorescence microscopy

Transfection results were evaluated qualitatively by fluorescence microscopy (Axiovert 40 CFL, Zeiss) and transgene expression measured by the mean fluorescent area in fluorescence microscopy images was determined using ImageJ software, v. 1.46r.

3. 8. *In vivo* transfection assays

To evaluate the *in vivo* transfection efficiency of polyplexes, C57BL/6 mice were injected with polyplexes (1 µL, equivalent to 32 ng of pDNA) by subretinal injection in the right eye, under a stereomicroscope (Nikon, Nikon Stereoscopic Microscope, Melville, NY, USA). The left eye was used as control (non-injected contralateral eye). To evaluate GFP expression in histological sections the animals were sacrificed at different time points post-injection, the eyes enucleated and fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight, immersed in 30% sucrose (Sigma-Aldrich) and after 6 h included in Optimal Cutting Temperature (OCT) compound (VWR, Radnor, PA, USA).

Serial cryosections (14 µm thick) were cut along the horizontal meridian and progressively distributed on slides. The analysis of GFP fluorescence and image acquisition was performed using a fluorescence microscope (Axio Imager Z2; Zeiss, Oberkochen, Germany). Transfection following subretinal delivery of polyplexes was evaluated as follows: images of retinal cryosections were captured under a fluorescence microscope using the 38He(FITC) filter to visualize GFP positive cells.

For each eye at least three independent sections and the corresponding GFP positive cells were counted at 200x magnification.

4. Statistical analysis

Results are presented as mean ± standard deviation of at least three independent experiments unless otherwise stated. Statistical analysis was performed with GraphPad Prism 5 software. Data were subjected to analysis of variance (One and Two-way ANOVA) and multiple comparisons tests using a confidence interval of 95%.

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Chapters III to VIII

Results

Chapter III

Transfection efficiency of chitosan and thiolated chitosan in retinal pigment epithelium cells: A comparative study

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1. ABSTRACT

Objective: Gene therapy relies on efficient vector for a therapeutic effect. Efficient non-viral vectors are sought as an alternative to viral vectors. Chitosan, a cationic polymer, has been studied for its gene delivery potential. In this work, disulfide bond containing groups were covalently added to chitosan to improve the transfection efficiency. These bonds can be cleaved by cytoplasmic glutathione, thus, releasing the DNA load more efficiently. **Materials and Methods:** Chitosan and thiolated chitosan nanoparticles (NPs) were prepared in order to obtain a NH_3^+:PO_4^- ratio of 5:1 and characterized for plasmid DNA complexation and release efficiency. Cytotoxicity and gene delivery studies were carried out on retinal pigment epithelial cells. **Results:** In this work, we show that chitosan was effectively modified to incorporate a disulfide bond. The transfection efficiency of chitosan and thiolated chitosan varied according to the cell line used, however, thiolation did not seem to significantly improve transfection efficiency. **Conclusion:** The apparent lack of improvement in transfection efficiency of the thiolated chitosan NPs is most likely due to its size increase and charge inversion relatively to chitosan. Therefore, for retinal cells, thiolated chitosan does not seem to constitute an efficient strategy for gene delivery.

KEYWORDS: Chitosan, gene therapy, non-viral vectors, thiolation, transfection efficiency

2. Introduction

Gene therapy has recently met success in several clinical trials for retinal degeneration, X-linked combined severe immunodeficiency (X-SCID) and others. [1-3] Yet, these trials use viral vectors since viruses have evolved to achieve high delivery efficiency while minimizing detection by the immune system of the host. While this renders virus extremely useful for gene therapy, they still present some limitations, such as limited size of gene load and the risk of activating oncogenes by random integration into the genome. [4]

Due to these limitations non-viral vectors have been studied as an alternative. Nevertheless, for these systems to become a viable therapeutic alternative it is imperative they can promote *i*) cellular membrane penetration, *ii*) escape from endo-lysossomal degradation and *iii*) translocation in to the nucleus, in a similar way to viral vectors. [5]

Generally, non-viral vectors are based on electrostatic interactions established between the phosphate groups of the DNA or RNA backbone and the cationic groups from a polymer or lipid. [6] Polymers are generally less immunogenic and easier to manipulate than lipids; moreover, it is possible to optimize these systems by combining different molecular weights (MWs) with other physico-chemical properties. Cationic polymers, are commonly used and can be either synthetic, like poly(L)lysine and polyethyleneimine or of natural origin like chitosan. [7-9]

Chitosan is an aminated polysaccharide derived from the deacetylation of chitin. [10] Its bioavailability, low immune, and toxicological response, both *in vitro* as *in vivo* and reactive functional groups that make it susceptible to chemical modification, along with the ability to promote endo-lysosomal escape and significant condensation of genetic material, have proven its potential as a gene therapy vector. [11, 12] However, its ability to promote gene transfer is low compared with viral vectors, and therefore, chemically modifying chitosan is regarded as a way to improve gene delivery.

It has been demonstrated that chemically added disulfide bonds improve gene delivery and expression when compared to unmodified polymers, since these can be

cleaved by the action of intracellular glutathione and therefore, promoting a faster release of the genetic material. [6, 13-18]

We have previously produced chitosan-pDNA nanoparticles (NPs) as vectors for retinal gene therapy, which displayed low gene expression in retinal pigment epithelial cells. [14] In an attempt to improve gene transfer and expression by chitosan, we covalently linked a group containing a disulfide bond to the amine group of chitosan. This disulfide bond should be cleaved in the presence of cytoplasmic glutathione, thus, releasing the genetic material near the nucleus, readily enabling nuclear translocation. [13, 15, 19] We have performed a direct comparison between the two polymers using two types of cells: Human embryonic kidney (HEK 293) cells and retinal pigment epithelium cells. In the eye, we aim to target RPE cells, due to their important role in the support of the retinal homeostasis and involvement in several retinal diseases.

3. Results

3. 1. CS-(AEDTP) synthesis

The synthesis of CS-(AEDTP) was carried out under nitrogen atmosphere in two distinct steps. The first reaction step was monitored by the decrease of SPDP (step 2, Fig. 3.1) by TLC on silica gel plates. In the second reaction step, the formation of CS-(AEDTP) (step 5, Fig. 3.1), is accompanied by the formation of pyridine-2-thione (step 6, Fig. 3.1).

Figure 3.1 – Reaction mechanism of N-succinimidyl-3-(2-pyridyldithio)-propionate (2) with chitosan, (1) resulting in chitosan-3-(2-pyridyldithio) propionyl (CS-PDP) (3) and hydroxysuccinimide. CS-PDP reacts with mercaptoetilamine salt, (4) producing chitosan-3-(2-Aminoethyldithio) propionyl- chitosan-3-(2-aminoethyldithio) propionyl (5) and pyridine-2-thione (6).

This by-product absorbs at 377 nm, which allows monitoring the reaction by UV. By consumption of the latter, we were able to determine the completion of the reaction and observed by NMR that chitosan was modified to incorporate the group containing the disulfide bond.

3. 2. CS and CS-(AEDTP) nanoparticle characterization

CS and CS-(AEDTP) NPs were characterized regarding size and zeta potential (Table 3.1). NPs presented a mean size of 456.28 ± 128.92 and 1105.01 ± 290.04 nm in diameter and a mean zeta potential of +19.9 ± 2.4 and +15.8 ± 2.9 mV, for CS and CS-(AEDTP), respectively. DNA loaded NPs presented a mean size of 254.61 ± 74.08 and 416.43 ± 160.02 nm in diameter and a mean zeta potential of +20.1 ± 2.3 and -20.1 ± 5.2mV, for CS and CS-(AEDTP), respectively. This inversion in charge for CS-(AEDTP) was directly related to the presence of pDNA in the NPs.

Table 3.1 – Characterization of chitosan and Chitosan-AEDTP: size, polydispersity and zeta potential data.

Particle	Z-Average (∅, nm)	Polydispersity	Zeta potential (mV)
CS	456.28 ± 128.92^{b}	0.273 ± 0.128^{a}	+19.9 ± 2.4^{c}
CS-(AEDTP)	1105.01 ± 290.04^{c}	0.399 ± 0.124^{b}	+15.8 ± 2.9^{b}
CSpDNA 5:1	254.61 ± 74.08^{a}	0.266 ± 0.068^{a}	+20.1 ± 2.3^{c}
CS-(AEDTP)pDNA 5:1	416.43 ± 160.02^{b}	0.464 ± 0.085^{b}	-20.1 ± 5.2^{a}

Values are presented as mean ± S.D. Mean values in each column followed by the same letter are not statistically different by Duncan Post-hoc tests ($p \leq 0.05$).

The NPs were further characterized regarding their pDNA loading efficiency and protection against DNAse I degradation (shown by pDNA integrity) by agarose gel retardation assays (Fig. 3.2). Both NPs effectively condensed pDNA, (NH_3/PO_4 ratio of 5:1) as shown by the absence of free DNA migration bands in the agarose gel (Fig. 3.2). The produced NPs remained stable and did not release pDNA, in detectable amounts, protecting pDNA from nuclease degradation when incubated with DNase I (Fig. 3.2).

To assess the capacity of glutathione to cleave the disulfide bonds incorporated in to chitosan, CS-(AEDTP) DNA loaded NPs were incubated with glutathione and DTT, a strong reducing agent. No detectable amount of pDNA was released from the NPs in either conditions, as observed in Fig. 3.3A and 3.3B.

Morphological characterization of NPs by TEM (Fig. 3.4) revealed a spherical morphology and no significant differences between the morphology of NPs and DNA loaded NPs (TEM micrographs shown for CS is representative of CS and CS-(AEDTP)).

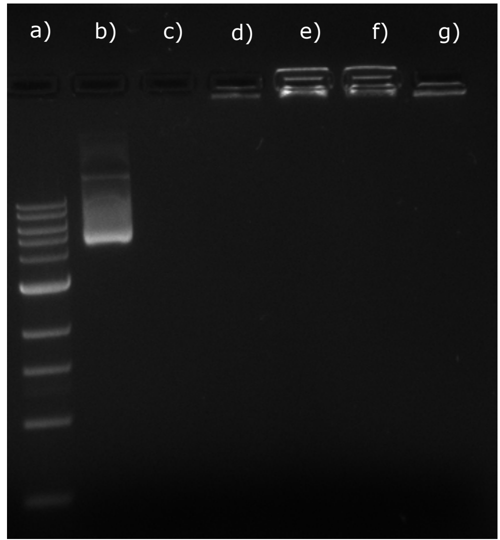

Figure 3.2 – pDNA complexation and protection. Both chitosan (CS) and chitosan-3-(2-aminoethyldithio) propionyl (CS-(AEDTP)) nanoparticles (NPs) effectively protect DNA from DNAse degradation, as analyzed in a 1% agarose gel electrophoresis, with DNA visualized by GreenSafe Premium. Lanes: (a) DNA marker, (b) Plasmid DNA (pDNA), (c) pDNA + DNAse I, d) CS-pDNA NPs, (e) CS-pDNA NPs + DNAse I, (f) CS-(AEDTP)-pDNA NPs, (g) CS-(AEDTP)-pDNA + DNAse I

3. 3. CS and CS-(AEDTP) cytotoxicity

NPs toxicity was evaluated using the MTT assay (Fig. 3.5). Cell survival was not affected by concentrations below 5.0 μg of polymer with viability values above 80%. However, at 10.0 μg, both cell lines have a significantly lower cell survival percentage both for CS and CS-(AEDTP) NPs (Fig. 3.5), in particular, ARPE-19 cells with 26.83 ± 23.06 and 25.92 ± 6.77, for CS and CS-(AEDTP) respectively. In the presence of 5.0 μg, D407 have a cell survival percentage of 66.16 ± 1.33 and 70.06 ± 9.30, for CS and CS-(AEDTP) respectively. HEK 293 (data not shown) results are similar to D407 cells for all tested concentrations.

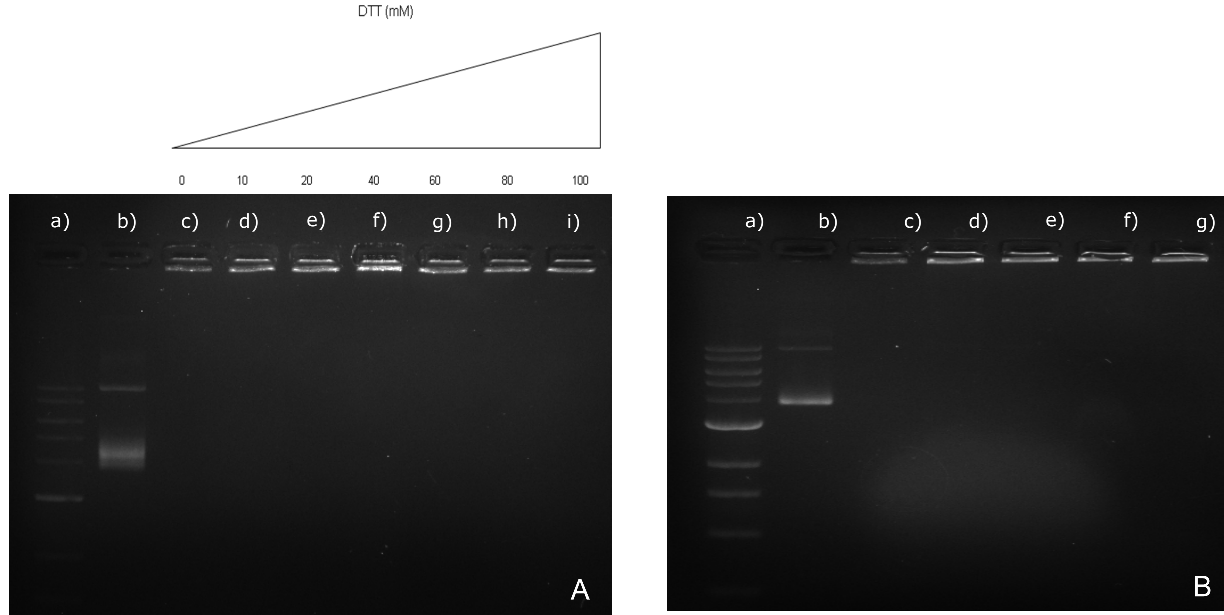

Figure 3.3 – DNA retention after incubation with reducing agents. DNA retention by chitosan-3-(2-aminoethyldithio) propionyl (CS-(AEDTP)) nanoparticles (NPs) after 24 h of incubation with: A) Increasing concentrations of dithiothreitol; Lanes: (a) DNA marker, (b) Plasmid DNA (pDNA), (c) CS-(AEDTP) NPs, (d) to i) range from 10mM to 100mM according to the image. B) 0.4 M gluthatione reduced-form; Lanes: (a) DNA marker, b) pDNA, (c) CS-(AEDTP) NPs, (d) CS-(AEDTP) NPs + NADPH + phosphate buffer, (e) CS-(AEDTP) NPs + glutathione reductase + NADPH + phosphate buffer, (f) CS-(AEDTP) NPs + PB + NADPH + phosphate buffer, (g) CS-(AEDTP) polyplexes + PB + GR+ NADPH + phosphate buffer. This was analyzed in a 1% agarose gel electrophoresis, with DNA visualized by GreenSafe Premium.

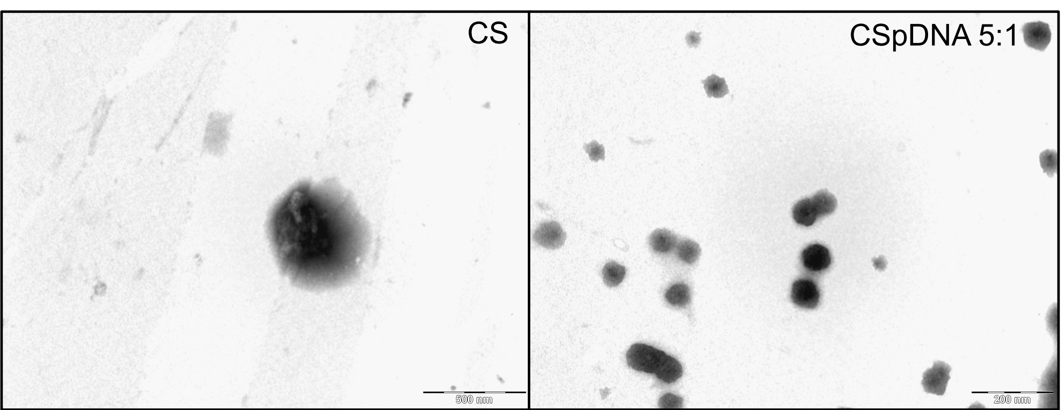

Figure 3.4 – Transmission electron microscopy microphotographs, chitosan (CS) nanoparticles and DNA loaded nanoparticles (CSDNA 5:1).

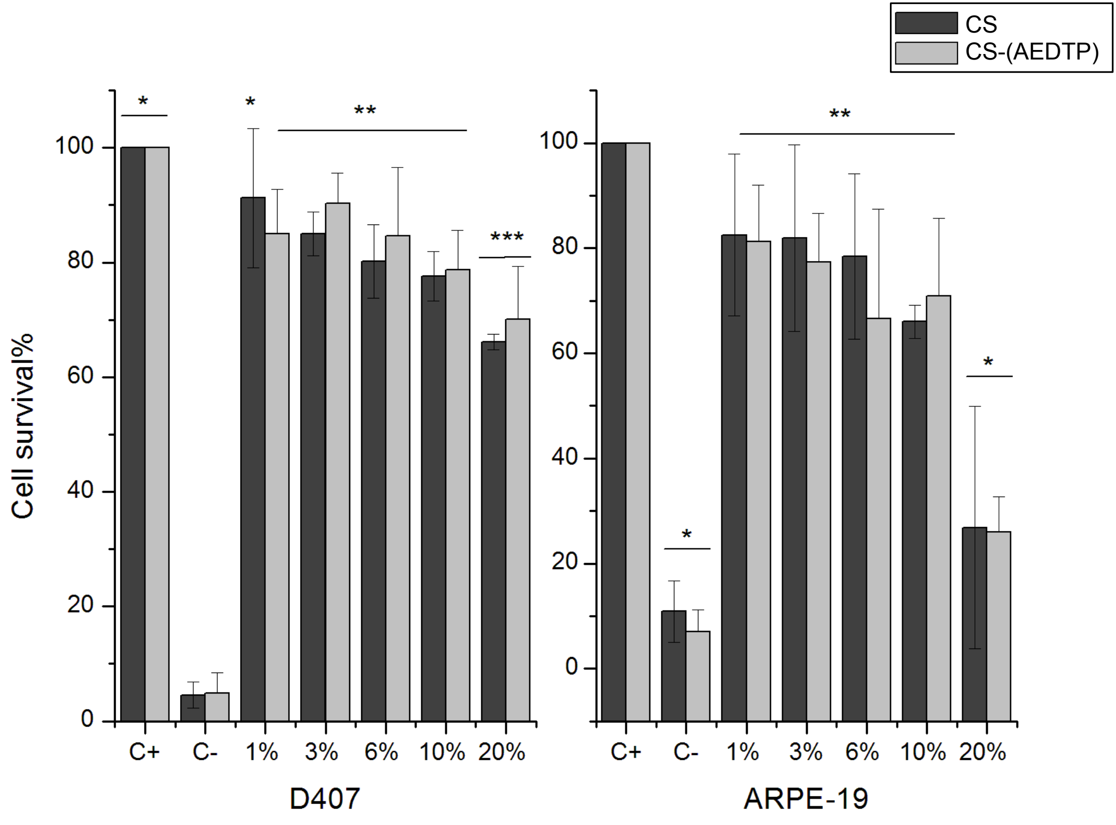

Figure 3.5 – Cell survival (%) as a function of CS and chitosan-3-(2-aminoethyldithio) propionyl nanoparticles amount (μg of polymer). D407 and ARPE-19 cells were incubated for 72 h with the various concentrations of nanoparticles; (C+) untreated cells, (C-) cells treated with latex extract. Vertical bars = S.D. The number of *indicates significantly different sets of data

3. 4. Transfection efficiency

Transfection efficiency was monitored by GFP expression using fluorescence microscopy and quantified by flow cytometry (Fig. 3.6). GFP expression was observed up to 72 h post-transfection and, as observed for other materials, was lower than FuGENE®, an optimized commercial transfection reagent, here used as a control (Fig. 3.6). To ensure that the transfection efficiency is dependent on cell type, HEK293 cells were used as a control cell line; due to their widespread use in similar studies [6, 20, 21]. Results show there is a difference in transfection efficiency between cell lines but not between CS and CS-(AEDTP). The differences in transfection efficiencies are more evident in comparison to FuGENE®, where the highest transfection was obtained with

HEK293 (72.58 ± 6.92) followed by D407 (49.13 ± 12.92) and ARPE-19 (30.14 ± 9.16). CS had transfection efficiencies of 0.36 ± 0.060; 0.33 ± 0.28 and CS-(AEDTP) 0.59 ± 0.45; 0.057 ± 0.021, for D407 and ARPE-19 cell line (data for HEK293 cells not shown).

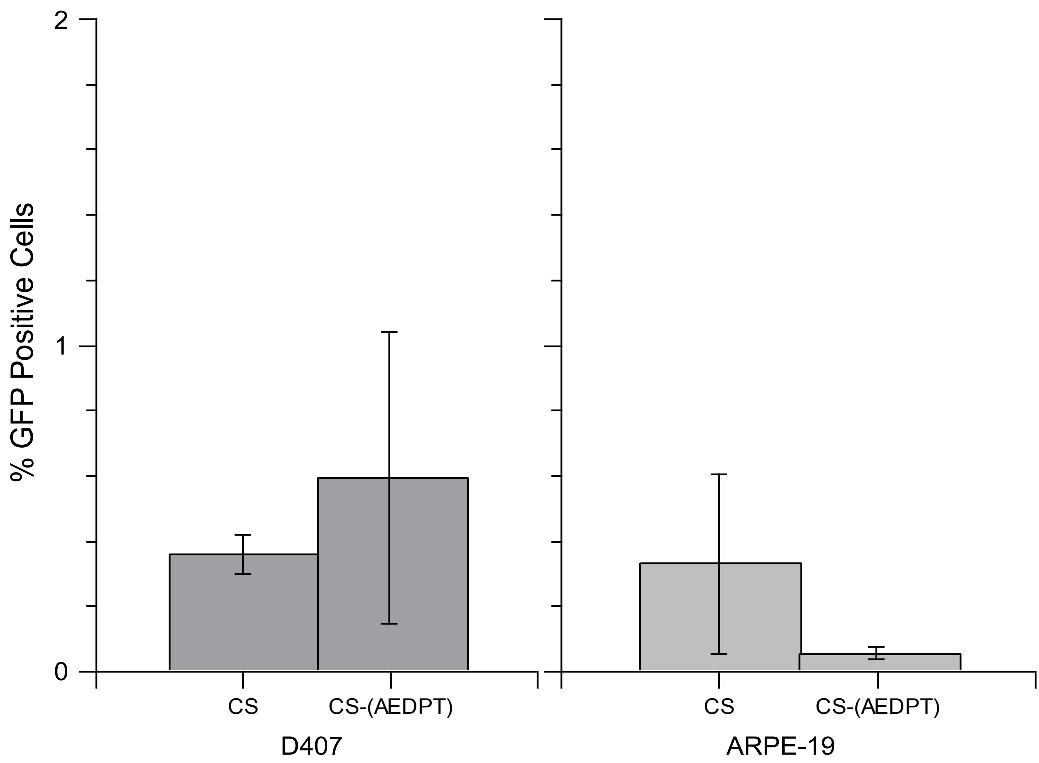

Figure 3.6: Transfection efficiency represented as GFP positive cell percentage as a function of polymer.

4. Discussion

The aim of this work was to modify CS to incorporate a disulfide bond with the goal of promoting endo-lysosomal escape as well as timely release of pDNA in the vicinity of the nucleus, in order to facilitate nuclear translocation and therefore, increase the gene delivery profile of CS.

TLC and NMR analysis confirmed the incorporation of the disulfide bond in CS, originating CS-(AEDTP). Regarding the pDNA complexation capacity, both CS and CS-(AEDTP) were able to complex DNA. This is shown by the decrease in size observed between CS-(AEDTP) NPs and DNA loaded NPs indicate that CS-(AEDTP) was able to form NPs, via electrostatic interactions, between anionic pDNA phosphates and the cationic polymer amine groups. The latter is also verifiable for CS. The formation of the NPs was reproducible, yielding NPs of defined size and charge, as verified by DLS (Table 3.1).

Both NPs formed by CS-(AEDTP) present a statistically significant increase in size when compared with CS NPs. This might be due to the increased size of the modified polymer, since the amine groups are not incorporated into the main polymeric chain, but are present as side groups, in the same amount as in CS. Also the presence of some residual tosylate counter-ions on CS-(AEDTP) polymer may somehow hinder the chain entanglement process during NPs formation and hence, the higher NP size. The tosylate counter-ion does not behave as other counter-ions, such as chlorine, and instead of being promptly released in to the solvent it tends to bind to micelles and particles. It does so by immersing its aromatic ring in the hydrocarbon polymeric chains and directing their charged part towards the surface of the NP. [22] In addition, CS-(AEDTP) NPs suffer an inversion of surface charge when compared to CS NPs (Table 3.1), which confirms the complexation of pDNA with CS-(AEDTP). This negative charge can also be partially attributed to the presence of the tosylate counter-ion as described previously. Additionally, due to the increased size of the polymer, while maintaining the same density of amine groups, pDNA complexation yields NPs with increased size (Table 3.1).

Notwithstanding, NPs formed with CS and CS-(AEDTP) performed very similarly in terms of pDNA encapsulation and protection from degradation (Fig. 3.2), suggesting no major differences in the packaging capability of the modified polymer. Both were able to efficiently incorporate and protect pDNA from DNAse I degradation, which means that they can be used to deliver pDNA to cells and protect it from extracellular and intracellular degradation.

The cytotoxicity of the NPs was tested in HEK293, D407 and ARPE-19 cell lines (Fig. 3.5). This is important to assess what will be the effect on cells of the polymer after DNA is released. Up to 5.0 μg of polymer, cell viability was unaffected. Above this value cell viability decreased for all cell lines, as expected, more markedly in ARPE-19. [23] Notwithstanding, the highest concentrations tested are several orders of magnitude higher than the ones used for the transfection assays or *in vivo* in mouse models, which indicate that these polymers are not cytotoxic for cells. Additionally, since NPs are not subjected to any purification or isolation step some of the cytotoxicity observed is due to the acetic acid present in the NPs dispersion and not to the NPs or the polymer itself (data not shown). Different ways to purify the NPs are currently being evaluated: dialysis, ultracentrifugation, and lyophilization. The former leads to an extensive dilution of the NPs, while the two latter need to be optimized to reduce aggregation.

Transfection studies with CS particles have been performed on several cell lines but never on retinal cells. [9, 24-27] Studies with HEK293 cells, where the same particle preparation method and pDNA amount were used, obtained a higher transfection efficiency than the one we here report for retinal cells. [9, 24] This might be attributed to differences in the CS used, namely in the MW and DD, since, this is known to influence pDNA release, thus, transfection results. [25, 28, 29] At a constant DD, higher MWs represent more extensive polymeric chains, with a higher number of positive charges able to interact electrostatically with nucleic acids and the sum of these interactions may hinder DNA release. Contrarily, lower MWs represent fewer cationic groups to establish electrostatic interactions, hence, neither efficiently complexing nor protecting genetic material from degradation. Furthermore, cell membrane penetration and endo-lysossomal escape are also influenced by the MW and DD. For this study, we have selected a CS with an average MW and DD based on the above – referred effects, in an

in an attempt to have a chitosan that complexes and protects nucleic acids and efficiently releases them.

It is widely accepted that the success of NPs transfection is limited by cellular uptake, endossomal escape and nuclear transport. CS NPs are believed to enter the cell through endocytosis and that cell binding may be dependent on polyplex charge. [28, 30-32] Cationic NPs, such as CS, bind electrostatically to negatively charged moieties in the cellular membrane due to the presence of negatively charged surface proteoglycans. These interactions are thought to play an important role in initial steps of polyplex cellular uptake. [33, 34] Kopatz *et al*. demonstrated the involvement of proteoglycans in this process using PEI particles. [35] In their study, cellular entry is mediated by syndecans (a transmembrane heparan sulfate proteoglycan) through an electrostatic zipper effect of the plasma membrane onto the particles. [35] In this way, the difference in charge between CS and CS-(AEDTP) (+20.1 ± 2.3 and -20.1 ± 5.2, respectively) can account for the lack of improvement in the transfection efficiency of CS-(AEDTP), due to hindrance in cell penetration due to poor interaction with the cellular membrane.

Additionally, the different sizes of CS and CS-(AEDPT) NPs might have led them through different paths into the cell. As demonstrated previously by Rejman *et al*. particle size can affect the internalization pathway via clathrin- and caveolae-mediated endocytosis. [36] Larger particles (>200 nm to <1 μm) enter cell in a slow manner and preferably through caveolae-mediated endocytosis or macro-pinocytosis whereas smaller particles (≤ 200 nm) enter the cell rapidly and preferably through clathrin-mediated endocytosis. [31, 36] Therefore, the size increase of the NPs might have slowed down their progression once inside the cell. Therefore, the observation at 72 h post-transfection would need to be extended further in order to confirm the hypothesis of slow progression of the NPs.

Chitosan-DNA interactions have been reported to be highly stable, which might be its major limiting step for pDNA intracellular release. [37] Thiolation was generally regarded as a way to improve release, with studies showing increased transfectability of thiolated CS both *in vitro* as *in vivo.* [13] This might be related to an increased mucoadhesion and cell permeation properties, and also due to the fact that thiolation

decreases the number of positively charged amine groups, hence, establishing less electrostatic interactions with pDNA, however, resulting in more prompt pDNA release. [13] However, in our study the number of amines capable of electrostatic interaction was similar in CS and CS-(AEDTP), therefore, the capacity of interaction with DNA was maintained, without improvement in the release.

In our study, thiolation did not seem to improve the transfection efficiency in retinal cells. This is most likely due to hindered cellular penetration and the inability of intracellular glutathione to access and reduce the disulfide bonds in CS-(AEDTP) since our results show that incubation with increasing amounts of DTT and cytoplasmic concentration of glutathione did not lead to the release of pDNA in our electrophoretic assays. Therefore, the amount of cytoplasmic glutathione in RPE cells might not be enough to reduce the disulfide bonds. Another explanation might be related to the negative surface charge of CS-(AEDTP) DNA loaded NPs. Glutathione is negatively charged at physiological pH and hence it would not easily interact with and penetrate the NPs, therefore, not causing the release of the pDNA. Current work of our lab uses intracellular tracking strategies to provide information about the route for cellular penetration and pathway to the nucleus of both CS and CS-(AEDTP). Results with CS confirm that the cellular penetration is not the limiting rate, but it might be the case with CS-(AEDTP) (results not shown).

6. Conclusions

The use of NPs as gene delivery vectors to target tissues has demonstrated great potential in the treatment of a large variety of pathologies, especially, as drug delivery systems. [38] Their application in gene therapy is, to this day, limited by their low transfection efficiency. In an attempt to improve the transfection efficiency of chitosan, we have modified chitosan to incorporate disulfide bonds, since it has been described as means to enhance gene delivery by this polymer. While other studies have shown a significant increase in the transfection efficiency, our results show that for retinal cells, thiolation does not seem to improve expression of genes delivered by chitosan. To the best of our knowledge, this is the first report of such a comparison in retinal cells, which indicates that for gene therapy of the retina, thiolation of chitosan does not constitute and advantageous modification. Nevertheless, this apparent drawback may eventually be circumvented by the replacement of mercaptoethylamine p- toluenessulfonate by its hydrochloride salt, since chloride ions do not present the same problems as tosylate.

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Chapter IV

Combining hyaluronic acid with chitosan enhances gene delivery

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1. Abstract

The low gene transfer efficiency of chitosan-DNA polyplexes is a consequence of their high stability and consequent slow DNA release. The incorporation of an anionic polymer is believed to loosen chitosan interactions with DNA and thus promote higher transfection efficiencies. In this work, several formulations of chitosan-DNA polyplexes incorporating hyaluronic acid were prepared and characterized for their gene transfection efficiency on both HEK293 and retinal pigment epithelial cells. The different polyplex formulations showed morphology, size and charge compatible with a role in gene delivery. The incorporation of hyaluronic acid rendered the formulations less stable, as was the goal, but it did not affect the loading and protection of the DNA. Compared with chitosan alone, the transfection efficiency had a 4-fold improvement, which was attributed to the presence of hyaluronic acid. Overall, our hybrid chitosan-hyaluronic acid polyplexes showed a significant improvement of the efficiency of chitosan-based non-viral vectors *in vitro,* suggesting that this strategy can further improve the transfection efficiency of non-viral vectors.

Keywords: Chitosan, Hyaluronic acid, Gene therapy, Retina, Non-viral vectors.

2. Introduction

Non-viral gene therapy is currently limited by the lack of vectors with gene transfer efficiency similar to viral vectors. Chitosan is one of the most studied cationic polymers for non-viral gene therapy, both *in vitro* and *in vivo*. [1-3] Although chitosan-based polyplexes (complexes of chitosan-nucleic acids) have desirable characteristics for gene therapy such as efficient nucleic acid encapsulation and protection against degradation, they show low gene transfer efficiency, which is the major obstacle to its use as a gene therapy vector. [1, 4] Several studies suggest a direct correlation between the stability of polyplexes and transfection efficiency, proposing that the high stability and strong interactions between chitosan and DNA are the cause for the low transfection results. [2, 5, 6] Polyplex stability is thus a crucial parameter when designing a polymer based gene delivery vector: it should be stable enough to withstand the cellular internalization process, but not too stable that once inside the cell it will not release its therapeutic load. [7, 8]

Approaches for improving the efficiency of chitosan-mediated gene transfer focus on chemical modification of chitosan and the incorporation of anionic biopolymers. We have previously modified chitosan to incorporate disulfide bonds that could be cleaved intracellularly, but the increase in transfection efficiency was moderate. [9] The incorporation of anionic polymers, which destabilize polyplexes and hence facilitate DNA release [4, 7, 8, 10, 11] is another strategy that has shown promising results. Competition-binding assays showed that the addition of alginate effectively reduces the interaction strength between CS and DNA, which improved DNA release, and consequently transfection. [10] Other studies have shown that coating polyplexes with hyaluronic acid (HA) enhanced internalization and transfection in association with other cationic polymers, such as polyethyleneimine. [12, 13] It has also been previously shown that the incorporation of HA into the polyplex formulation increased green fluorescent protein (GFP) expression and the authors suggest this increase to be related to 1) improved internalization due to interactions with the cellular surface, 2) HA function as a transcription activator and 3) HA accumulation in the perinuclear region and cell nuclei. [14] Furthermore, several studies hypothesized that HA could be used to improve targeting through the specific HA/CD44 receptor interaction, and could be of value to both gene and drug delivery strategies. [14, 15]

In this work, we designed CS polyplexes incorporating HA (CSHA), with two different molecular weights (MW) in several CS to HA ratios and evaluated their potential *in vitro* for gene delivery. While other studies have used CSHA polyplexes targeting the anterior part of the eye [12, 14, 16], this is, to our knowledge, the first report in the posterior part of the eye. We have tested these formulations in cells of the retinal pigment epithelium, a cell layer of the retina that supports the overall health of the retina, [17] and whose function, when compromised, is implicated in several retinal pathologies such as age-related macular degeneration, among others.

3. Results

3. 1. Size, Polydispersity, Surface charge and Morphology

The initial step in polyplex characterization was the determination of their size, polydispersity (PdI) and surface charge (by zeta potential, ZP), and the results are presented in Table 4.1. For some of the tested CS:HA ratios (3:1, 4:1 and 10:1) we observed aggregation, and consequently large sizes and high PdI. These ratios were not further tested (data not shown).

Polyplexes formulated without pDNA had sizes between 334.7 ± 62.76 nm and 402.8 ± 94.10 nm while those formulated with pDNA revealed sizes between 241.8 ± 64.04 nm and 328.1 ± 50.25 nm. The observed decrease in size is statistically different between CS polyplexes and pDNA loaded polyplexes CSDNA, CSHA4 and CSHA8; differences were also found between CSDNA polyplexes and pDNA unloaded CSHA formulations.

All mean PdI values are under 0.300, reflecting homogeneous preparations. The only statistically significant differences found were between formulations CSHA3 and CSHA4, CSHA3 and CSHA8 ($p \leq 0.05$). These might reflect minor differences in the homogeneity of the polyplex suspensions mainly due to the presence of an additional anionic compound in the mixture. Polyplexes were produced as a single homogeneous preparation as illustrated by the DSL size distribution graphs (Fig. 4.1), where no secondary peaks were observed. Regarding the surface charge of the polyplexes, all displayed a positive charge with ZP values between 18.20 ± 2.30 and 24.29 ± 3.19. The ZP values that were found to be statistically different were only for CSHA2 versus CSHA3 and CSHA3 versus CSHA5 ($p \leq 0.05$).

The morphology of the polyplexes was also analysed by TEM and the microphotographs revealed near spherical polyplexes with sizes consistent to the ones determined by DSL (Fig. 4.2).

3. 2. pDNA complexation and protection from DNase degradation

The second step in the characterization of the polyplexes was to evaluate if the presence of HA in the different formulations affected pDNA complexation and protection against nuclease degradation.

Table 4.1 – Composition, size, polydispersity and zeta potential of CS and CSHA polyplexes.

Particle	pDNA§	HA (kDa)	CS:HA ratio	Size (∅, nm)	Polydispersity	Zeta potential (mV)
CS	-	-	-	402.8 ± 94.10	0.273 ± 0.128	19.9 ± 2.38
CSpDNA	+	-	-	241.8 ± 64.04****	0.266 ± 0.068	20.09 ± 2.28
CSHA1	-	132	5:1	351.3 ± 24.79##	0.245 ± 0.092	23.57 ± 1.19
CSHA2	+			328.1 ± 50.25	0.260 ± 0.088	24.18 ± 3.06
CSHA3	-		7:1	334.7 ± 62.76#	0.157 ± 0.066	18.20 ± 2.30
CSHA4	+			267.7 ± 30.52****	0.293 ± 0.080	19.08 ± 3.56
CSHA5	-	214	5:1	360.5 ± 69.66##	0.183 ± 0.064	24.29 ± 3.19
CSHA6	+			322.5 ± 69.94	0.270 ± 0.097	23.04 ± 2.31
CSHA7	-		7:1	345.6 ± 43.05#	0.243 ± 0.068	20.84 ± 4.48
CSHA8	+			284.9 ± 34.31***	0.298 ± 0.065	20.00 ± 2.60

§Presence or absence of pDNA in the formulation is indicated by + or -, respectively. Values marked with asterisks are statistically different to CS value, *** $p<0.001$, **** $p<0.0001$;Values marked with cardinals are statisticaly different to CSDNA value, # $p<0.05$, ## $p<0.01$ (statistical differences determined by Tukey’s multiple comparisons test).

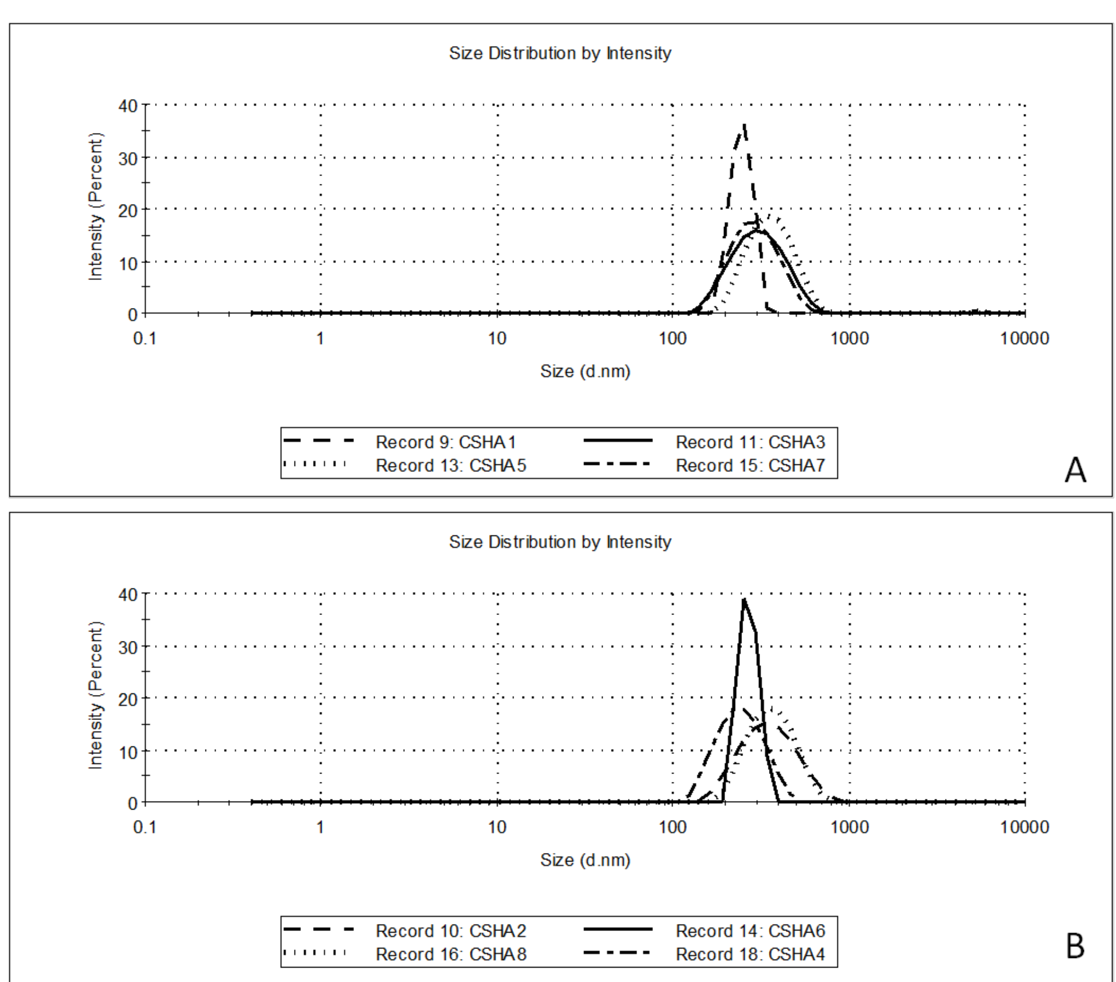

Figure 4.1 – Dynamic light scattering raw data. Representative graphs for CSHA polyplexes: A) without pDNA and B) with pDNA,

All formulations complexed pDNA successfully, as observed by the absence of pDNA migration in an agarose gel (Fig. 4.3A and B). Polyplexes without pDNA (CS and CSHA) were used as controls and, as expected, did not produce any detectable signal. These results were further confirmed by performing the same experiment in a 0.3% agarose gel (Fig. 4.4). Regarding pDNA protection against nuclease degradation, all formulations showed a similar behavior, with no detectable pDNA degradation (Fig. 4.3C and D). Uncomplexed pDNA was used as a control and, as expected, was completely degraded by DNase I.

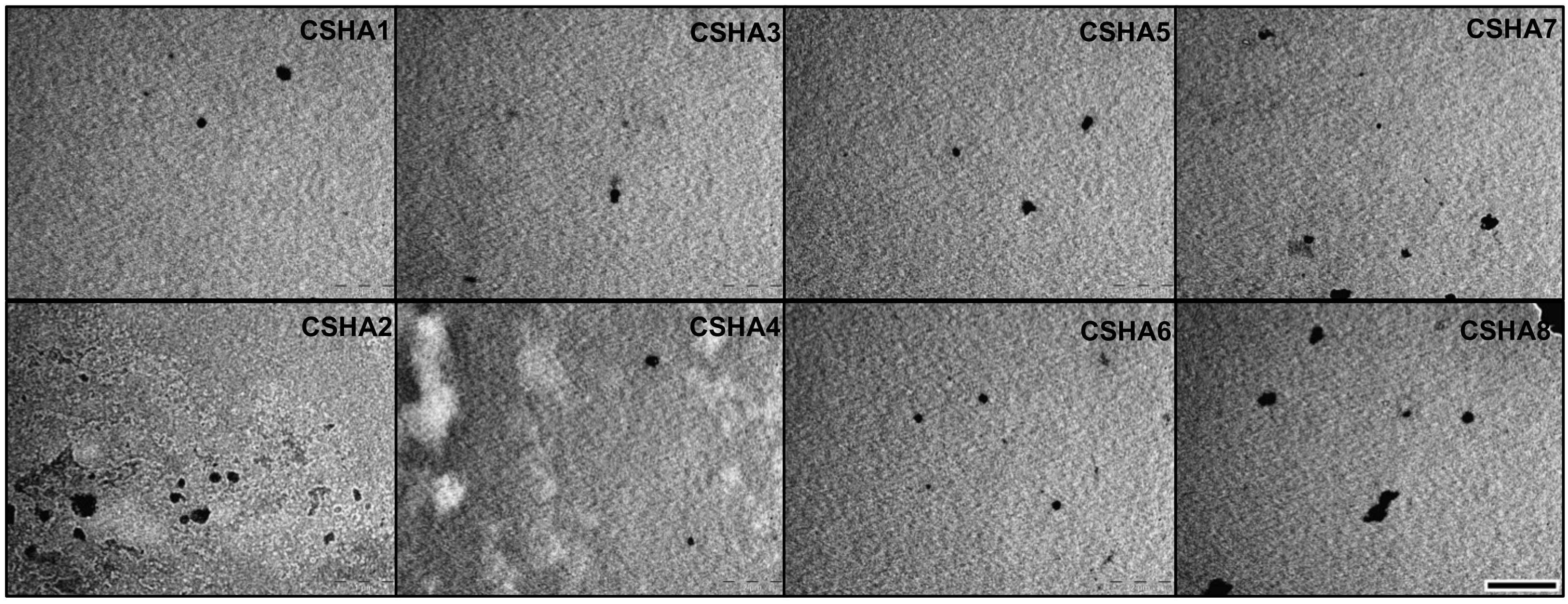

Figure 4.2 – TEM microphotographs of CSHA polyplexes. The different formulations have a regular, close to spherical morphology (amplification: 100,000x, scale bar represents 2μm).

3. 3. Polyplex stability

Stability at physiological conditions is an important factor since polyplexes should be stable enough to protect pDNA and only release it once it has reached the intracellular milieu. We therefore tested the stability of the polyplexes at physiological pH and in the presence of serum, to mimic *in vivo* conditions, by incubating the polyplexes with PBS (pH 7.4) or DMEM with 10% FBS, respectively. No detectable pDNA release was observed regardless of the formulation and period of incubation (Fig. 4.5).

The shelf life of the produced polyplexes is also an important factor to consider. Long-term stability of the polyplexes was evaluated at 4 and 37ºC by analysis of their size and PdI (Fig. 4.6). Results depicted in Fig. 4.6 were evaluated by variance analysis (1 way-ANOVA) and Dunnett's post-hoc test to compare values to the starting time point (t=0). All CSHA formulations performed very similarly and therefore the results shown for formulations CSHA7 and 8 are representative of the other CSHA formulations.

The results showed that both size and PdI directly increased with temperature and that all tested formulations remained stable at 4ºC for an extended period of time (up to 13 weeks). The size of CSDNA polyplexes remained constant throughout time. The presence of pDNA in the formulation seemed to increase stability in CS formulations. Although there were no statistical differences in the size of CSDNA polyplexes, their PdI at 37ºC started to increase, reflecting their decreasing stability at this temperature, after 49 days (Fig. 4.6). Also, measurements of some formulations were discontinued due to visible aggregation and/or insufficient volume, hence the difference in the depicted time points.

As aimed, formulations containing HA and pDNA were less stable probably due to increased repulsion between charged groups. The stability of CSHA8 formulation decreased after 119 days at 4ºC and much earlier at 37ºC (63 days), as can be observed by the increase in size and PdI (Fig. 4.6)

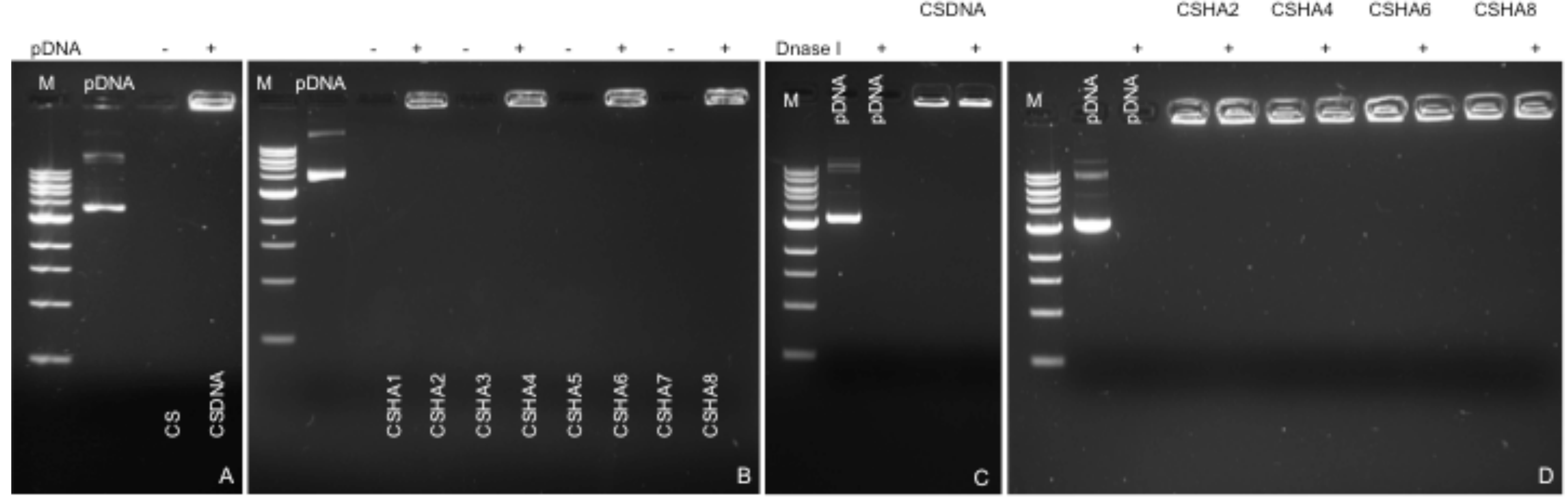

Figure 4.3 – Polyplexes encapsulation efficiency and nuclease protection. Analysis by 1% agarose gel electrophoresis, DNA visualized with ethidium bromide, A) and B) pDNA encapsulation in CSpDNA and CSHApDNA polyplexes, respectively; lanes positive for polyplexes but negative for pDNA represent unloaded polyplexes. DNA is protected against DNase I digestion: C) CSpDNA and D) CSHApDNA polyplexes after a 1 h incubation with DNase I.

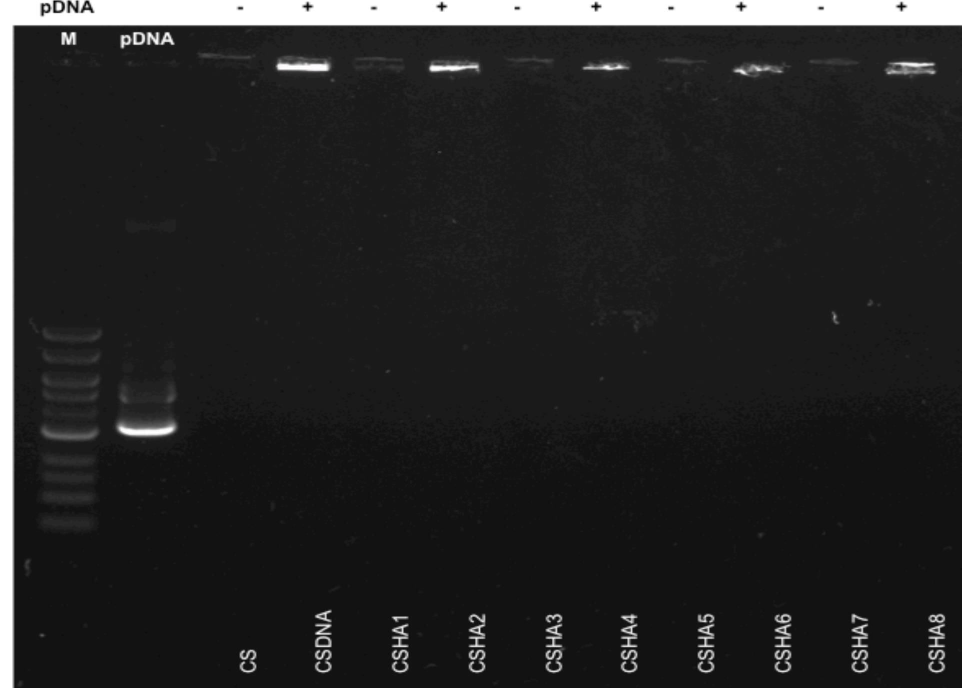

Figure 4.4 – Polyplexes encapsulation efficiency analyzed by 0.3% agarose gel electrophoresis. DNA visualized with ethidium bromide, lanes positive for polyplexes but negative for pDNA represent unloaded polyplexes.

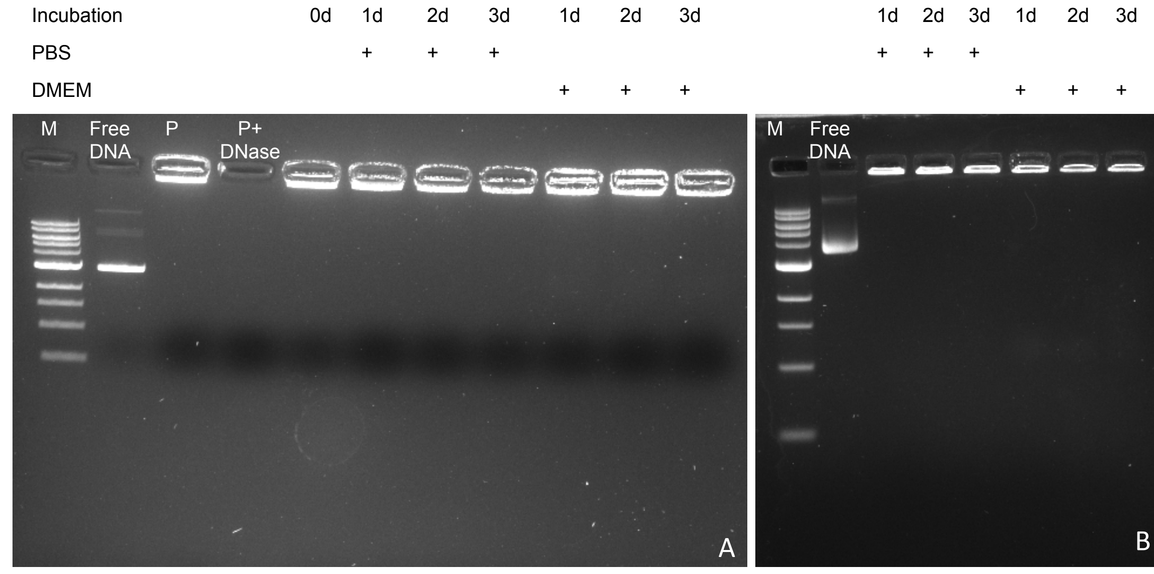

Figure 4.5 – Polyplexes remain stable at physiological conditions. A) CSpDNA and B) CSHApDNA polyplexes after incubation in PBS or DMEM with 10% FBS at 37ºC. Polyplex pDNA retention analyzed by 1% agarose gel electrophoresis and pDNA visualized with ethidium bromide (P-polyplexes, P+DNase- polyplexes incubated with DNase I, data showed only for CSHA8 but representative of all CSHA polyplexes).

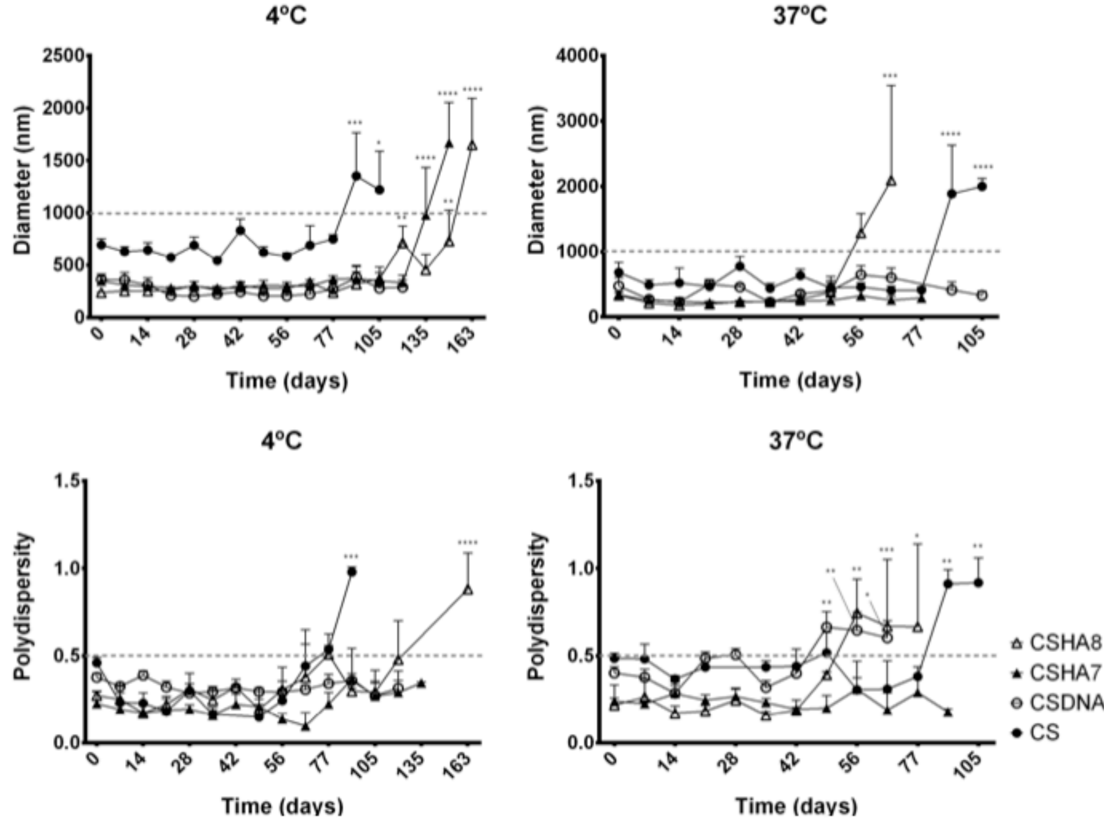

Figure 4.6 – Particle stability at 4ºC and 37ºC; as verified by continued monitoring of their size (upper row) and polydispersity (lower row). Significant differences to initial value were determined by Dunnett's multiple comparisons test, (**** p<0.0001, *** p<0.001, ** p<0.01 * p<0.05)

3. 4. *In vitro* studies

Cytotoxicity of the developed polyplexes was evaluated using an MTT assay in the retinal cell line (ARPE-19, Fig. 4.7). For HEK293 cells, our previous studies have indicated absence of cytotoxicity. [9] Statistical analysis revealed no differences between CS and CSHA formulations. Cell viability was above 75% regardless of formulation and concentration, except for the highest concentration tested, which caused a decrease in cellular viability, with values below 50% for all formulations. However, this concentration is above the range to be used *in vivo*.

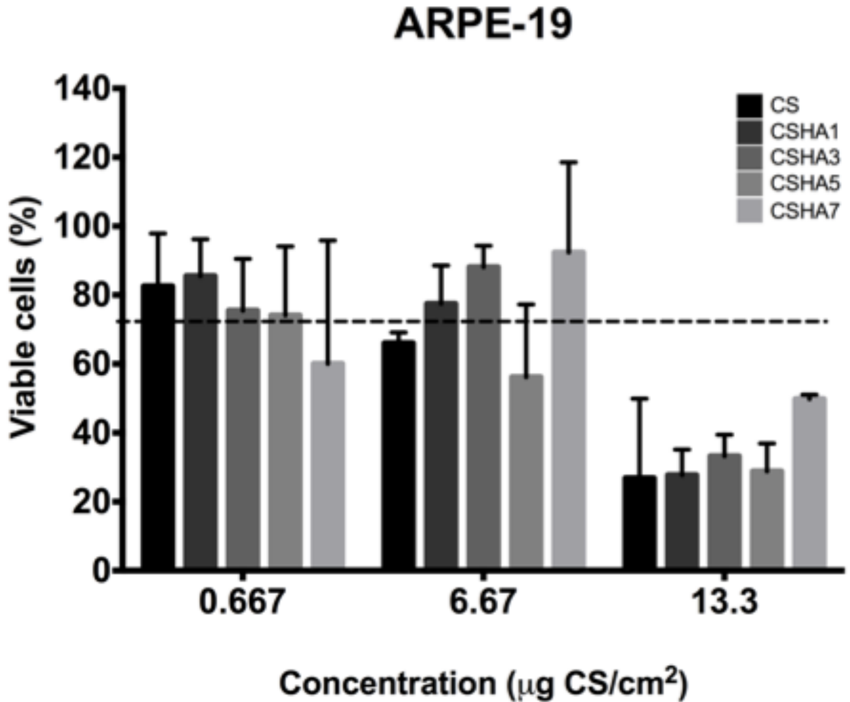

Figure 4.7 – Cytotoxicity in RPE cell line (ARPE-19), given as percentage of viable cells after 72h incubation with polyplexes at increasing concentrations. Polyplex concentration is given in total CS μg in polyplexes per cm^2 of growth area.

One of the key points of our study was to evaluate if the introduction of HA would affect the stability of the polyplexes to facilitate DNA release and hence increase their transfection efficiency. CS and CSHA formulations with pDNA coding for GFP were tested both in the retinal cell line ARPE-19 and in HEK293 cells. The latter were used as a transfection control, as their permissibility in terms of transfection is widely accepted. As a control of the GFP expression system we used FuGene® HD, a commercial reagent. The percentage of GFP positive cells 72h post-transfection is presented in Fig. 4.8. Results showed that the transfection efficiency varied with cell line and type of formulation. HEK293 cells displayed higher levels of GFP expression when compared with the RPE cell line, as expected, based on their different mitotic rates. All formulations incorporating HA present increased GFP expression when compared to CS polyplexes, with CSHA polyplexes showing a near 4-fold increase in transfection efficiency in HEK293 cells.

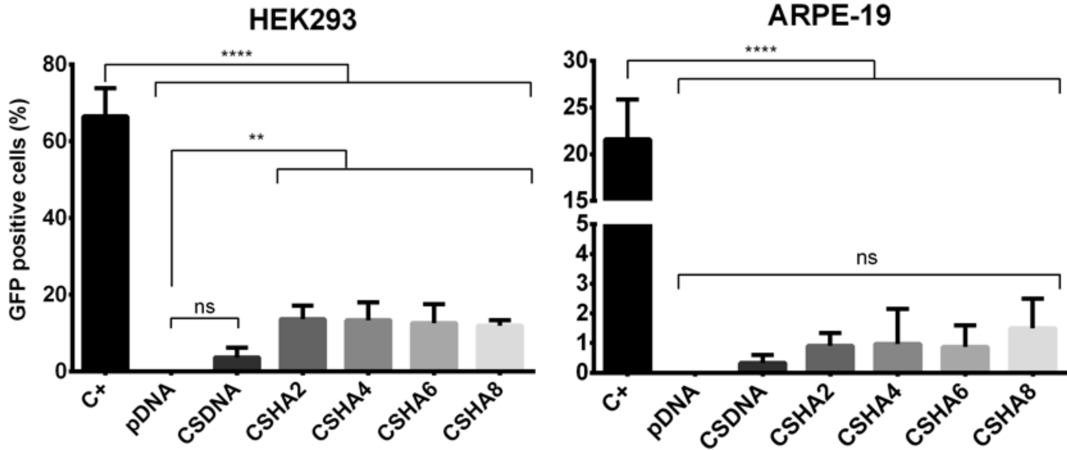

Figure 4.8 – Transfection efficiency 72h post transfection as percentage of GFP positive cells. Statistical differences were calculated using Tukey and Dunnett's multiple comparisons test (**** $p<0.0001$, ** $p<0.01$, ns – not significant).

4. Discussion

4. 1. Polyplexes

This work aims to evaluate the effect that incorporating HA into CS polyplexes would have in the stability of the polyplexes and in their transfection efficiency. In summary, the characterization of the polyplexes revealed differences in size for pDNA loaded and unloaded polyplexes, but not between CS and CSHA polyplexes. This size difference between polyplexes with and without pDNA has been previously observed and is in accordance with the literature, since CS establishes strong interactions with negatively charged pDNA that contribute to a higher chain entanglement thus producing smaller polyplexes. [9]

Variations in size and surface charge were expected after the incorporation of HA into the formulation, since it has been shown to occur an increase in size and decrease in surface charge associated with HA content. [12, 14] In our study no effect of HA was observed in either size or surface charge. When addressing the effect of the MW of HA on the size of polyplexes, studies have shown the formation of smaller polyplexes with HA of increasing MWs, and also with the use of higher CS:HA ratios. [11] In this aspect, our results are well correlated with the literature, since using HA with a high MW produced polyplexes with smaller size and positive surface charge, whereas the use of low ratios increased sizes and decreased surface charge, causing aggregation (data not shown) as shown by Duceppe *et al.* (2009). [11]

It has also been suggested that the MW of the anionic polymer can influence the morphology of the polyplexes. [11] This was not observed in our study, since no morphological differences were noticeable either among CSHA formulations or between CS and CSHA polyplexes. We attribute this dissimilarity of our results with the literature to differences in the characteristics of the polymers and polyplexes such as MW and CS:HA ratios used in our study. Also, this difference in polyplex behavior might be related to differences in the localization and degree of incorporation of HA in the polyplexes. [14] During the chain entanglement process HA chains may have

been trapped in the interior of the polyplex, thus not contributing to a significant decrease in surface charge.

4. 2. Stability

Previous studies have shown that HA can influence the stability of polyplexes without affecting pDNA binding to CS. [8] Our results support these findings, and also show that polyplexes remain stable at physiological conditions and are able to protect pDNA from nuclease degradation. Only in the long-term stability assay a decrease in polyplex stability was observed in CSHA polyplexes, more pronounced at 37ºC.

4. 3. Cytotoxicity

Polyplex charge is also an important parameter due to its relation to toxicity and transfection efficiency, as some authors have shown that a reduction in the positive charge results in an increase in transfection efficiency associated with a decrease in toxicity. [18, 19] A decrease in CS polyplex cytotoxicity with increasing amounts of HA in their formulations has been reported by Fuentes *et al.*(2008), but our results show no such differences either when different CS:HA ratios or MW were used. [14] Since no decrease in surface charge was observed, we expected the incorporation of HA into the polyplexes to have no significant effect in the cytotoxicity. Nevertheless, considering the concentrations to be used *in vivo* our polyplexes can be considered a safe option for gene delivery.

4. 4. Transfection

It is believed that one of the major causes of low transfection with polyplexes is their high stability and consequently inefficient and slow DNA delivery. [8, 11] Considering that all formulations displayed similar size, surface charge, and cytotoxicity, this suggests that the introduction of HA affected the interactions between CS and pDNA and modulated the release behavior, thereby resulting in a more efficient transfection. The observed

transfection efficiency improvement over CS formulations may also be related to increased cellular internalization since it has been showed previously by de la Fuente that HA containing polyplexes can be internalized via interactions with the membrane CD44 receptor. [14] Recent studies with the same cell lines but using a lipid-based delivery system also containing HA corroborate our results and also supports the idea that HA enhances the transfection efficiency by modulating the DNA condensation degree in the vector. The same study also states that the participation of the CD44 receptor in the internalization of the vectors is an important factor for increased transfection. [20]

Similarly to the results observed for HEK293 cells, a trend of higher GFP expression levels was observed when ARPE-19 were transfected with CSHA polyplexes, but it is not statistically significant. One possible explanation for this is their dividing rate, which is considerably lower than the one of HEK293 cells. The fact that cell lines with different mitotic rates were transfected demonstrates the versatility of our vectors and paves the way to possible future applications in other tissues. Additionally, it is known that the expression of CD44, the receptor for HA, in RPE is affected both by their proliferative state and confluence, which might explain these results. [21] It has also been described that CD44 is increased in pathological conditions such as RPE wound repair and choroidal neovascularization. [22, 23] This would suggest that polyplex CD44-dependent internalization *in vivo* and in a pathological situation could be greatly increased when compared with *in vitro* results. Based on this, our results lay the foundation for the use of CSHA polyplexes for gene delivery in animal models of retinal diseases.

5. Conclusions

In our study we describe the characterization of a hybrid polyplex for retinal non-viral gene therapy. The polyplexes containing both cationic (CS) and anionic (HA) polymers display an improved performance over the previously developed chitosan-based polyplexes. The incorporation of HA into the polyplexes resulted in a decrease in polyplex stability that most likely enabled a more rapid pDNA release whilst still protecting pDNA from degradation.

CSHA formulations showed an increase in *in vitro* cell transfection, which suggests that this strategy might be very effective *in vivo,* especially in retinal cells expressing CD44 and in pathological situations where CD44 is known to be involved, allowing cellular targeting by HA-containing formulations through CD44 interaction.

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Chapter V

Enhancement of chitosan-mediated gene delivery through combination with PhiC31 integrase

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1. Abstract

Gene transfer efficiency and expression stability are key factors to a successful gene therapy approach. In the present work we have developed a combined system for gene transfer that integrates well established non-viral polymeric vectors based on chitosan particles with the properties of phiC31-integrase that promotes a relatively non-immunogenic, site-specific integration, with sustained gene expression. Simultaneously, to overcome one of the major limitations in adeno-associated virus mediated gene transfer - the delivery of large genes – we have tested the capacity of our non-viral vectors to incorporate a large (8 Kb) transgene. Polyplexes were extensively characterized for their size, surface charge, morphology, pDNA complexation, transfection efficiency and transgene expression *in vitro* using HEK293 cells. Co-transfection with integrase was done by complexation in a single polyplex preparation or the use of two separate polyplex preparations. Transgene expression, GFP and CEP290 (1Kb and 8Kb, respectively), was evaluated by fluorescence microscopy, flow cytometry and western blot analysis. DNA complexation efficiency, particle size and morphology were consistent with gene delivery for all formulations. In contrast, transfection efficiency and transgene expression varied with polymer and polyplex size. Following delivery by chitosan polyplexes, high levels of GFP expression were still visible 16 weeks post-transfection and over-expression of the large transgene was detected at least 6 weeks post-transfection. Polyplexes incorporating phiC1 integrase demonstrate prolonged gene expression of both small (GFP, 1 Kb) and large genes (CEP290, 8Kb). This approach, using a combined strategy of polymers and integrase may overcome the size limitation found in commonly used adeno-associated virus mediated gene transfer techniques, while maintaining a high safety profile and prolonged, sustained gene expression, thus constituting an alternative for gene delivery.

Keywords: Polymers; chitosan; gene therapy; gene delivery; integrase.

2. Introduction

Gene therapy shows great promise regarding treatment of several inherited diseases and, in recent years, gene therapy has been featured in numerous clinical trials with encouraging outcomes. [1-4] Currently, there are two general methodologies used to deliver transgenes: a viral mediated and a non-viral mediated approach. The first has shown great success through the use of viruses such as adeno-associated viruses while the latter uses, for example, cationic polymers and lipids to deliver genes with so far modest results.

Despite the success of viral vectors, the use of viruses still raises several issues. Retroviral vectors can randomly integrate into the host genome and promote long-term gene expression but are unable to transduce non-dividing cells and can cause insertional mutagenesis. [5] Alternatively, lentivirus can be used to transduce non-dividing cells; however insertional mutagenesis is also a concern. [5] Adenoviruses and adeno-associated viruses (AAV) have also been evaluated for gene delivery but have limited use due to cytotoxiciy and limited transgene size packing, respectively. [5] The great advances in ocular gene therapy in the past decade can be attributed to the use of AAV vectors. As such AAV vectors have been prominently used in the most recent gene therapy clinical trials, especially in retinal disease, that have shown overwhelming safety and in some trials, efficacy. [3, 4, 6] The main limitation of this virus considering ocular applications is its limited packaging capacity (less than 5 kb), although some clinical trials have used lentivirus to overcome the limited packaging issue of AAV, safety and efficacy concerns continue. Also, AAV transduction of terminally differentiated post-mitotic cells, like retinal cells, is limited by insufficient dsDNA production. Despite the fact that these dsDNA genomes can integrate into the host cell, integration occurs at low frequency and randomly and so transgene expression tends to decline with time due to silencing or degradation of the episomal agent. [5]

In contrast, non-viral vectors are biocompatible, with low or no immunogenicity, have less handling safety requirements and have greater potential to deliver large genes. [7] However, their lower efficiency and short-term transgene expression compared to viruses has limited their use. This is the major rationale behind our study, which aims to demonstrate that the aforementioned shortcomings can be improved on.

Any successful gene therapy strategy requires long-term sustained transgene expression. Recently, several strategies have been evaluated in order to promote safe integration and long-term expression. One of the most promising exploits a site-specific recombinase, the phage phiC31 integrase. PhiC31 integrase is a member of the serine site-specific recombinase family that catalyzes recombination between attachment sites on phage and bacterial genomes, attP and attB, respectively. [8, 9] Recombination at these sites results in the formation of hybrid sites, known as attL and attR that prevent further interaction with the integrase recombinase. In the absence of an excisionase protein, the reaction results in unidirectional integration. [9] PhiC31 integrase has also been shown to promote a safer, site-specific integration in mammalian cells using pseudo attP sites endogenous to the mammalian genome. [8, 10] Recent studies have shown that the phiC31 integrase can be successfully included in gene therapy strategies promoting integration and prolonged expression *in vivo* in mouse lungs [11], mouse liver [12], rat retina [13] and human skin [14, 15].

The use of non-viral gene delivery systems has gained importance over the years, especially with the use of natural cationic polymers, such as chitosan. Chitosan is a deacetylated derivative from chitin that effectively encapsulates and protects therapeutic nucleic acids [16], and has been used for gene delivery in prior *in vitro* [16-22] and *in vivo* [23-25] studies.

This study reports efficient gene transfer, by a novel combination of two well-reported methods, the non-viral phiC31-integrase mediated gene transfer with delivery to the cell using chitosan polyplexes. This method promotes stable transgene integration and sustained transgene expression. Additionally, we demonstrate the ability for stable and efficient gene transfer of large genes (> 8kb) that exceed the carrying capacity of commonly used adeno-associated viral vectors. This would enable gene therapy protocols for inherited degenerative diseases caused by mutations on large genes such as the blindness-leading Leber Congenital Amaurosis type 10 (LCA10) and Stargardt disease that affect the retina, among others.

3. Results

3. 1. Polyplex characterization

Polyplexes were characterized for their size, polydispersity and surface charge (given by the zeta potential). C1-based (80 kDa chitosan) polyplexes had smaller sizes than C2-based polyplexes, ranging between 371.7 – 444.1 nm, while C2 polyplexes had sizes between 594.9 – 661.3 nm (Table 5.1). The same trend was observed for the surface charge with C1-based polyplexes displaying lower values than C2 polyplexes (Table 5.1). Regarding their polydispersity, all polyplexes had similar polydispersity values consistently around 0.2 with no statistically significant differences.

Table 5.1 – Size, polydispersity and zeta potential of polyplexes.

Particle	Diameter (∅, nm)[a]	Polydispersity	Zeta potential (mV)
C1i	372.3 ± 48.3**	0.25 ± 0.09	17.9 ± 1.2
C1GFP	419.1 ± 84.6**	0.24 ± 0.11	16.1 ± 3.5
C1GFPi	444.1 ± 92.0	0.27 ± 0.12	18.3 ± 3.1
C1CEP	371.7 ± 35.3*	0.21 ± 0.09	18.2 ± 2.6
C1CEPi	422.6 ± 72.6	0.22 ± 0.04	19.3 ± 2.2
C2i	613.3 ± 123.2**	0.14 ± 0.07	22.4 ± 2.1
C2GFP	607.3 ± 130.9**	0.21 ± 0.10	20.4 ± 2.8
C2GFPi	597.8 ± 120.7	0.21 ± 0.13	21.3 ± 2.5
C2CEP	594.9 ± 16.4*	0.26 ± 0.13	20.3 ± 5.9
C2CEPi	661.3 ± 73.7	0.12 ± 0.06	23.5 ±3.4

[a] Mean ± standard deviation, n≥3 , *p<0.05, **p<0.00

Morphological analysis of polyplexes by TEM revealed a spherical morphology and no significant differences between the morphology of the different formulations (Fig. 5.1). Polyplexes were also characterized for their effectiveness in complexing DNA. No free DNA was detected during analysis of polyplexes containing pGFP and pCEP alone, or pGFP and pCEP combined with the integrase plasmid (Fig. 5.2). All polyplexes successfully complexed pDNA regardless of the

polymer used, plasmid size and number of plasmids used in the preparation (Fig. 5.2).

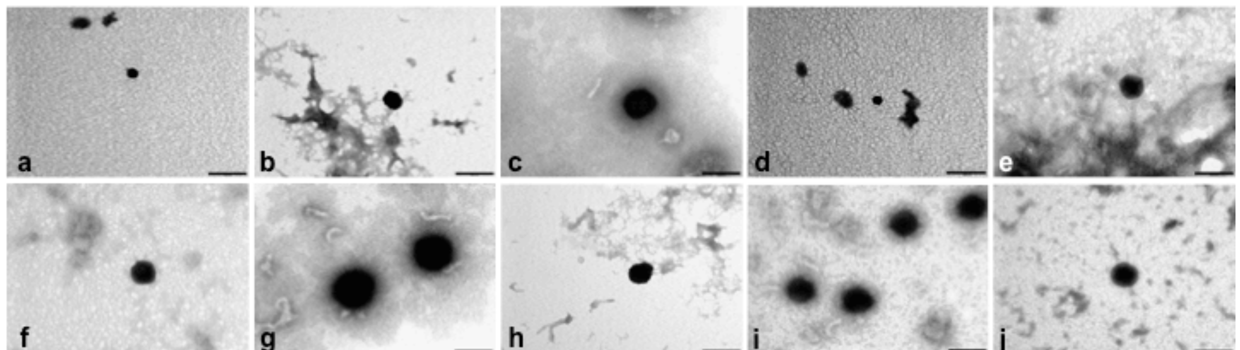

Figure 5.1 – TEM microphotraphs of polyplexes (Amp. 100,000x, scale bar represents 200 nm): (**a**) C1i, **(b)** C1GFP, **(c)** C1GFPi, **(d)** C1CEP, **(e)** C1CEPi, **(f)** C2i, (**g**) C2GFP, **(h)** C2GFPi, **(i)** C2CEP, **(i)** C2CEPi.

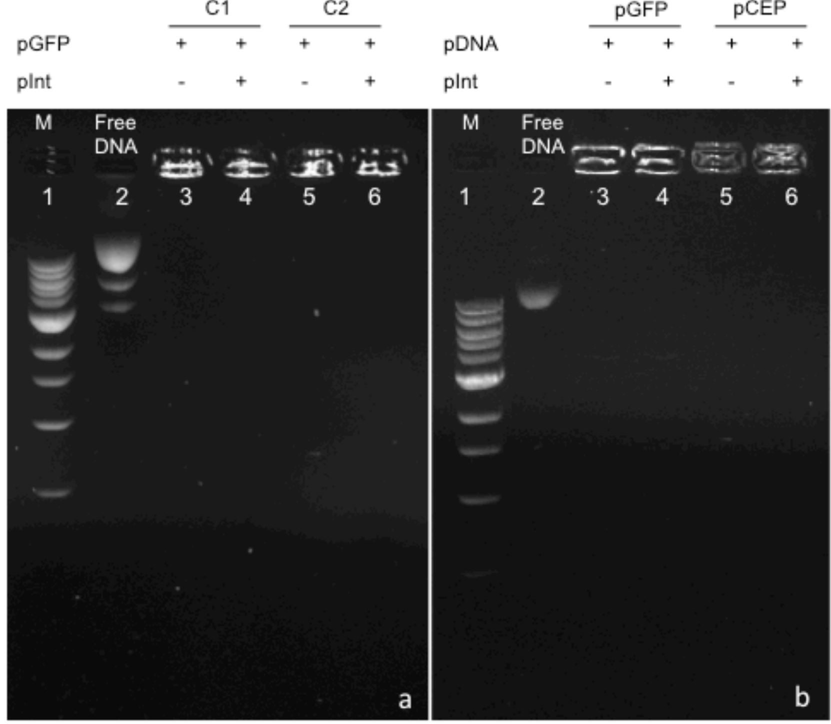

Figure 5.2 – DNA encapsulation in polyplexes. Analyzed by 1% agarose gel electrophoresis and DNA visualized with ethidium bromide; **(a)** using chitosan with two different sizes, C1 polyplexes with one or two plasmids in lane 3 and 4, C2 polyplexes with one or two plasmids in lane 5 and 6, respectively; **(b)** using plasmids of different sizes, C1 polyplexes with pGFP or pGFP+pINT in lane 3 and 4, and pCEP or pCEP+pINT in lane 5 and 6, respectively.

3. 2. Transfection studies

To demonstrate the potential for integrase to improve transfection efficiency and transgene expression, a series of transfections were performed in HEK293 and HEK293T cells using the smaller C1- and and larger C2-based polyplex formulations (Fig. 2.2, Chapter II). Transfection efficiency and transgene expression were analyzed by flow cytometry, fluorescence microscopy and western blot analysis at consecutive time points.

The short-term transfection efficiency of the different transfection conditions (CGFP, CGFPi and CGFP+Ci) was evaluated by flow cytometry 72 h post-transfection. Transfection efficiency was highest in the positive control (commercial transfection reagent FuGENE® HD) with a mean percentage of transfected cells of 57.79 ± 9.47 (data not shown). This reagent is optimized for *in vitro* transfection and is not recommended for *in vivo* experiments. C1-based polyplexes – C1GFP, C1GFPi and C1GFP+C1i had transfection efficiencies of 7.31 ± 1.12, 5.89 ± 1.49 and 9.00 ± 3.42, respectively. For C2-based polyplexes – C2GFP, C2GFPi and C2GFP+C1i the transfection efficiencies were 2.45 ± 0.90, 1.46 ± 1.16 and 2.13 ± 0.65, respectively. These results show that transfection is more efficient with C1-based polyplexes than with C2-based polyplexes, *i.e* transfection efficiency is greater with lower (80 KDa) than high (260 kDa) molecular weight chitosan (Fig. 5.3).

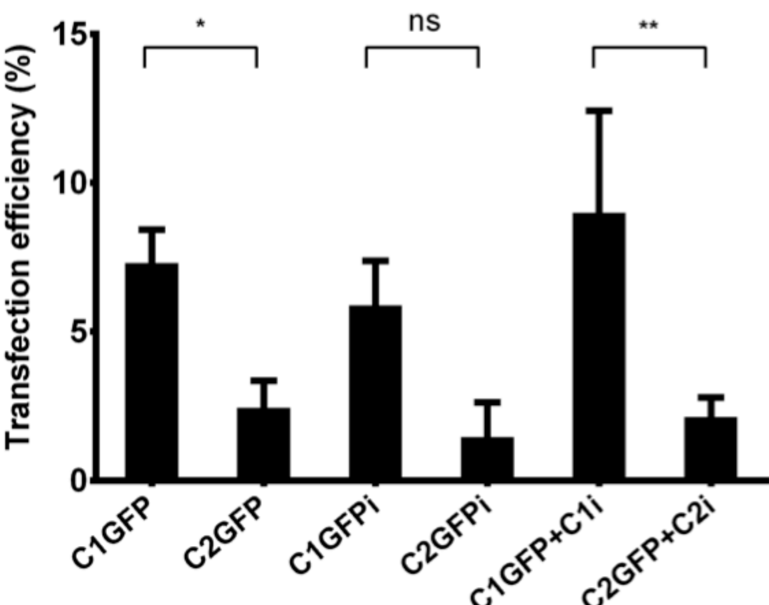

Figure 5.3 – Transfection efficiency 72h post-transfection in HEK293 cells as percentage of GFP positive cells. Statistical differences and their significance are indicated by the star (*) symbol, with * $P < 0.05$, ** $P < 0.01$, ns – non significant.

Transfected cells were kept in culture for several weeks to assess sustained long-term, GFP expression that was monitored by fluorescence microscopy and western blot analysis. GFP expression remained visible under fluorescence microscopy up to 16 weeks after transfection (Fig. 5.4) and GFP protein expression detected 14 weeks post-transfection (Fig. 5.5a). As expected, cells transfected with pGFP and integrase are more numerous and present higher intensity of GFP fluorescence than cells transfected with just pGFP (Fig. 5.4), but transgene expression had a decrease over time. However, when compared with traditional, non-integrative non-viral strategies it is clear that our strategy shows a significant improvement in long-term gene expression.

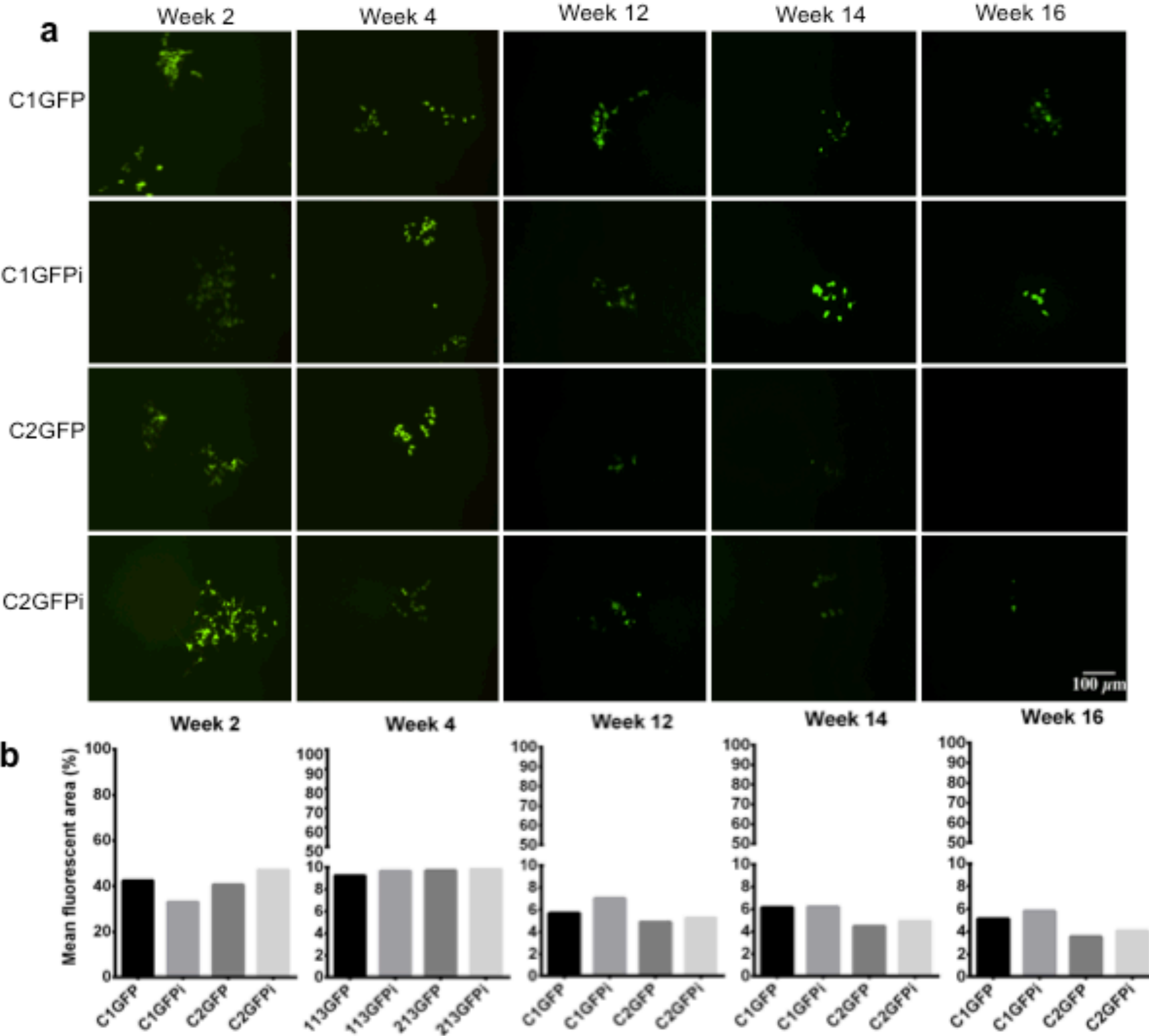

Figure 5.4 – GFP expression in cells transfected with C1 and C2 polyplexes with and without integrase over a period of several weeks: (**a**) fluorescence microphotographs (maginification.100x) and (**b**) mean fluorescent area of images in panel a) quantified by ImageJ.

To demonstrate efficient gene transfer of a large gene (>8 kb), CEP290 expression was also evaluated by western blotting. Since CEP290 is an endogenously expressed gene in the HEK cell line that was used, efficient transfection was measured as over-expression, which was consistent for at least 14 days post-transfection (Fig. 5.5b and c). This confirms our hypothesis of effective large gene delivery by our formulations.

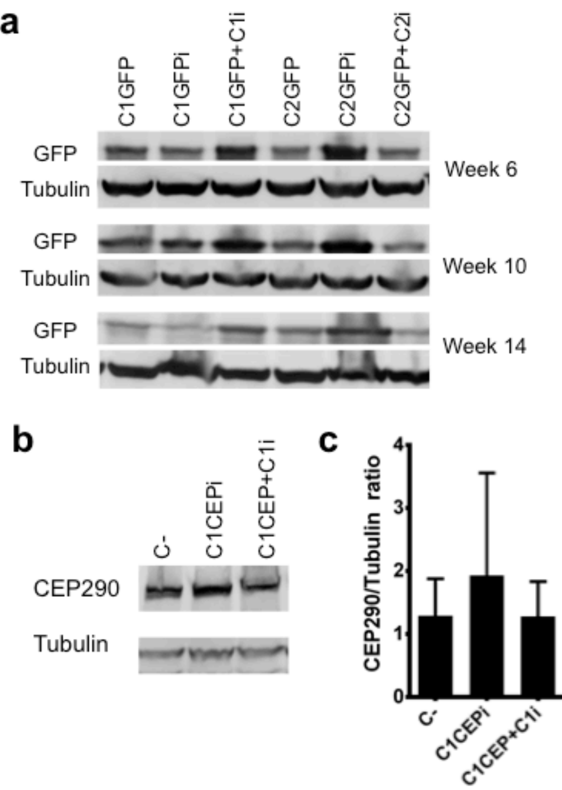

Figure 5.5 – Transgene expression over time in cells transfected with polyplexes analyzed by Western blot. (**a**) GFP expression, C+: Fugene HD transfected cells (120 µg total protein per gel lane); (**b**) CEP290 expression C- control, non-transfected cells (50 µg total protein per gel lane; (**c**) Densitometric ratio CEP290/Tubulin for the different transfection conditions of the Western blot shown in panel A. Horizontal dotted line represents value for the control (non-transfected cells).

In order to test for long-term expression, transfection parameters were tested followed by antibiotic selection, which selects for stably transfected cells, which will express the gene of interest for longer periods of time. After two weeks of geneticin selection the cells were cultured in standard culture medium and analyzed as described previously. As observed before, GFP expression decreased over time; however, 16 weeks post-transfection GFP levels are still high, especially for transfection conditions that include integrase (C1GFPi, C1GFP+C1i and C2GFPi) (Fig. 5.6 and 5.7a).

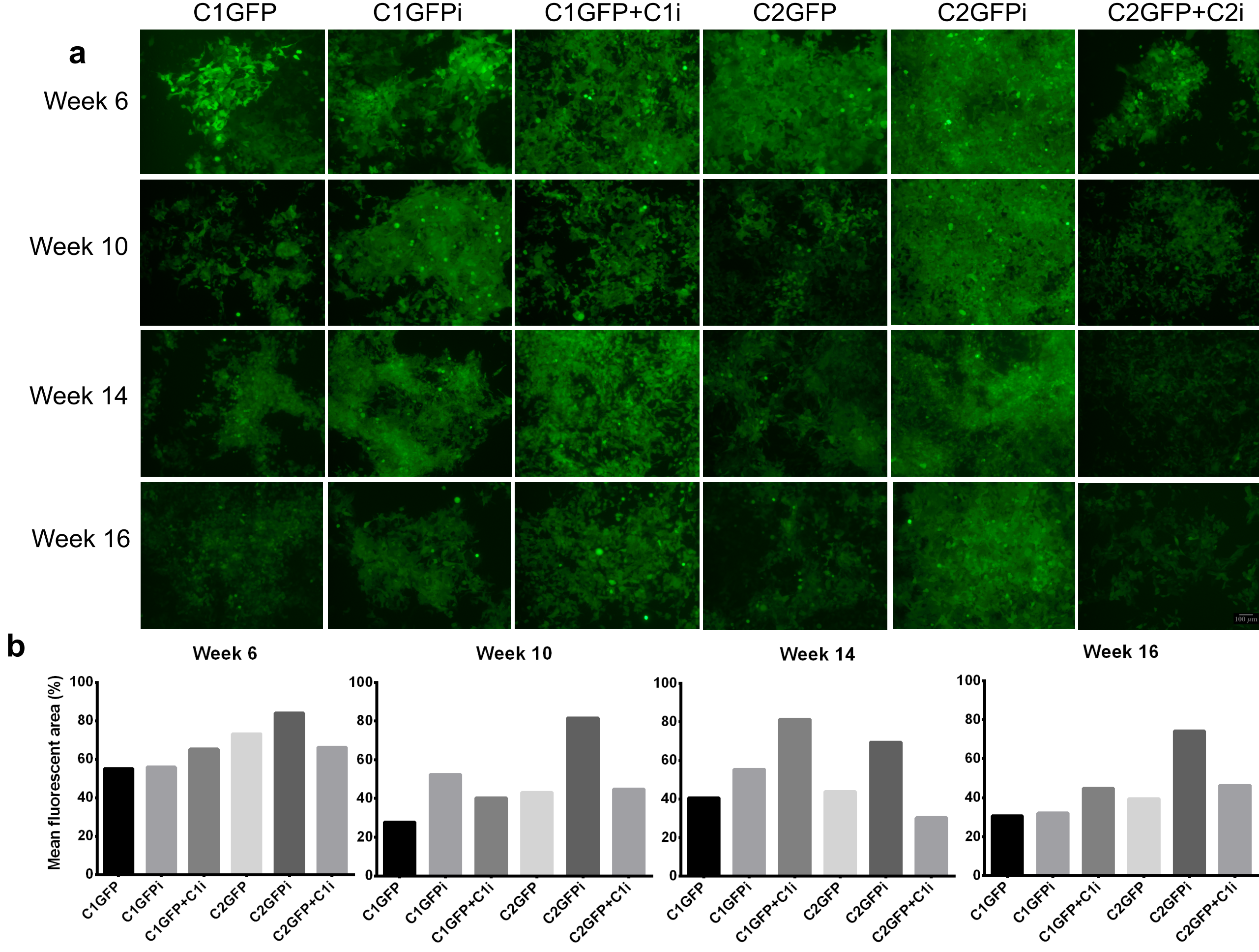

Figure 5.6 – GFP expression in cells transfected with CS1 and 2 polyplexes over a period of several weeks, after antibiotic selection. (**a**) fluorescence microphotographs (magnification100x) and and (**b**) mean fluorescent area of images in panel a) quantified by ImageJ.

Large gene (>8 Kb, CEP290) expression was also evaluated after antibiotic selection and western blot analysis shows a clear over-expression pattern even after 6 weeks post-transfection (Fig. 5.7b and c).

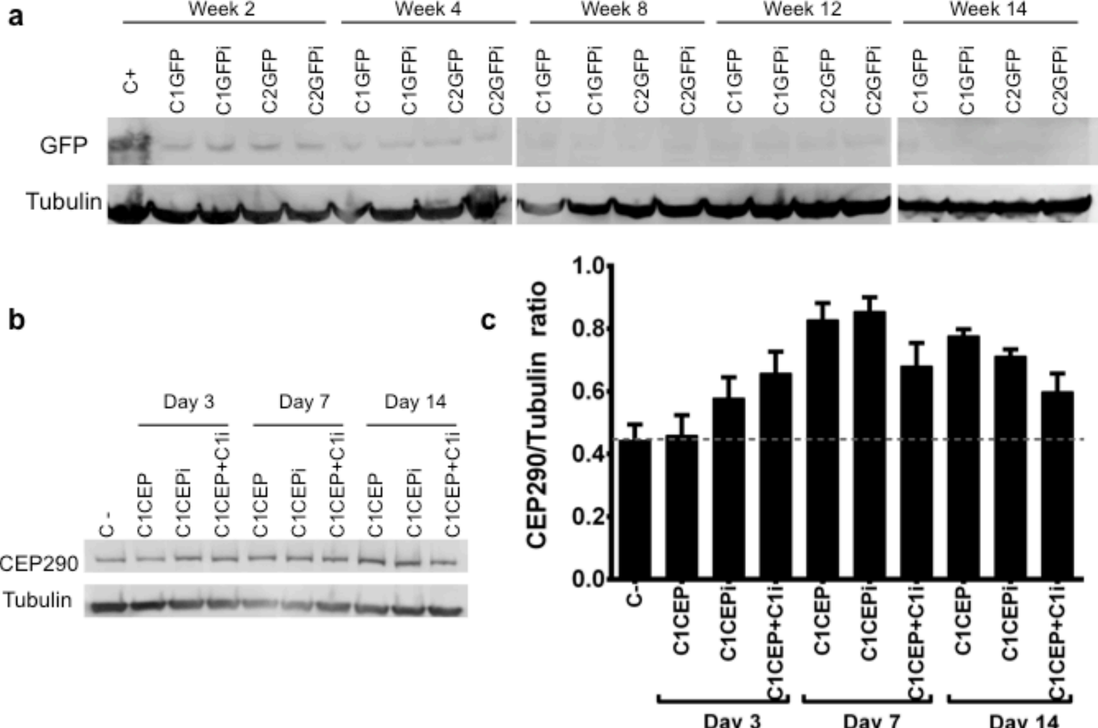

Figure 5.7 – Transgene expression over time in cells transfected with polyplexes analyzed by Western blot after antibiotic selection. (**a**) GFP expression, (120 µg total protein per gel lane); (**b**) CEP290 expression C- control, non-transfected cells (50 µg total protein per gel lane; (**c**) Densitometric ratio CEP290/Tubulin for the different transfection conditions of the Western blot shown in panel A.

4. Discussion

4. 1. Polyplex characterization

A successful gene therapy strategy needs stable and long-term transgene expression. Here, we investigated the use of phiC31 integrase mediated gene transfer following delivery by chitosan polyplexes to promote efficient and sustained transgene integration and expression.

All the formulations of chitosan polyplexes are similar in terms of size, polydispersity and surface charge, even when 2 plasmids are combined in the same polyplex preparation (Table 5.1). Nevertheless, C2-based (260 kDa chitosan) polyplexes were larger in size and displayed higher surface charge than C1-based (80 kDa chitosan) polyplexes. However, these differences are only statistically significant for size: C1GFP versus C2GFP, C1i versus C2i, C1CEP versus C2CEP.

The difference in size was expected since it is known that the molecular weight of chitosan can affect both the size and surface charge of polyplexes, as well as their stability. [18, 19] Molecular weight can influence polyplex formation through the chain entanglement effect. The process of polyplex formation with lower molecular weight chitosan has a smaller contribution from the chain entanglement because a larger number of polymer chains are necessary to achieve the same N:P ratio, which may not be energetically favorable to polyplex formation. On the other hand, longer chitosan chains can entangle free pDNA with greater ease after the initial electrostatic interactions have occurred. [18]

Our differences in surface charge were consistent with current literature. [19] While there is a trend toward an increase in surface charge with the increase in MW, the differences found are not statistically significant. Also, the size and surface charge of polyplexes were not affected by the size of the encapsulated plasmid, as demonstrated by the lack of statistical difference between polyplexes containing pGFP (with a total plasmid size of 5 kb) and pCEP (with a total plasmid size of 10.2 kb). These results are corroborated by analysis of TEM images, where once again no significant differences were found. Also, there was no tendency to polyplex aggregation and polypex sizes

were comparable to polyplex sizes determined by DSL. Our results are in accordance with prior studies, that report that encapsulation of various size plasmids (5.1-11.9kb), had no effect on final polyplex sizes. [16]

Regarding the co-encapsulation of two plasmids, no differences were found between polyplexes complexing two plasmids (GFP and integrase or CEP290 and integrase) and polyplexes complexing only one plasmid (either GFP or CEP290), which indicate that the complexation of more than one plasmid had no effect on the physicochemical properties of the polyplexes.

Polyplexes also had similar behavior regarding their complexation efficiency. Both polymers complexed DNA effectively regardless of the plasmid size and number of plasmids used in the preparation (Fig. 5.2). Previous studies have indicated that multiple plasmids could be co-encapsulated by adding them to the chitosan solution before coacervation, this is consistent with the methodology of this study [17, 26], without affecting the structural or functional integrity of the encapsulated plasmids. [26]

Although it has been previously shown that the complexation efficiency and DNA loading efficiency varies with the molecular weight of the plasmid [17], we found no differences in complexation efficiency and loading capacity of the polyplexes. This could be due to the size of the plasmids used in this study, were not sufficiently different to cause differences in these parameters. This is consistent with Leong *et al*., with plasmid sizes between 7 and 12 kb, similar in size to the ones used in our study, found only a 3% difference in loading levels and no difference in encapsulation efficiency. [17] The same study also found no differences between release patterns of polyplexes loaded with one or two plasmids so we did not expect any differences to be observed in our study, which was confirmed as described above.

4. 2. Transfection efficiency and GFP expression

Initial transfection studies involved the determination of the transfection efficiency measured 72 hours post-transfection. In all the formulations tested, C1-based polyplexes performed better than C2-based polyplexes. Transfection with formulations C1GFPi and C2GFPi, where polyplexes contained the two plasmids simultaneously, did not show statistically different

results. Since this study intended to be a proof of concept for retinal gene therapy, initial transfection studies were also preformed in ARPE-19 cells (Fig. 5.S1). However due to low number of transfected cells that did not permit the long-term evaluation and selection assays for GFP-expressing cells.

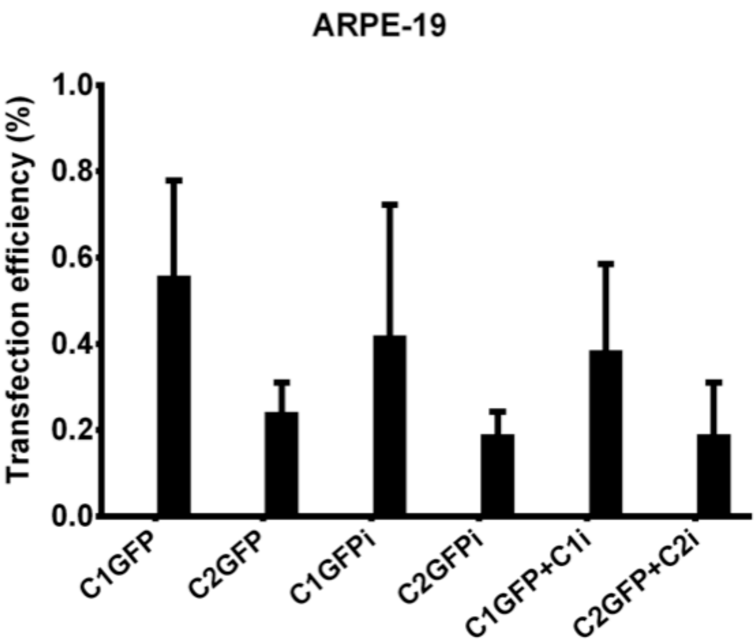

Figure 5.S1 – Transfection efficiency 72h post-transfection in ARPE-19 cells as percentage of GFP positive cells. No statistical differences were found between tested conditions.

Differences in transfection efficiency could be attributed to the size differences in polyplexes or the molecular weight of the polymer used. In fact, C1-based polyplexes had better transfection efficiencies than C2-based polyplexes for transfection conditions C1GFP and C1GFP+C1i, although the C2-based polyplexes used had a smaller size. The decreased transfection efficiency of C2-based polyplexes could be due to the tendency of high molecular weight polyplexes towards high stability, thus limiting plasmid release and leading to limited transfection efficiency. [27, 28] Some authors suggest that this high stability promotes a more controlled and prolonged release, which may result in sustained gene expression [17, 26], similarly to what is desirable in classic drug delivery systems. Furthermore, it has been shown that chitosan polyplexes with high molecular weight released DNA in a sustained manner that gradually increased transfection *in vivo.* [26]

Additionally, 72 hours post-transfection no differences were found between the different formulations (GFP, GFPi and GFP+integrase). Therefore the presence of integrase does not seem to have an effect in the transfection efficiency, irrespective of being delivered simultaneously or separately. The absence of an initial improvement in GFP expression may be explained by two factors: the slow release of the DNA and the short time period elapsed since transfection that did not allow for integrase to be expressed and start its activity.

Differences in GFP expression are noticeable only several weeks post transfection (Fig. 5.4). After 12 weeks, GFP expression is higher in cells transfected with transfection condition GFPi. The difference in GFP expression is especially noticeable when C2-based polyplexes are used. GFP expression is very reduced after 14 weeks when the GFP plasmid is transfected without the integrase plasmid, whereas GFP is still clearly visible when integrase was co-transfected (GFPi).

Regarding GFP expression after antibiotic selection, as observed before, there was also a decrease in GFP expression, but at a much slower rate. GFP expression levels remained relatively high even after 16 weeks post transfection, especially for formulations C1GFPi, C1GFP+C1i and C2GFPi (Fig. 5.6). Although there were no significant differences in the initial transfection between the different conditions, it is clear that GFP expression is higher in the formulations with integrase. The decrease in GFP expression is most likely related to the dilution phenomenon that naturally occurs with dividing cells in culture and not to instability in transgene expression. In tissues where cell division does not occur – such as in the retina – we do not expect this to happen.

4. 3. CEP290: delivery and expression

Current delivery approaches for large genes include the use of viruses with a larger capacity such as lentivirus or helper-dependent adenovirus vectors, but are complicated by random integration and immune mediated complications. Another approach involves trans-splicing where the gene is split into several vectors. [29] However, this strategy has had limited success

and needs further refinement. One of the advantages non-viral vectors have over several viral vectors is the very large packaging capability. In order to test our vectors for delivery of large transgenes, we have chosen the CEP290 gene. This gene codes for a centrosomal protein with 290 kDa and the gene is approximately 8 kb, which is over the packaging limit of AAV vectors. Mutations in this gene have been associated with several pathologies and recently linked to the most common form of Leber's congenital amaurosis, a retinopathy that leads to early childhood blindness. [30] To date, gene therapy strategies in animal models deficient in CEP290 have not been successful, probably due to the difficulties associated with transfecting and expressing a large gene in photoreceptors. [30] Therefore, an effective and long lasting gene delivery strategy for this gene would be highly beneficial for retinal diseases.

Our results show expression of this large gene at least 6 weeks post–transfection, in combination with integrase (Fig. 5.7). CEP290 over-expression was detected up to 2 weeks (Fig. 5.5), but at 6 weeks the levels are reduced (Fig. 5.7). This is most likely due the low transfection efficiency that resulted in low transgene expression. Since this is an endogenously expressed gene and transgene expression was low, it was not possible to detect over-expression. Nevertheless, in CEP290 deficient cells or animal models, even a low expression level can result in a therapeutic benefit, as observed in other studies for treatment of retinal degeneration. [31]

Similarly to what was described for GFP, transfection and selection assays were also performed in ARPE-19 (data not shown) with similar results, limiting the continuation of the study. Moreover, the over-expression of CEP290 in these cells seemed to have a deleterious effect on their viability, suggesting that CEP290 expression needs to be tightly regulated. Despite this result, we expect that in a mouse model with a CEP290 mutation, coupled to the *in vivo* enhanced cellular uptake of RPE cells, we can observe a therapeutic effect. In parallel, chitosan polyplexes tend to generate a more controlled, slower plasmid release and hence controlled transgene expression. [32] The present results, alongside with our preliminary *in vivo* results using chitosan polyplexes suggest that this strategy will be able to

deliver both transgenes to RPE cells and obtain sustained transgene expression for long periods of time. [33]

Although few studies have been published for large gene delivery, one study demonstrated that a large plasmid could be successfully complexed with chitosan into polyplexes. However, they were not able to detect transgene expression and attributed that to the plasmid size that could be affecting gene transfer to cells. [34] In our lab, plasmid release studies are currently being conducted to better understand the influence of plasmid size in the transfection process.

5. Conclusions

Our results demonstrate that the type of polymer and size of the polyplex affect transfection efficiency and transgene expression. We have also shown that co-transfection of integrase with either GFP or CEP290 originated sustained expression for over 16 weeks for GFP and expression of CEP290 for at least 6 weeks post-transfection. Moreover, the results with CEP290, a large transgene (>8Kb), indicates that our non-viral approach can be used to deliver large genes, thus overcoming the limitation of restricted carrying capacity found in viral vectors.

Overall, this gene delivery approach has several desirable characteristics such as 1) delivery of multiple plasmids, 2) prolonged transgene expression and 3) the possibility of ligand conjugation or polymer modification to increase cell-targeting. This set of characteristics makes it suitable for gene delivery of both small and large genes such as CEP290, thus widening the range of gene therapy applications and future therapies.

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6. References

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Chapter VI

Modification and characterization of hyaluronic acid: a new polymer for retinal gene delivery

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1. Abstract

Purpose: A successful gene therapy approach can prevent or treat congenital and acquired diseases. However, there is still no ideal non-viral vector for gene delivery in a safe and timely manner. In this report the anionic polymer hyaluronic acid (HA) was investigated as a potential vector for gene therapy. Due to its intrinsic characteristics it constitutes an excellent candidate to deliver therapeutic genes, pending the modification of its surface charge.

Methods: To modify its charge, HA was modified with cystamine. Several formulations were prepared using modified HA combined with sodium sulphate, sodium triphosphate, K-carrageenan and chitosan. Vectors were characterized with respect to size, charge, DNA load and its protection, and effect on cell viability. The better performing formulations were further evaluated *in vitro* for their transfection efficiency in HEK293T and ARPE-19 cells.

Results: Cell viability assays showed low cytotoxicity for both polymers. Gene transfer efficiency depended on cell line and formulation, but no increased transfection efficiency was observed with the modified polymer.

Conclusions: HA has great potential as a gene therapy vector, but further optimization, including incorporation of a higher percentage of positive groups in HA, is needed before its use as a gene delivery vector.

Keywords

Gene therapy, Hyaluronic acid, Polymer modification, Retina, Biocompatibility.

2. Introduction

Gene therapy is an area that has been growing rapidly and revolutionizing the world of research. [1] An essential requirement for the success of this technique is an efficient method for gene transfer, since DNA molecules cannot enter cells efficiently because of their large size, hydrophilic nature and susceptibility to degradation mediated by nucleases. [2, 3] Considering the great diversity of diseases targeted by gene therapy, it is implausible that a single gene delivery system (vector) is suitable for all applications. However, the main requirements are common to all systems: vectors should transfer the genetic material to the tissue of interest and induce the proper level of therapeutic gene expression with no side effects. [4]

Currently, vectors used in gene therapy can be divided into two main categories: viral and non-viral. Recombinant viruses such as retrovirus, lentivirus, adenovirus, adeno-associated viruses, and herpes viruses have been widely used for genetic material transfer. [2] Viruses have as main advantage their high transfection efficiency, however, viral vectors entail many intrinsic problems such as difficulties in production, limitations concerning repeated administrations that can lead to acute inflammatory responses, immune responses of the host to the virus and induction of mutagenesis by some viruses that integrate into the genome of host cells. [2] On the other hand, non-viral vectors have a higher safety profile due to their low toxicity and low immunogenicity. Additionally, non-viral vectors have the ability to carry larger genes and present lower production costs. Despite this appealing feature from the safety standpoint, non-viral vectors have little clinical importance due to low transfer and expression of transgene. [2] However, due to problems in clinical trials using viral vectors in the recent past, the interest in non-viral technologies has been renewed, particularly in the release properties of the non-viral vectors that resemble traditional drugs. [4]

Non-viral vectors include synthetic or naturally occurring chemical compounds, such as lipids and cationic polymers. These can form complexes with the negatively charged DNA through electrostatic interactions that allow the therapeutic transgene across the cell membrane via endocytosis. [2, 3, 5] The complexes protect DNA from nuclease-mediated degradation and facilitate cell entry as well as gene transferring into the nucleus. [2]

Polymers have chemical flexibility, which enables the design and construction of polymers with multiple functionality, and thus more efficient in gene delivery, while maintaining the characteristics of biocompatibility, easy production and stable formulation. [4] One of the key points for the construction of more efficient vectors relates to various biological barriers of cells that must be overcome to achieve higher transfection rate. These barriers include the attachment of the polymer to the cell surface, then entering the cell through the cell membrane, displacement throughout the cytoplasm, escape endosomal degradation and ending with the passage of the nuclear envelope and nuclear entry. [3] In order to overcome different barriers, functional groups can be introduced into polymers, such as ligands to enhance cell entry via receptor-mediated endocytosis, membrane peptides to allow endosomal release and nuclear localization signals to increase nuclear entry of the transgene. [3]

In this paper, hyaluronic acid (HA) was evaluated as a possible retinal gene therapy vector. HA is a biocompatible, non-toxic, non-immunogenic, non-inflammatory anionic biopolymer that has been widely used in various biomedical applications. [6] Multiple studies on the biological function of HA have revealed that there is a strong relationship between the presence of HA and the migration and proliferation of cells as well as an involvement in wound healing, cell motility, angiogenesis, and extracellular matrix formation. Another important feature of HA for its use as a vector of therapeutic genes is the ability to interact with various cell receptors. [6] The negatively charged carboxyl group of HA is responsible for the interaction with membrane receptors allowing the connection with HA. [7]

Despite it’s advantageous features, HA exists in the form of an aqueous gel, has a short lifetime and quickly degrades after administration. In order to increase the lifetime of HA for long-term clinical applications, several strategies have been developed, particularly modifications of the polymer at the level of carboxyl and hydroxyl groups. These new polymers, although they have different physico-chemical properties compared to unmodified HA, they maintain essential biological properties that allow their use in non-viral therapies. [6] Among possible chemical modifications to perform, the more relevant for gene therapy is the inclusion of amine groups. [6] A higher amount of amine groups may lead to greater DNA loads and increase the efficiency of transfection and transgene expression. In this study, HA was modified with cystamine through a coupling reaction with the carboxyl groups of

HA. This modification with cystamine not only adds amine groups to the polymer but it also contains a disulfide bond that can be cleaved in the presence of intercellular glutathione, thereby promoting a more rapid release of genetic material. [8, 9]

In this work we compare the transfection efficiency between modified and unmodified HA using two types of cells: human embryonic kidney (HEK293T) cells and retinal pigment epithelium cells (ARPE-19). The former are a commonly used cell line in transfection studies, while the latter are a model of retinal pigmented epithelial (RPE) cells, our target in the retina, due to their important role in the support of the retinal homeostasis and involvement in several retinal diseases. [8]

3. Results

3. 1. Characterization of the modified polymer: degree of modification

The first step in the characterization of the polymer was to determine the extent of modification by quantification of the added thiol groups. After treatment with an excess of DTT it was possible to quantify the thiol groups present in the polymer through Ellman's test. The average percentage of thiol substitution to the total carboxylic acid groups of HA was 9.88% ± 0.34.

The behavior of $HASSNH_2$ as a function of pH was then evaluated by titration, where the carboxylic group (COO^-) was considered to be the protonatable group. Comparing the unmodified and modified titration curves it is possible to observe differences in the equivalence points present confirming the success of the modification reaction (Fig. 6.1).

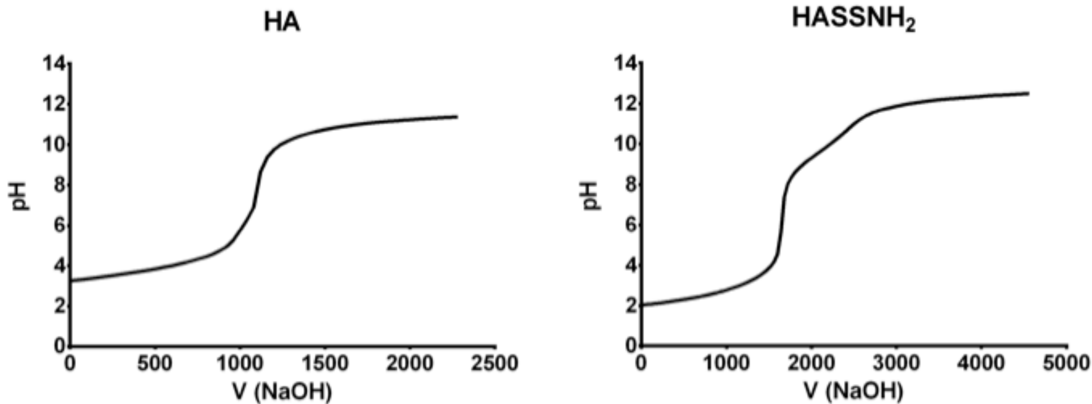

Figure 6.1 – Potentiometric titration curves of HA and $HASSNH_2$ in water. The initial polymer solutions were prepared to contain a fixed amount of protonable amine groups (0.100 mmol) in a total initial solution volume of 10.0 ml and the pH of the solution was adjusted to 2.00-3.00 with 2.00 N HCl. The solution was then titrated using 0.0800 N NaOH (The x-axis label of the plot denotes the total volume of added NaOH in µl).

3. 2. Physical characterization of $HASSNH_2$ polyplexes

Several conditions were tested in order to produce vectors formulations with adequate characteristics. Firstly, formulations based on $HASSNH_2$ and different concentrations of anionic agents were tested (Na_2SO_4, TPP, HA and κ-carrageenan) but did not yield polyplexes with the appropriate size (< 500 nm) or polidispersity (< 0.5). Then, formulations with the cationic polymer

chitosan and the modified polymer or HA were examined, yielding positive results as described in Fig. 6.2. Only formulations $CSHASSNH_25N$ and $CSHASSNH_27N$ presented size and polydispersity above average. Regarding polyplexes prepared in water (CSHA5H, CSHA7H, $CSHASSNH_25H$ and $CSHASSNH_27H$) these presented much higher zeta potential values with approximately a 2-fold increase in the surface charge.

Next, polyplexes were evaluated regarding their DNA complexing capacity and nuclease protection. Polyplexes were capable of efficient DNA complexion and protection against DNAse-induced degradation, as verified by agarose gel retardation assays (Fig. 6.3). Also, polyplexes remained stable and did not release DNA in detectable amounts, even after incubation for several days at physiological conditions. However, upon incubation in media with serum in some of the formulations it is possible to observe some DNA release (Fig. 6.4) indicating a less stable polyplex. Nevertheless it is noteworthy that the DNA released by the polyplexes is still undamaged whereas the free DNA used as control is clearly degraded indicating the polyplexes did protect the DNA.

3. 3. *In vitro* studies: cytotoxicity and transfection

The cytotoxicity evaluation was performed by an MTT assay and aimed to study possible changes in the cytocompatible profile of the modified polymer by comparing it with unmodified HA. The results are presented in Fig. 6.5, and show no differences among tested concentrations and polymers.

After verifying that there were no cytotoxicity associated with the polymers, the polyplexes were evaluated regarding their transfection efficiency in two cell lines, HEK293T and ARPE-19, the first are a commonly used cell line in transfection studies, while the latter are a model of retinal pigment epithelial (RPE) cells, our target in the retina.

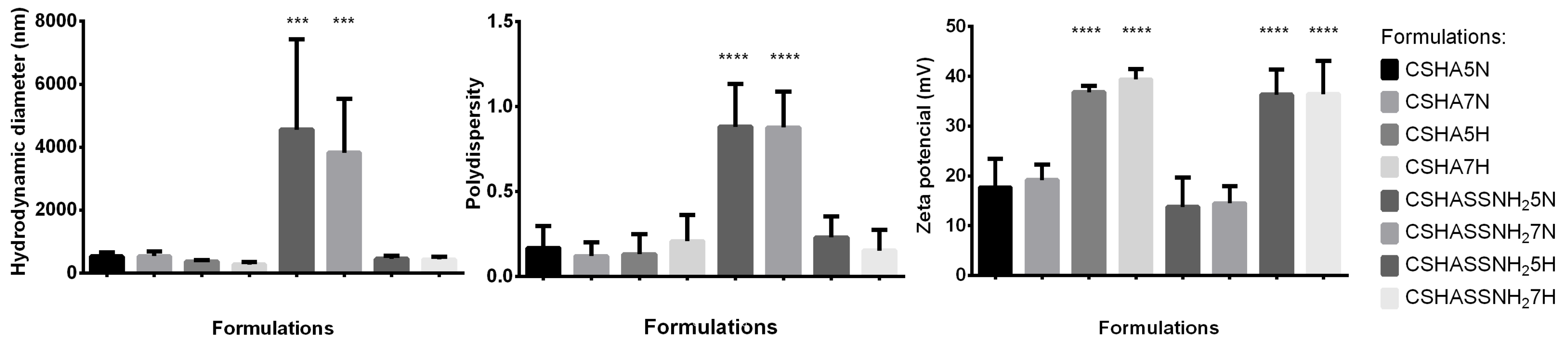

Figure 6.2 - Size, polydispersity and zeta potential of polyplexes. Statistical differences and their significance are indicated by the star (*) symbol, with *** P < 0.001, **** P < 0.0001, ns – not significant.

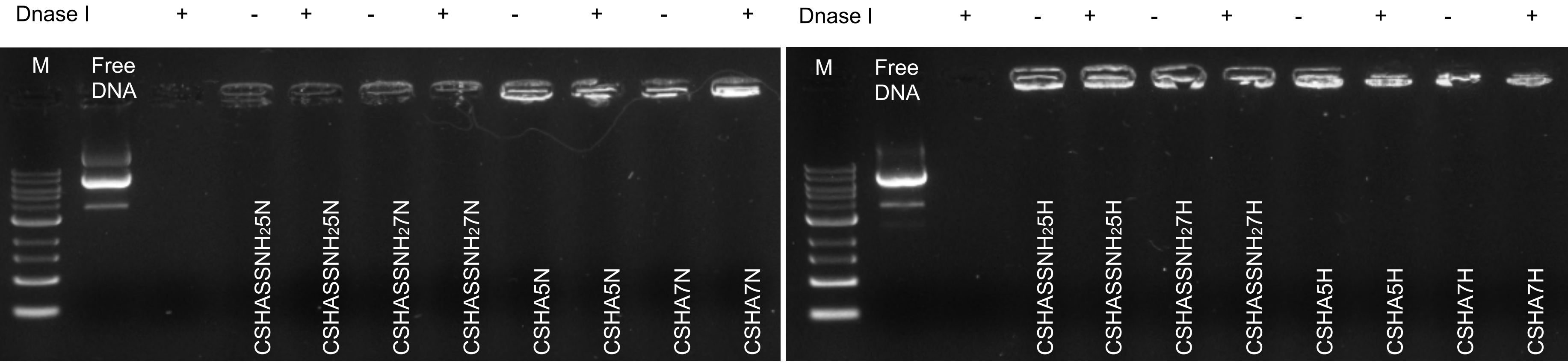

Figure 6.3 - DNA complexation and Dnase I protection in polyplexes. Analyzed by 1% agarose gel electrophoresis and DNA visualized with GreenSafe Premium after incubation with Dnase I for 15 min at 37ºC (+).

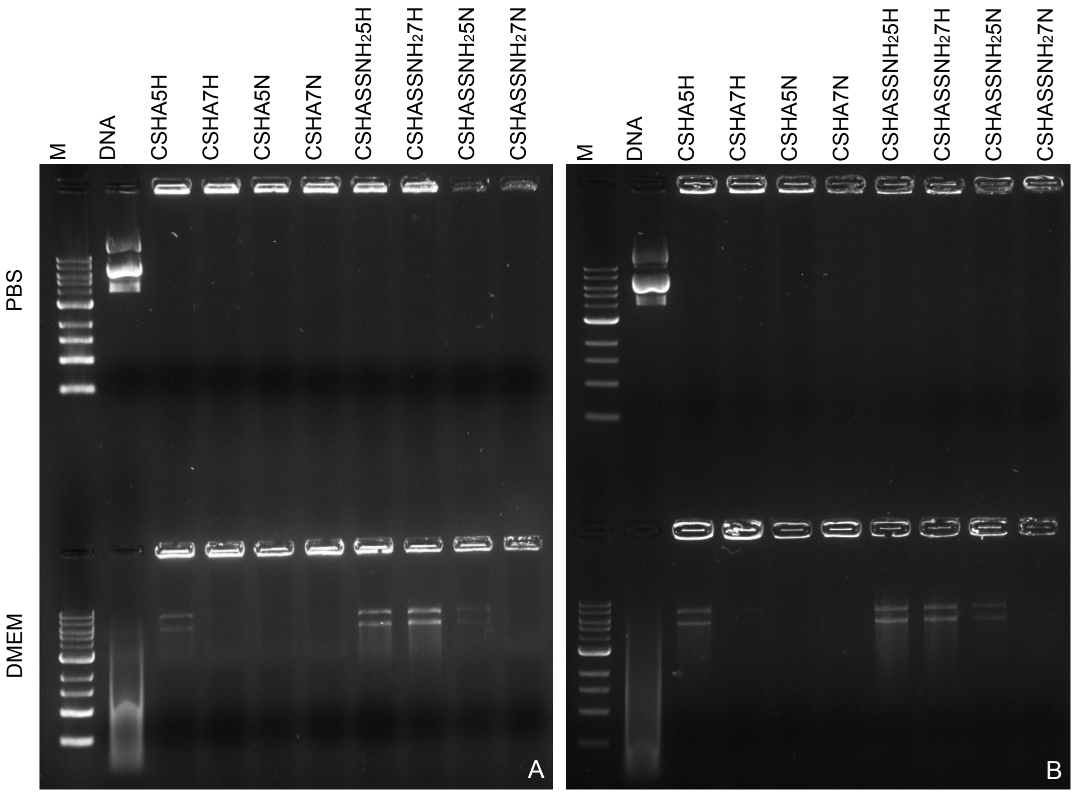

Figure 6.4 – DNA complexation in polyplexes at physiologic conditions. Analyzed by 1% agarose gel electrophoresis and DNA visualized with GreenSafe Premium after incubation with PBS or DMEM with 10% FBS for A) 3 and B) 7 days at 37ºC.

In ARPE-19 cells no differences between different formulations were found whereas in HEK293T there were statistically lower transfection efficiency when $HASSNH_2$ polyplexes were prepared in sodium sulphate (Fig. 6.6). Nonetheless, polyplexes prepared in water, with both HA and $HASSNH_2$, had similar transfection results.

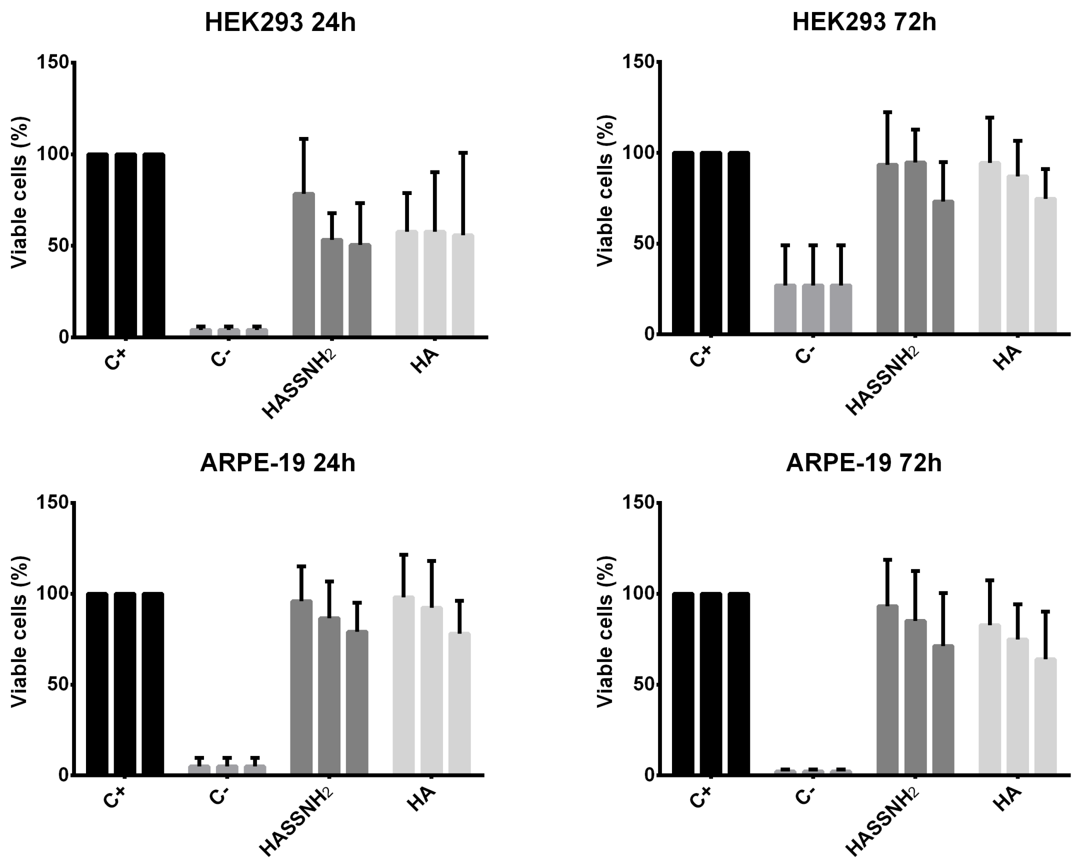

Figure 6.5 – Cytotoxicity in HEK293T and ARPE-19 cell lines; given as percentage of viable cells after 24 or 72 h incubation with polymer at increasing concentrations (0.01, 0.05 and 0.1 mg/ml per well). Un-challenged cells and cells incubated with latex extracts were used as positive and negative controls of cellular viability.

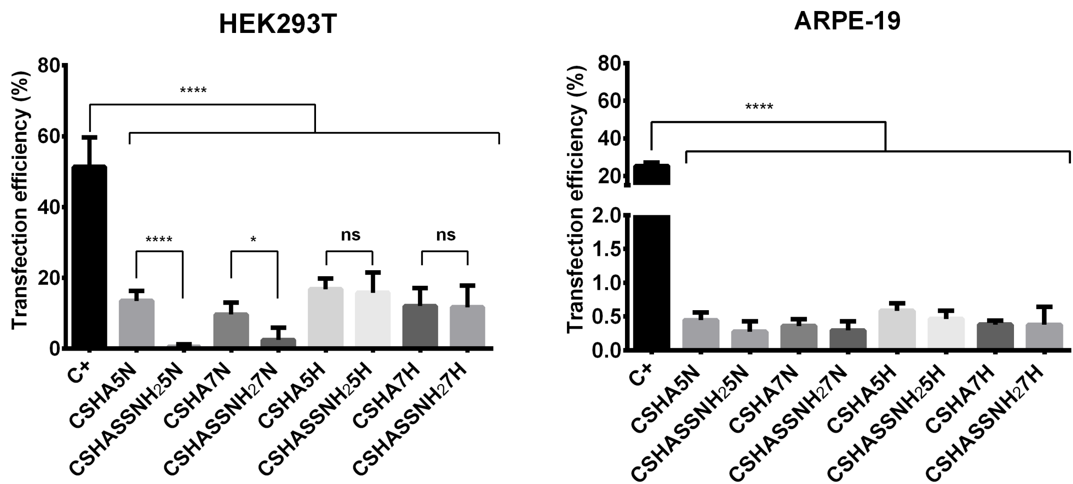

Figure 6.6 – Transfection efficiency 72h post-transfection in HEK293T and ARPE-19 cells as percentage of GFP positive cells. Statistical differences and their significance are indicated by the star (*) symbol, with * $P < 0.05$, **** $P < 0.001$, ns – not significant.

4. Discussion

4. 1. HA was successfully modified with cystamine

Hyaluronic acid was modified by reaction of the carboxylic groups of HA with the amine groups of cystamine dihydrochloride using EDAC activation. Later, a portion of the modified polymer was treated using an excess amount of the DTT to cleave the disulfide bonds. The resulting product (HASH) was purified by dialysis and isolated after freeze-drying. The average percentage of thiol substitution (approximately 10%) was determined by Ellman's test. This percentage of substitution is in agreement with values in the literature since it is considered that a partial modification (10-20%) is desirable to achieve polyplexes with well-defined sizes. [10]

The presence of two equivalence points in the potentiometric titration curve of $HASSNH_2$ further confirmed the success of the modification reaction and indicated the pH range more adequate for polyplex preparation. At pH values much below the first equivalence point the carboxyl groups are predominantly deprotonated. This may hinder the preparation of polyplexes because in this case carboxyl groups may interact with amine groups in the polymer, leaving very few amine groups available to interact with DNA phosphate groups. On the other hand, at pH values exceeding the second equivalence point all amine groups are deprotonated and there are fewer positive charges available to interact with DNA phosphate groups, establishing less electrostatic interactions. Thus it is preferable to prepare polyplexes at pH values between 6 and 9, which is also desirable for later *in vitro* and *in vivo* experiments.

4. 2. $HASSNH_2$ does not produce polyplexes with anionic agents

Initially nanoparticles were evaluated and characterized with respect to their size and polydispersion. Several formulations with $HASSNH_2$ and different concentrations of anionic agents were tested (Na_2SO_4, TPP, HA and κ-carrageenan) with no satisfactory results. The modified polymer was unable to generate polyplexes by itself or with the addition of other anionic agents,

probably due to insufficient electrostatic interactions. This may be related to the degree of substitution of the modified polymer that seems to have a low number of positive charges available to interact.

4. 3. CSHA and $CSHASSNH_2$ polyplexes: a comparison

Previous studies in our lab had shown positive results in terms of transfection with formulations based on chitosan and hyaluronic acid. [11] In this study we wanted to evaluate and compare the performance of polyplexes prepared with normal unmodified hyaluronic acid and polyplexes prepared with $HASSNH_2$. Different formulations were prepared and the first step in their evaluation was to characterize them for their size, polydispersity, and zeta potential (Fig. 6.2). Polyplexes were compared through a statistical multiple comparison analysis. Among the various comparisons, those with higher significance for the interpretation of the results were comparisons 1) between formulations with different solvents but the same polymer and ratio; 2) between formulations with different ratios while maintaining the same polymer; and 3) between formulations with different polymers but keeping the same ratio and solvent. Given the comparisons described above, in Fig. 6.2 are represented all comparisons with a significant statistical difference. All other comparisons show no statistical difference.

The different formulations yielded polyplexes with similar sizes except for $CSHASSNH_25N$ and $CSHASSNH_27N$. These two formulations also presented high polydispersity and a slightly lower surface charge (given by the zeta potencial values). The zeta potential can be used to predict the stability of the dispersion so this reduced surface charge might have caused aggregation of the polyplexes hence the large size and polydispersity. [12] Since chitosan is a polycation, a positive zeta potential was expected, which occurred in all cases. However, statistical differences were observed between polyplexes with the same polymer and ratio but different solvents (Na_2SO_4 and H_2O). This may be due to the presence of the negative charges from sulphate anions, which reduce the overall surface charge.

4. 4. Polymer modification did not affect cytotoxicity

The cell viability assay main objective was to evaluate the possible cytotoxic effect of the polymers in the cells after DNA release and to assess if the modification changed the polymer cytotoxicity profile. HA is known as a biocompatible polymer and studies have shown it is safe for concentrations up to 1-2% (W/V). [13, 14] Comparing the results obtained for the HA and $HASSNH_2$ polymers, no statistical differences were observed in all four tested situations. This is possibly due to the fact that the difference in composition of the two polymers is small, since only 10% modification was achieved, and this did not sufficiently alter the toxicity profile of the polymer.

However, after 24 hours of incubation HA and $HASSNH_2$ showed a reduction of cell viability in HEK293 cells for all tested concentrations when compared to the control. This was not observed for the other tested conditions and this could be related to the fact that at 24 h the cells underwent acute exposure while at 72 h it resembles a chronic exposure. The fact that the cell viability had decreased slightly in some cases may also be related to the fact that the polymer solutions have not been extensively purified before addition to cells, and so the observed cytotoxicity may be due to residual impurities of the modified polymer. Nevertheless, at concentrations to be used in future *in vitro* and *in vivo* transfection studies, no cytotoxicity was observed.

4. 5. Polyplex stability at physiological conditions varies with temperature and presence of serum

All polyplexes were capable of efficient DNA complexion and protection against DNAse-induced degradation when exposed to DNase I (Fig 6.3). This is a critical aspect for all non-viral vectors, since a successful gene therapy vector needs to protect its load until it reaches the target and only unload it at the appropriate time. [15, 16] Our stability assay results shown that all polyplexes remained stable up to 7 days at physiological temperature and pH (polyplexes incubated with PBS). However, when polyplexes were incubated in DMEM with serum some DNA release was observed, particularly in polyplexes with the modified polymer. It is hypothesized that serum

components establish electrostatic interactions with the polyplexes, destabilizing them, and causing DNA release. The fact that polyplexes prepared with the modified polymer appear less stable can actually be advantageous since several studies have shown a negative correlation between polyplex stability and transfection efficiency. [11, 17-19]

4. 6. Transfection efficiency is formulation-dependent

One of the main objectives of this study was to evaluate if the modification of HA would affect the stability of the polyplexes and consequently their transfection efficacy. In our study, different formulations of $CSHASSNH_2$ polyplexes showed very similar transfection efficiencies. Analyzing the results obtained for ARPE-19 and HEK293 cells, the latter had much higher transfection efficiency in all formulations due to their higher permissibility to transfection, associated with their higher mitotic rate. Despite the higher transfection values there were only differences in the transfection efficiency between CSHA and $CSHASSNH_2$ polyplexes prepared in sodium sulphate. These polyplexes ($CSHASSNH_2$5N and $CSHASSNH_2$7N) were expected to have a reduced efficacy since their size was too large for an efficient and timely cellular internalization and this was indeed confirmed.

Moreover, less stable formulations, with visible DNA release in the stability assay, displayed higher transfection values as expected, based the aforementioned relation between polyplex stability and transfection efficiency.

Also, polyplex charge is a key parameter due to its relation to toxicity and transfection efficiency. Other studies have shown an association between a reduction in positive charges and increased transfection efficiency as well as a decrease in toxicity. [20, 21] In this study polyplexes prepared in water displayed nearly a 2-fold increase in surface charge that correlated with higher transfection values, irrespective of the polymer used. So it is clear that the preparation method used is as important as the polymer used.

5. Conclusions

This work had as main objective the characterization of a novel polymer, $HASSNH_2$, for subsequent use as a retinal gene therapy vector. After confirming the success of the modification reaction and determining its extent (~10%), several strategies were tested in order to yield polyplexes with adequate characteristics. The resulting polyplexes were capable of effective DNA complexation though their stability at physiological conditions varied. Transfection studies using HEK293 and ARPE-19 cells showed relatively modest transfection efficiency where no differences were observed between polyplexes with modified or unmodified HA. Higher transfection values correlated with lower polyplex stability as well as higher surface charge.

In the future different degrees of modification can be tested in order to modulate the stability and DNA release from the polyplexes, to allow further improvement of the transfection efficiency of these gene therapy vectors.

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Chapter VII

Chitosan-based vectors mediate long-term gene expression in the retina

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1. Abstract

One of the major limitations to the success of non-viral gene therapy has been the low gene transfer efficiency and short transgene expression. Chitosan has shown great promise as a non-viral carrier due to its biocompatibility, biodegradability and low cost, but limited by its low transfection efficiency. Our goal is to develop chitosan-based non-viral vectors optimized for retinal gene therapy and continued gene expression. Chitosan-pDNA polyplexes gene transfer efficiency and biocompatibility was evaluated both *in vitro* on retinal pigment epithelial (ARPE-19) cells and human embryonic kidney (HEK293T) cells and *in vivo* in the mouse retina.

Our results show that chitosan polyplexes have size and surface charge consistent with gene delivery. When considering off the shelf applications, vector stability is crucial, and our vectors are stable both in storage (4ºC) and physiological conditions (37ºC), and remain stable after several freeze-thaw cycles.

Despite moderate *in vitro* efficiency, *in vivo* sub-retinal administration shows sustained transgene expression in RPE cells at least 6 months post- injection. These results show that chitosan-based vectors can successfully transfer genes to the retina and provide persistent transgene expression.

Keywords: Chitosan, Gene delivery, Retina, *In vitro*, *In vivo*, Gene expression.

2. Introduction

Presently two approaches are used to deliver transgenes: a viral and a non-viral approach. The first has shown great success while the second, which uses physical or chemical methods, such as cationic polymers and lipids, to deliver genes with so far modest results. [1] Despite the success of viral vectors, the use of viruses still raises several issues, namely safety concerns due to the possibility of insertional mutagenesis. For example, adeno-associated viruses (AAVs) are one of the most promising in the field of ocular gene therapy but have limited use due to limited transgene size packing. [2]

On the other hand, non-viral vectors have better safety profiles and the capacity to deliver large genes along with low handling safety requirements and low production costs. [1, 3] So far their relatively low gene transfer efficiency and short-term transgene expression have hampered their clinical usefulness. However, with problems in clinical trials using viral vectors the use of non-viral gene delivery systems has gained a renewed interest specially the use of natural cationic polymers, such as chitosan. Chitosan is a cationic polymer obtained inexpensively by the deacetylation of chitin and one of the most used polymers in non-viral gene delivery. [4] It has been previously established that chitosan is able to *i)* encapsulate and condense a desired amount of therapeutic nucleic acids; *ii)* internalize the cell; *iii)* promote endo-lysossomal escape, all without originating an immune or toxic response. [5]

The success of a gene therapy approach greatly depends not only on the delivery vector but also the target organ. Regarding the target, the immune-privileges of the eye due to its anatomical compartmentalization and it is affected by several well-understood genetic diseases that could benefit from a gene therapy treatment approach present unique advantages for gene therapy. [6, 7] The optical transparency of the eye enables therapeutic effects on structure and function to be readily observed using a variety of techniques. [8] These characteristics make the eye an ideal system to test the performance of new gene therapy vectors. However, to date few studies have focused on the use of polymer-based gene delivery to the retina. Our goal is to develop chitosan-based non-viral vectors optimized for retinal gene therapy with continued gene

expression. Chitosan-pDNA polyplexes were formulated and characterized regarding their physical properties, gene transfer efficiency on human embryonic kidney (HEK293T) cells and retinal pigment epithelial (ARPE-19) cells and, and *in vivo* by subretinal injection on C57BL6 mice. Since transfection efficiency is related to particle stability, we also investigated the stability in physiological conditions and the long-term stability in storage conditions.

3. Results

3.1. Polyplex characterization: Size, Polydispersity and Zeta Potential

The same methodology described previously [9] was used to prepare chitosan-based polyplexes using chitosan with two different molecular sizes (CS113 and 213 with 80 and 260 kDa, respectively). Due to the larger molecular weight chitosan a range of different sodium sulphate concentrations was tested (data shown only for the ratios with better results). The first step in the characterization of the polyplexes was to determine their size, polydispersity and surface charge (given by the zeta potential). Polyplexes prepared with the longer polymer (CS213) were slightly bigger then the ones prepared with the smaller polymer (CS113) but have similar characteristics such as sizes below 1000 nm, low polydispersity and positive zeta potencial (Table 7.1). Also, polyplexes with pDNA tend to have smaller sizes and this is more evident in polyplexes prepared with CS213 where significant statistical differences were found (denoted by asterisks (*) in Table 7.1).

3.2. Polyplex complexation and stability

Polyplexes were also characterized regarding their pDNA loading efficiency and protection (shown by pDNA integrity) by agarose gel retardation assays. All pDNA was condensed by the particles, as shown by the absence of DNA bands in the agarose gel (Fig. 7.1). Furthermore, polyplexes remained stable and did not release pDNA, in detectable amounts, even after being incubated for 72 h at physiological conditions (Fig. 7.2). For the following assays only CS113/40 and CS213/90 formulations were further analyzed due to their characteristics (either smaller size or polydispersion).

Long-term stability of unloaded and pDNA loaded vectors was evaluated by measuring their size, polidispersity and zeta potential during incubation at 4 or 37ºC for several weeks.

Table 7.1 – Size, polydispersity and zeta potential of chitosan-based polyplexes.

Particle name	CS size (kDa)	Na_2SO_4 concentration (mM)	Size (∅, nm)		Polydispersity		Zeta potential (mV)	
			Mean	SD	Mean	SD	Mean	SD
CS113/20	80	20	566.97	84.72	0.241	0.107	15.05	4.38
CS113/20GFP			366.15	27.65	0.355	0.202	14.85	3.32
CS113/40		40	548.67	233.60	0.285	0.094	14.94	3.99
CS113/40 GFP			396.60	119.76	0.243	0.140	15.92	3.41
CS213/90	260	90	841.87	200.42	0.162	0.060	15.70	1.35
CS213/90 GFP			351.57**	93.74	0.297	0.097	15.05	2.92
CS213/100		100	820.17	283.62	0.284	0.042	16.05	0.92
CS213/100 GFP			412.75*	37.69	0.248	0.070	15.80	3.68

Obs: Statistical differences between formulations with and with out pDNA are denoted by asterisks (* $P < 0.05$, ** $P < 0.01$).

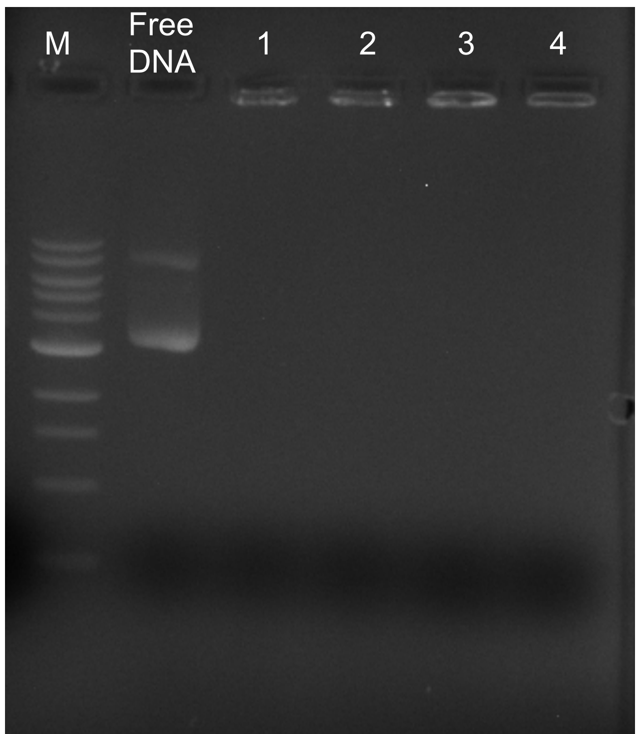


Figure 7.1 – DNA encapsulation in chitosan polyplexes analyzed by 1% agarose gel electrophoresis. DNA visualized with GreenSafe Premium, M- DNA marker,1- CS113/20GFP , 2- CS113/40GFP, 3- CS213/90GFP, 4- CS213/100GFP.

Results show that temperature plays an important role in particle stability as well as the molecular weight of the polymer (Fig. 7.3). This effect is especially noticeable in the rapid decrease in zeta potential observed for particles kept at 37ºC. The change in surface charge causes particles to aggregate as observed by the increase in polidispersion at early time points.

Also, the size of the polyplexes remained stable after freeze-thaw following resuspension with cryoprotectants (glucose and sucrose, Fig. 7.4). The only observable differences were a slight tendency to higher polidispersion and lower zeta potential values (only statistically significant after six cycles of freeze-thaw).

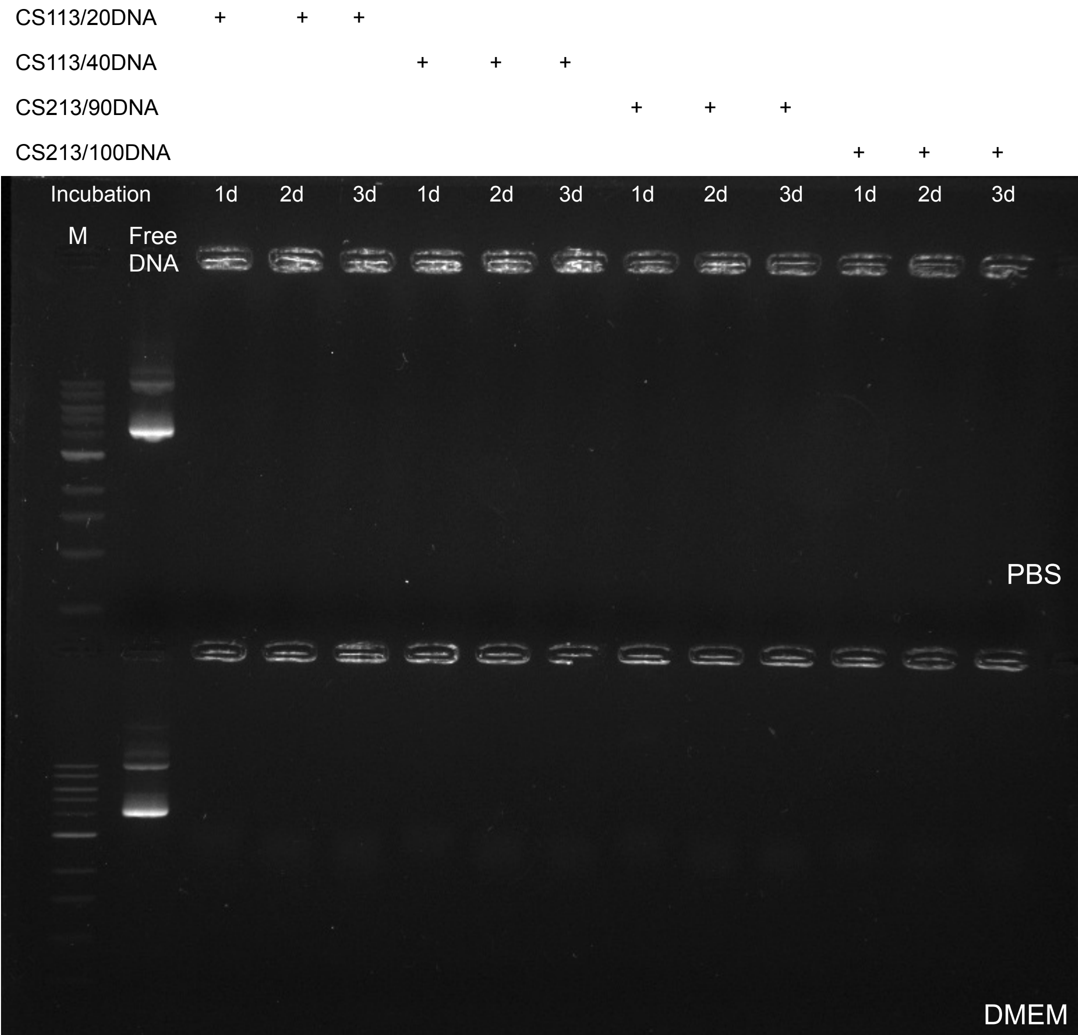
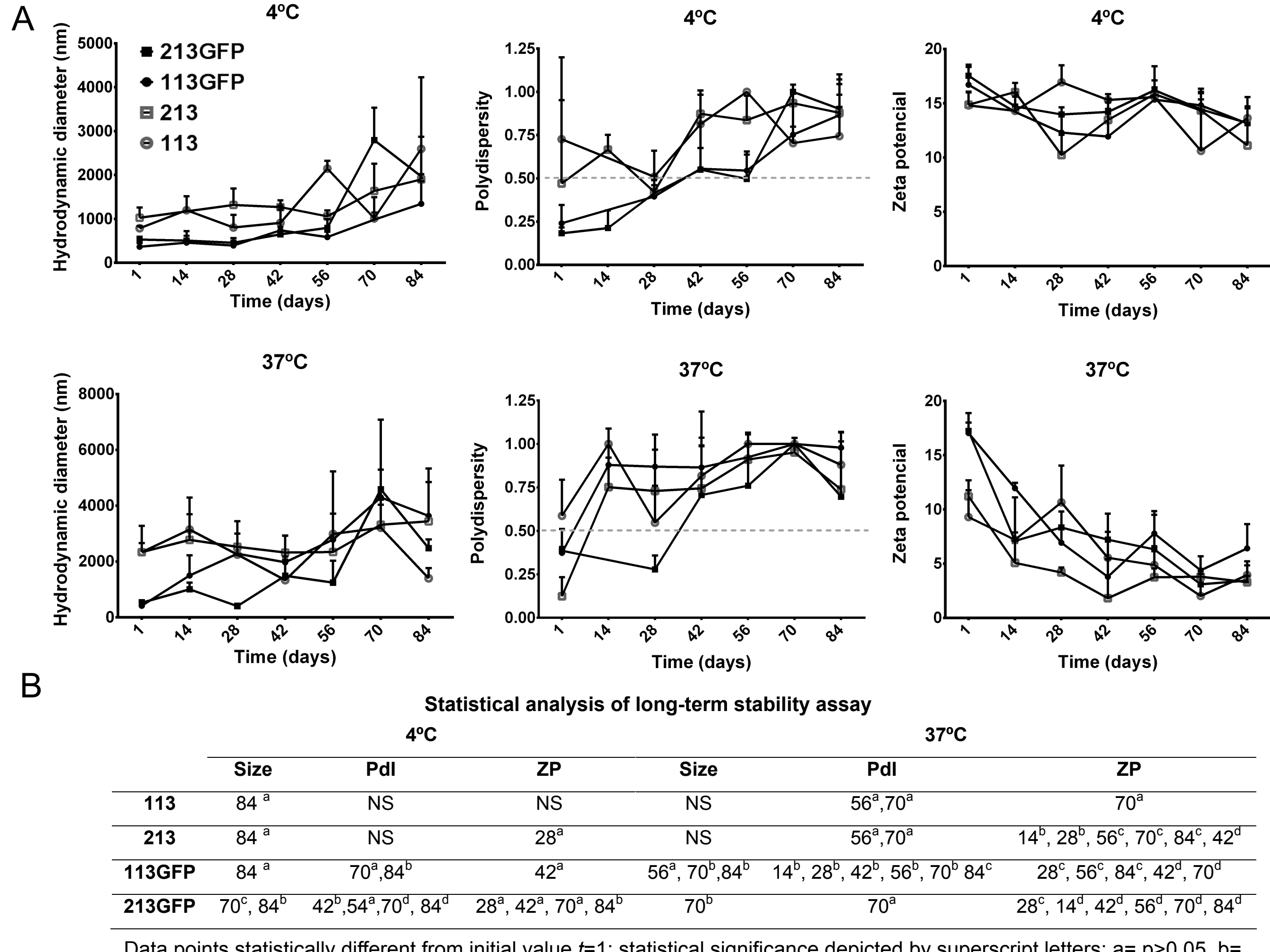


Figure 7.2 – Chitosan polyplex stability over time in PSB and DMEM with 10% FBS. Polyplexes analyzed by 1% agarose gel electrophoresis, DNA visualized with GreenSafe Premium. Incubation time indicated in days (d).

3.3. *In vitro studies*

Polyplex cytotoxicity was evaluated using an MTT assay in two cell lines (HEK293T and ARPE-19, Figure 7.5). Statistical analysis revealed differences between polymers, with the smaller polymer (CS113) performing slightly better than the longer polymer (CS213), but no differences between cells lines were observed.



Statistical analysis of long-term stability assay

	4ºC			37ºC		
	Size	**PdI**	**ZP**	**Size**	**PdI**	**ZP**
113	84^{a}	NS	NS	NS	56^{a},70^{a}	70^{a}
213	84^{a}	NS	28^{a}	NS	56^{a},70^{a}	14^{b}, 28^{b}, 56^{c}, 70^{c}, 84^{c}, 42^{d}
113GFP	84^{a}	70^{a},84^{b}	42^{a}	56^{a}, 70^{b},84^{b}	14^{b}, 28^{b}, 42^{b}, 56^{b}, 70^{b} 84^{c}	28^{c}, 56^{c}, 84^{c}, 42^{d}, 70^{d}
213GFP	70^{c}, 84^{b}	42^{b},54^{a},70^{d}, 84^{d}	28^{a}, 42^{a}, 70^{a}, 84^{b}	70^{b}	70^{a}	28^{c}, 14^{d}, 42^{d}, 56^{d}, 70^{d}, 84^{d}

Data points statistically different from initial value *t*=1: statistical significance depicted by superscript letters: a= p>0.05, b= p>0.01, c= p>0.001, d p>0.0001, NS = not significant

Figure 7.3 – Polyplex stability at 4 and 37ºC throughout time: A) size, polydispersity and zeta potential measurements over time and B) statistical analysis of data presented in panel A.

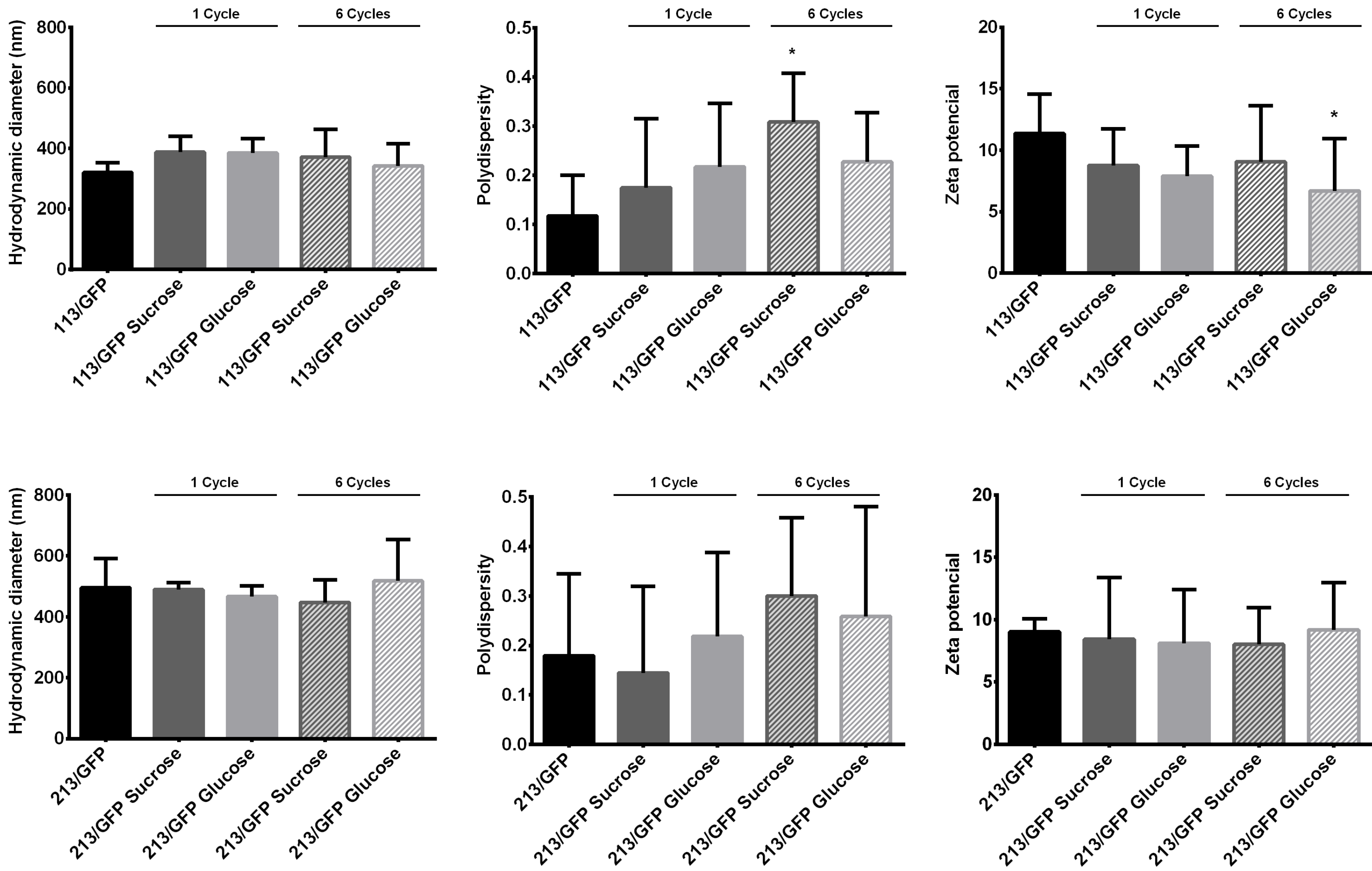


Figure 7.4 – Polyplex stability evaluated by measuring their size, poydispersity and zeta potential after treatment with cryoprotectants (glucose or sucrose) and different freeze-thaw cycles. Statistical differences to the original formulation (113/GFP or 213/GFP) and their significance are indicated by the star (*) symbol, with * $P < 0.05$.

Cell viability was above 75% regardless of formulation and concentration, except for the two highest concentrations tested, with values below 70% for all formulations. This effect is especially noticeable after the 72 h incubation. However, these concentrations are above the range to be used *in vivo* and were only tested to determine the cytotoxicity threshold.

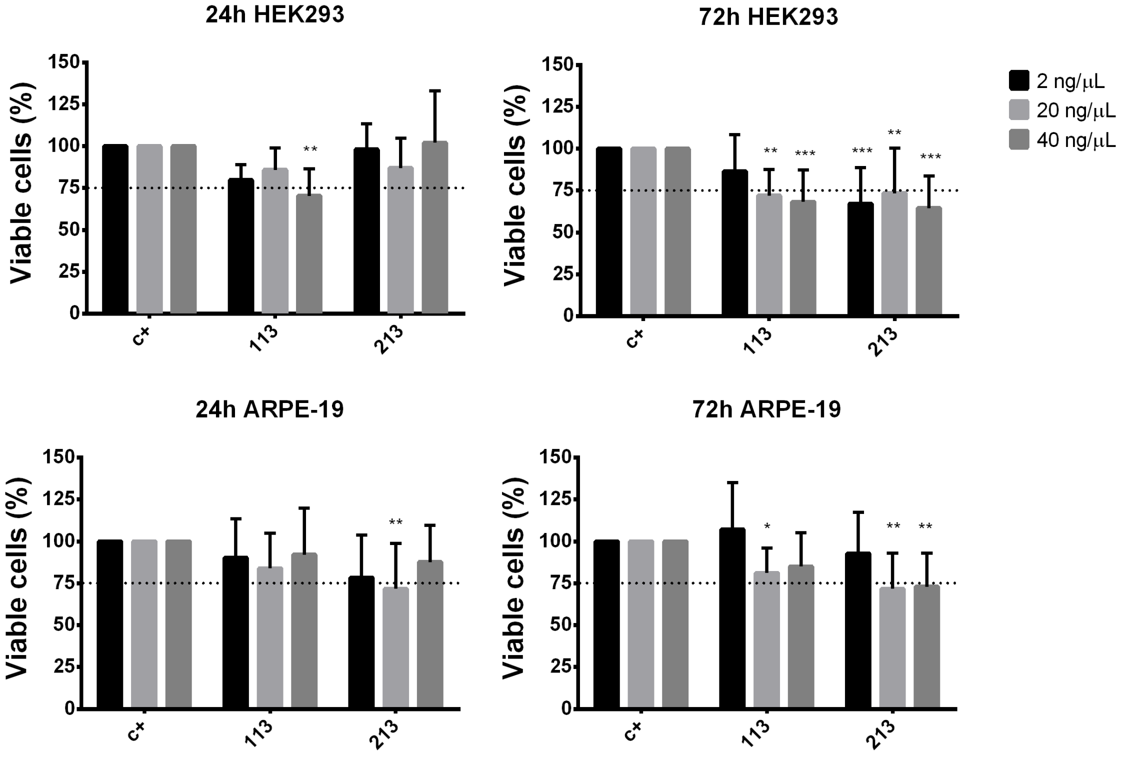


Figure 7.5 – Cell viability (%) as a function of polyplex amount (ng/uL of polymer) after 24 and 72 h of incubation with polyplexes. As a positive control of viability untreated cells (C+) were used. Statistical differences to the positive control and their significance are indicated by the star (*) symbol, with * P < 0.05, ** P < 0.01, *** P < 0.001.

Transfection efficiency was quantified by flow cytometry 72 h post-transfection (Fig. 7.6). GFP expression in cells transfected with polyplexes, as observed previously [9], was lower than in cells transfected with FuGENE HD, here used as a positive transfection control. Transfection studies also show that transfection efficiency is affected by cell line and molecular weight but remains unaltered by freeze-thaw cycles (Fig. 7.6). Transfection efficiency values obtained are in agreement with the literature and others have also reported that

results are cell line dependent and often inferior to commercial reagents used as controls. [10]

Since CS113 polyplexes performed better in both cell lines only these were further tested (freeze-thaw transfection assay and *in vivo* studies).

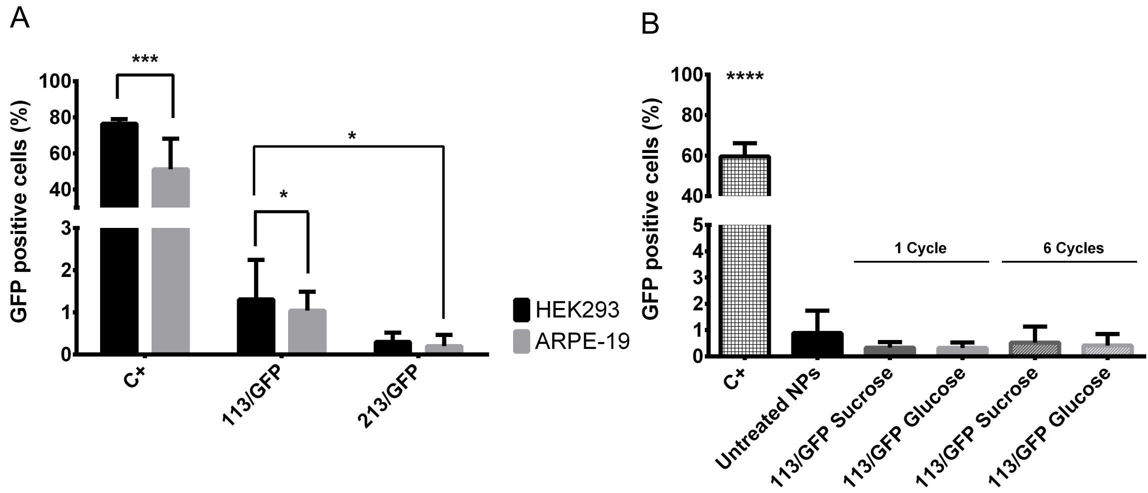


Figure 7.6 – Transfection efficiency 72h post-transfection as percentage of GFP positive cells, A) using two polyplexes 113/GFP and 213/GFP in two cells lines; B) using 113/GFP polyplexes treated with cryoprotectants in HEK293 cell line. As a positive control (C+) FuGENE HD transfected cells were used. Statistical differences are indicated by the star (*) symbol, with * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

3.4. *In vivo* transfection and GFP expression

In vivo transfection after subretinal injection can be observed after several months (Fig. 7.7). GFP expression increased over time probably as a result of slow plasmid release resulting in a sustained expression. Levels of GFP expression and number of transfected cells varied within injected animals, as expected, resulting in larger standard deviations than desired. However polyplexes seem to preferentially transfect RPE cells, as expected following subretinal injection

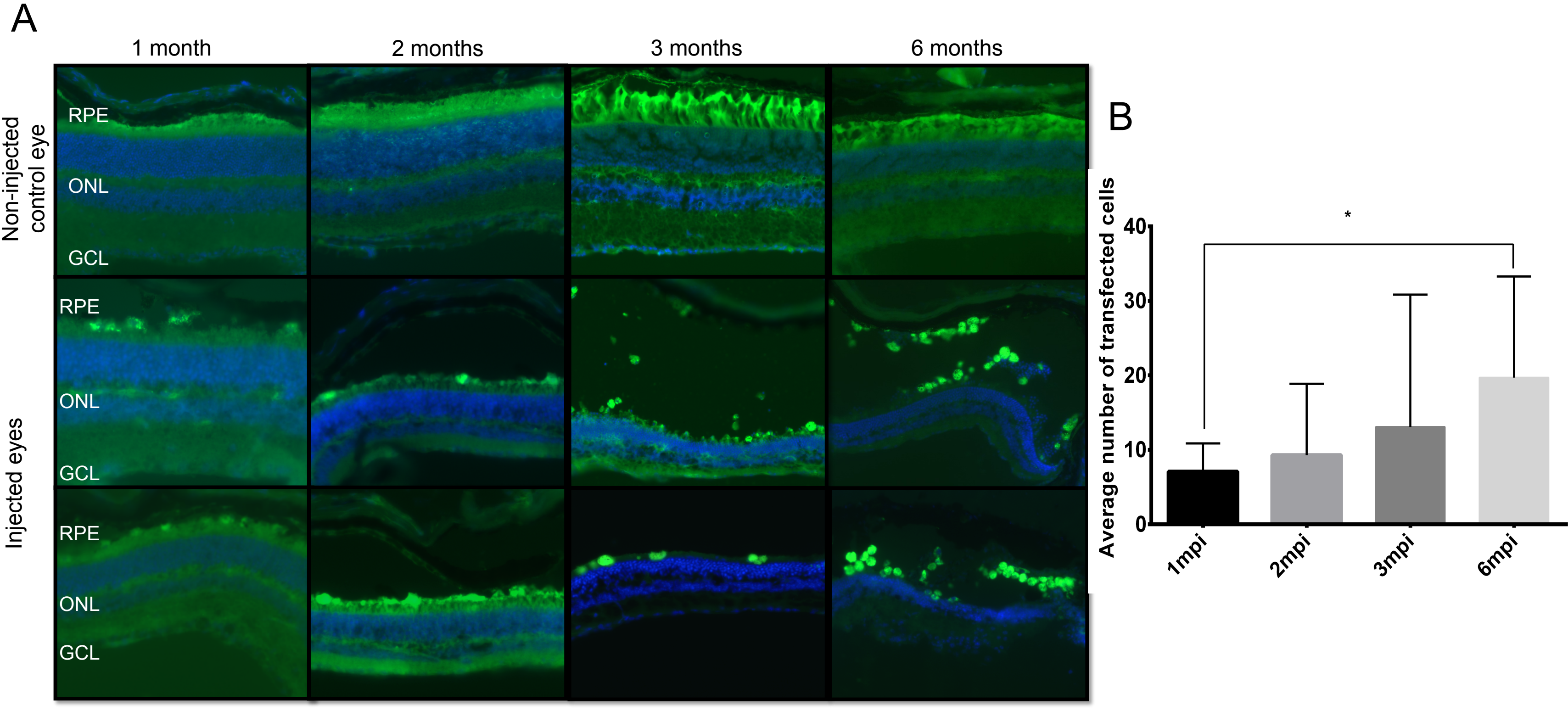


Figure 7.7 – Representative images of fluorescence microscopy of cryosections of injected and uninjected C57BL6 mice, A) one to six months after subretinal injection (Amplification 200x, RPE-retinal pigmented epithelium, ONL-Outer nuclear layer, GCL-Ganglion cell layer). B) Average number of transfected cells per field quantified by manual counting of at least 12 images per condition. Statistical differences are indicated by the star (*) symbol, with * P < 0.05.

4. Discussion

4.1. Effect of polymer molecular weight on polyplex characteristics

The goal of this study was to develop chitosan-based polyplexes with adequate properties for retinal gene delivery. The prepared polyplexes had very similar characteristics regarding polydispersity and zeta potential but showed some differences in size. Polyplexes prepared with the high molecular weight polymer (CS213) were on average larger then the ones prepared with the lower molecular weight polymer (CS113). This difference in size was more evident when comparing polyplexes with and with out pDNA, where DNA loaded polyplexes tend to have smaller sizes (Table 7.1). This agrees with the literature and with what was previously described by our group [11] since the molecular weight of the polymer can affect size, surface charge and stability of polyplexes. [12-14]

4.2. Effect of polymer molecular weight on *in vitro* transfection efficiency

One of the major obstacles for a successful gene delivery strategy is cellular uptake. Chitosan polyplexes are thought to enter the cell via endocytosis in a process that may be dependent on polyplex charge. [15, 16] Since there were no differences in charge in our polyplexes the differences observed in transfection efficiency may be related to differences in pDNA release. It has been shown that the molecular weight of the polymer can influence polyplex formation through the chain entanglement effect [12, 17] and this can also affect the pDNA release properties. [18] The pDNA release from the polyplexes is a critical step for the transfection and the success of any gene delivery vector. [5, 19] It has also been demonstrated that as molecular weight increases, the encapsulation efficiency tends to decrease. [20] Therefore, while no differences in pDNA complexation where noticeable, the lower transfection efficiencies obtained with the high molecular weight chitosan polyplexes (Fig. 7.6A) may be related both to

a lower pDNA encapsulation and a slower pDNA release.

4.3. Effect of polyplex stability on transfection efficiency

Since transfection efficiency is related to particle stability, it is important to investigate not only the stability at physiological conditions but also the long-term stability at storage conditions. All polyplex formulations had very similar pDNA complexation efficiencies and there was no visible pDNA release even after incubation at physiological conditions (Fig. 7.2 and 3). So we further analyzed them by incubation of polyplexes at 4 and 37ºC and determining the changes in physical characteristics overtime. The same tendency towards higher sizes in pDNA-unloaded polyplexes (symbols in grey in Fig. 7.3) is seen in this assay. Also, these polyplexes tend to show a more rapid deterioration in their overall characteristics that can be explained by the establishment of stronger electrostatic interactions in the presence of pDNA that contribute to the stability of the polyplex. [11]

As mentioned previously, temperature plays an important role in particle stability and its effect is evident since day 1, for example by comparing normal values of freshly prepared polyplexes (Table 7.1) with values for day 1 (Fig. 7.3) some changes were already noticeable. Results also show that the molecular weight of the polymer affects polyplex stability (Fig. 7.3). On average polyplexes prepared with high molecular weight chitosan show alterations earlier than polyplexes prepared with the lower molecular weight chitosan.

The lower stability of polyplexes prepared with high molecular weight chitosan could be advantageous in the context of transfection because ideally polyplexes should readily release their cargo once they enter the cells. [21, 22] However, this was not the case and in both cell lines these polyplexes resulted in lower transfection efficiency results.

4.4. *In vivo* transfection

The use of chitosan polyplexes for ocular drug delivery as been extensively reviewed; however few studies have been made on ocular gene delivery [23-25] and more precisely the retina. [26-28] Recent studies using low molecular weight chitosan have shown effective gene delivery to RPE cells using sub-retinal injections in rats. RPE cells transfection is an important step for ocular gene therapy since RPE cells can secrete transgene products to the neuronal retina as well as choroid vasculature and are known to play a significant part in ocular diseases associated with photoreceptor dystrophies. [27, 28]

So far studies only report *in vivo* chitosan-mediated gene expression 72 h or 2 weeks post-injection. [26, 28] To our knowledge, this is the first study where transgene expression is shown several months after injection. However results may have been hampered by the technique used for delivery. Subretinal injection despite being considered the best way to deliver to RPE cells is an invasive method that can cause complications such as inflammation, damage to the lens and retinal detachment. [29] No signs of inflammation were visible in the injected eyes but retinal detachment was evident in most transfected areas even months after the injections. We hypothesize that the retinal detachment was greatly caused by the technique itself since it is very challenging. The invasiveness of a subretinal injection may not be well tolerated in a degenerated retina and so alternative methods should be pursued. Recent studies using electroporation have successfully delivered transgenes to choroidal and RPE cells with no ocular complications, namely no retinal detachment. [30, 31] Currently, we are combining chitosan-mediated delivery with electroporation to obtain better transfection results with less ocular complications.

5. Conclusions

In this study we describe and characterize chitosan-based gene delivery vectors for retinal gene delivery. The effect of polymer molecular weight was evaluated in several polyplex characteristics including their stability and *in vitro* transfection efficiency. Despite modest *in vitro* transfection results chitosan polyplexes were able to successfully transfect mouse retina and GFP expression was observable up to 6 months after subretinal injection.

The developed chitosan polyplexes have adequate characteristics to be considered a good candidate to retinal gene therapy and should be further evaluated namely regarding other delivery methods to the retina and longer transgene expression periods.

Acknowledgements

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Chapter VIII

Chitosan and hyaluronic acid polyplexes achieve efficient retinal delivery

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Manuscript in preparation

1. Abstract

Non-viral gene delivery strategies need further addressing in order to achieve efficient transfection efficiencies. Chitosan is one of the most promising candidates with several reports supporting its usefulness in drug and gene delivery. However, its applicability is hindered by its low transfection efficiency. Based on previous results that showed an improved performance with the incorporation of hyaluronic acid, we here perform a pair-wise comparison between chitosan and chitosan-hyaluronic acid polyplexes. Their *in vitro* transfection efficiency was assessed in two cell lines: human embryonic kidney cells (HEK293T) and human retinal pigmented epithelium cells (ARPE-19), as well as *in vivo* in the retina of C57Bl6 mice. Polyplexes containing hyaluronic acid showed a marked improvement in the *in vitro* and *in vivo* transfection efficiency.

Keywords: Chitosan, Hyaluronic acid, Gene therapy, Retina.

2. Introduction

A successful ocular gene therapy strategy requires efficient gene transfer. The eye presents several advantages as a gene delivery target such as accessibility, immune privileged status due to the existence of the blood-retinal barrier and existence of several animal models of disease. [1] Currently, non-viral gene delivery approaches have limited clinical applicability mainly due to their low gene transfer and expression. However, there is a growing interest in the field mostly due to the advantages non-viral vectors have over viral vectors, such as better safety profile, ability to deliver large genes and lower production costs. [2]

Non-viral gene delivery employs several different methods to deliver the desired genetic information such as naked DNA delivered by electroporation or iontophoresis, liposossomes and nanoparticles composed of synthetic or natural polymers. [1, 3] Chitosan (CS) is one of the most studied cationic polymers for non-viral gene therapy having shown efficient nucleic acid encapsulation and protection against degradation while spontaneously forming polyplexes by complex coacervation. [4] Although CS has been used for several years in biomedical applications, for it to be considered a successful delivery vector further optimization is needed; several strategies have been used to improve its cell targeting, gene transfer and expression capacity (as described in [5]).

It has been suggested that the low transfection results obtained with CS polyplexes are caused by strong interactions between CS and DNA leading to high polyplex stability. [6, 7] One of the strategies to improve the efficiency of CS-mediated gene delivery focuses on the incorporation of anionic biopolymers. The combination of CS with anionic polymers destabilizes polyplexes and hence facilitates DNA release resulting in an increased transfection. [8-11]

Our group has previously shown that the incorporation of hyaluronic acid (HA) into CS polyplexes resulted in improved transfection efficiencies when compared with formulations without HA. [12] In this study, we further optimized CS-HA polyplexes based on the previously developed polyplexes using ultrapure CS. The newly developed polyplexes were evaluated for their *in vitro* transfection efficiency in two cell lines: human embryonic kidney cells (HEK293T) and human retinal pigmented epithelium cells (ARPE-19). Gene expression was also evaluated *in vivo* one month after administration, showing prolonged and sustained gene expression.

3. Results

3.1. Size, polydispersity and surface charge

Chitosan (CS) and chitosan-hyaluronic acid (CH) polyplexes were prepared by coacervation using the same methodology described previously. [12] In addition to preparation in sodium sulfate, polyplexes were also prepared in MilliQ water. All formulations yielded polyplexes with an average size below 500 nm, polydispersity lower than 0.3 and positive zeta potential (Fig. 8.1). When comparing the different formulations, polyplexes prepared in water displayed a tendency towards smaller sizes that was statistically significant between CH4 N and CH4 H, CH6 N and CH6 H. Also, when comparing CS formulation with all the other formulations containing HA, it was only found statistically different from formulation CH6 N.

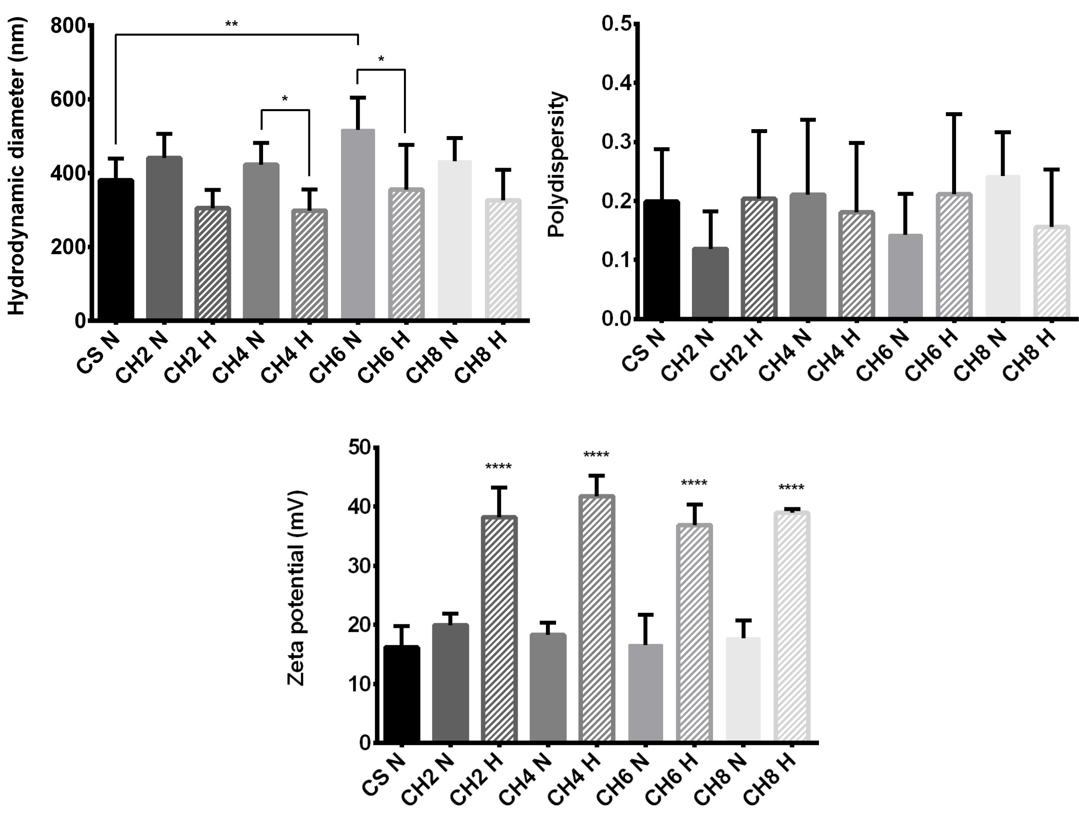


Figure 8.1 - Size, polydispersity and zeta potential of polyplexes. Statistical differences and their significance are indicated by the star (*) symbol, with * P < 0.05, **P < 0.01 **** P < 0.0001.

There were also differences in the zeta potential of the polyplexes, which displayed a higher surface charge when prepared in water. This difference was statistically significant between all formulations prepared in water (denoted with H) and all formulations prepared in sodium sulfate (denoted with N).

Regarding the size range of the polyplexes, the polydispersity index was low for all polyplexes and no statistical differences were found, indicating that all formulations are homogenous preparations.

3.2. pDNA-complexation and polyplex stability evaluation

Polyplexes were further characterized regarding their pDNA loading efficiency by an agarose gel retardation assay. All CH polyplexes showed efficient complexation regardless of the formulation tested, as observed by the absence of free DNA migration into the gel (Fig. 8.2). Efficacy of pDNA complexation by CS has been previous shown by our group. [13]

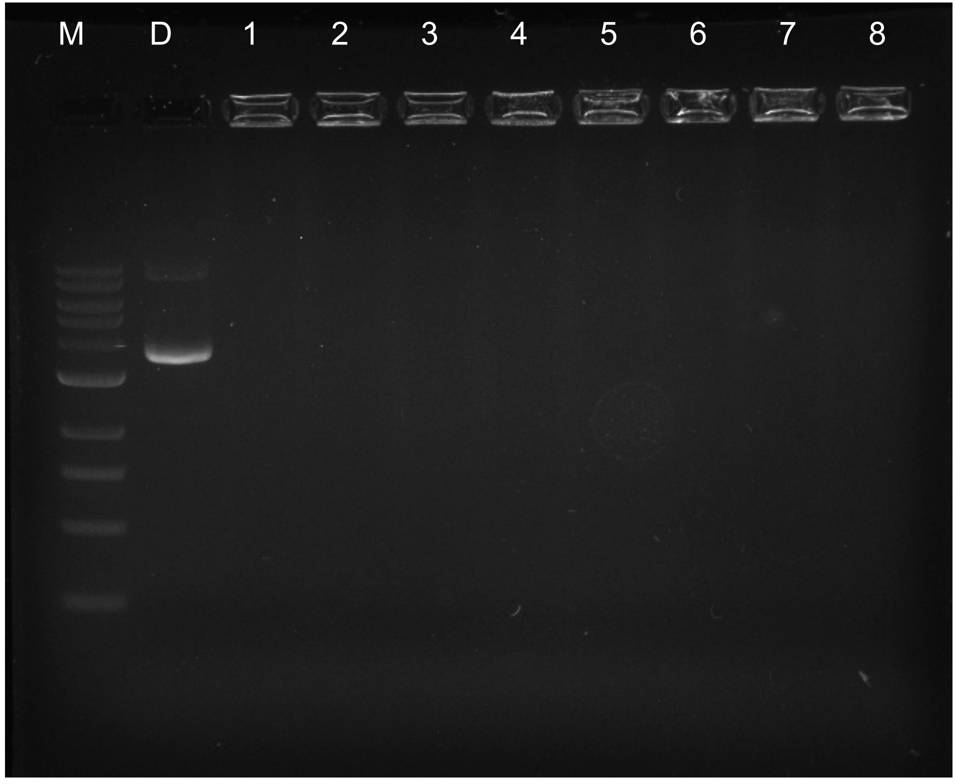


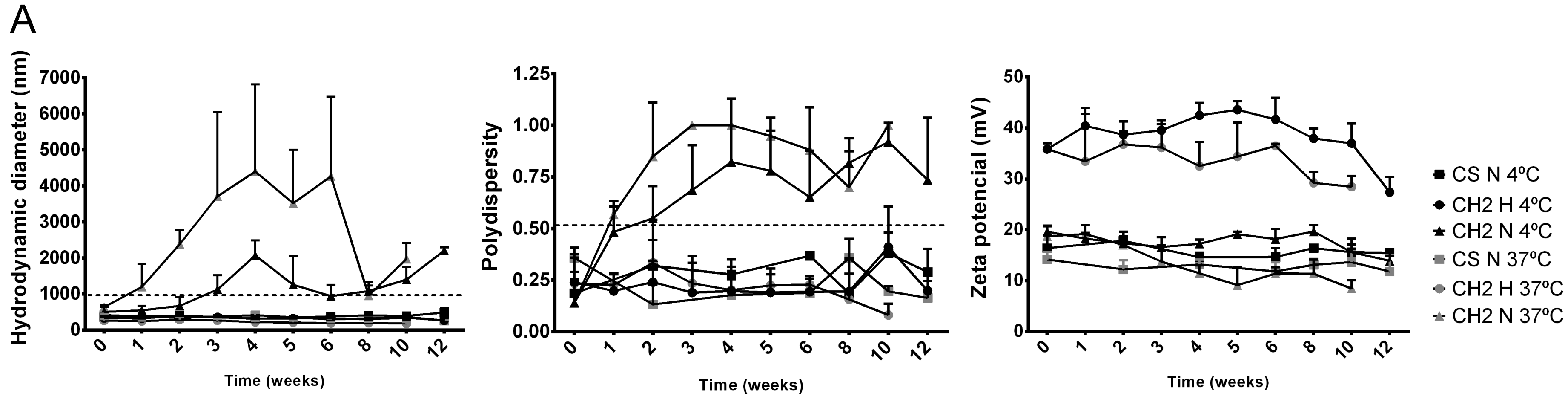
Figure 8.2 - DNA encapsulation in CS and CH particles analyzed by 1% agarose gel electrophoresis, DNA visualized with GreenSafe Premium. Lanes: M – DNA marker, D – plasmid DNA, 1-CH2 N, 2 – CH4 N, 3 – CH6 N, 4 – CH8 N, 5 – CH2 H,6 – CH4 H, 7 – CH6 H, 8- CH8 H.

Long-term stability was assessed at physiological and storage temperature by monitoring size and charge of polyplexes over time. As polyplexes start to degrade an increase in size and polydispersion along with a decrease in zeta potential, indicative of aggregation, was expected. In fact after only one week it was already noticeable an increase in size and polydispersion in formulations containing HA and prepared in sodium sulfate (formulation CH2 N) regardless of the incubation temperature (Fig. 8.3). Other formulations (CS and CH2 H) remained stable for longer periods of time.

3.3. *In vitro* studies

It had been previously shown that the incorporation of HA into CS polyplexes enhanced transfection, probably by modulating polyplex stability and pDNA release. [12] In this work, we also evaluated the effect of the solvent used in the preparation of the polyplexes with the incorporation of HA into the formulation. Formulations containing a plasmid coding for GFP expression were used to transfect two cells lines HEK293T and ARPE-19 (Fig. 8.4). Transfection efficiency varied with cell line and formulation. In ARPE-19 cells regardless of the formulation tested transfection efficiency was low (approximately 1%) while in HEK293T values varied greatly with the formulation tested (approximately from 10 to 40%). As expected, formulations containing HA performed better than CS polyplexes. The same tendency was observed at later times when transfected cells were kept in culture for 1 month and assessed for GFP expression (Fig. 8.5). Also, formulations prepared in water showed a tendency to perform better than formulations prepared in sodium sulfate. By comparison of transfection values for the different formulations and the positive control there are two formulations whose transfection efficiencies are statistically equal to the positive control: CH2 H and CH4 H.

A



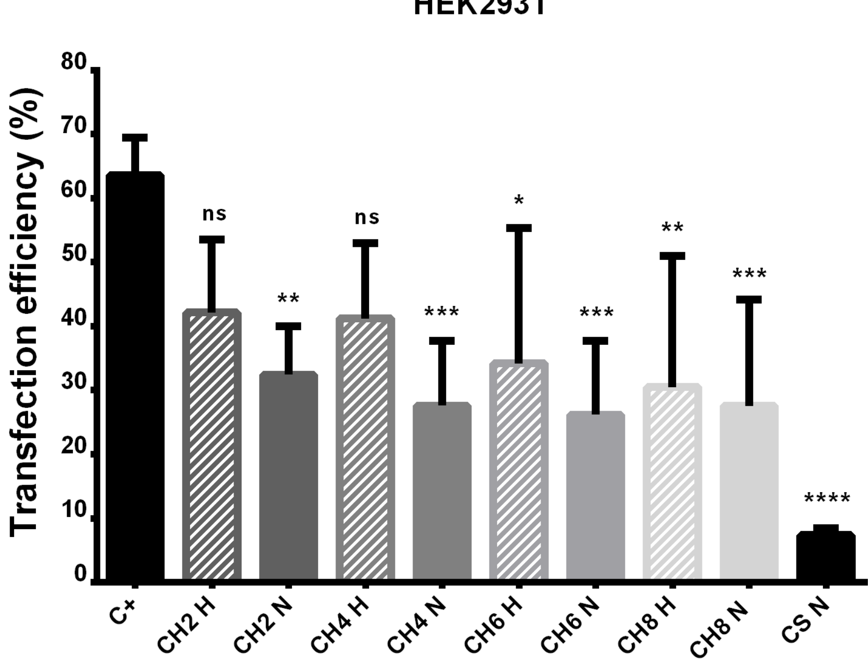
Statistical analysis of long-term stability assay

B

	4ºC			37ºC		
	Size	**PdI**	**ZP**	**Size**	**PdI**	**ZP**
CS N	NS	NS	NS	NS	NS	NS
CH2 N	4^{b},10^{a}, 12^{b}	3^{a},4^{b},5^{b},6^{a},8^{b},10^{c},12^{b}	10^{b},12^{c}	4^{a}	1^{a},2^{c},3^{d},4^{d},5^{c},6^{c},8^{b},10^{d}	4^{a},5^{b},8^{a},10^{b}
CH2 H	NS	NS	12^{a}	NS	NS	NS

Data points statistically different from initial value *t*=0, statistical significance depicted by superscript letters: a= p>0.005, b= p>0.001, c= p>0.0001, d p>0.00001

Figure 8.3 – Polyplex stability at 4 and 37ºC throughout time: A) size, polydispersity and zeta potential measurements over time and B) statistical analysis of data presented in panel A.



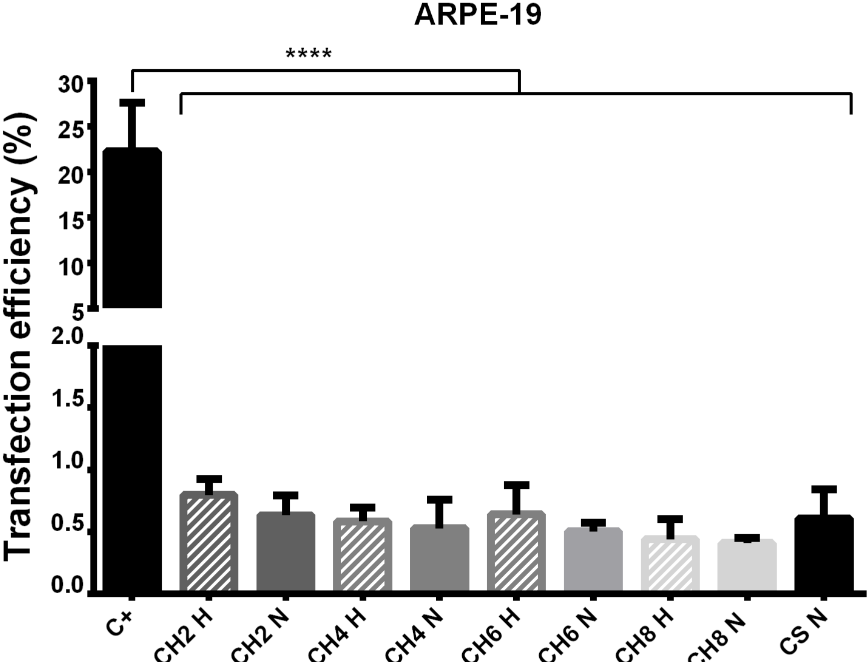


Figure 8.4 - Transfection efficiency 72h post-transfection as percentage of GFP positive cells. FuGENE HD transfected cells were used as a positive control (C+). Statistical differences to positive control are indicated by the star (*) symbol, with * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns – non significant.

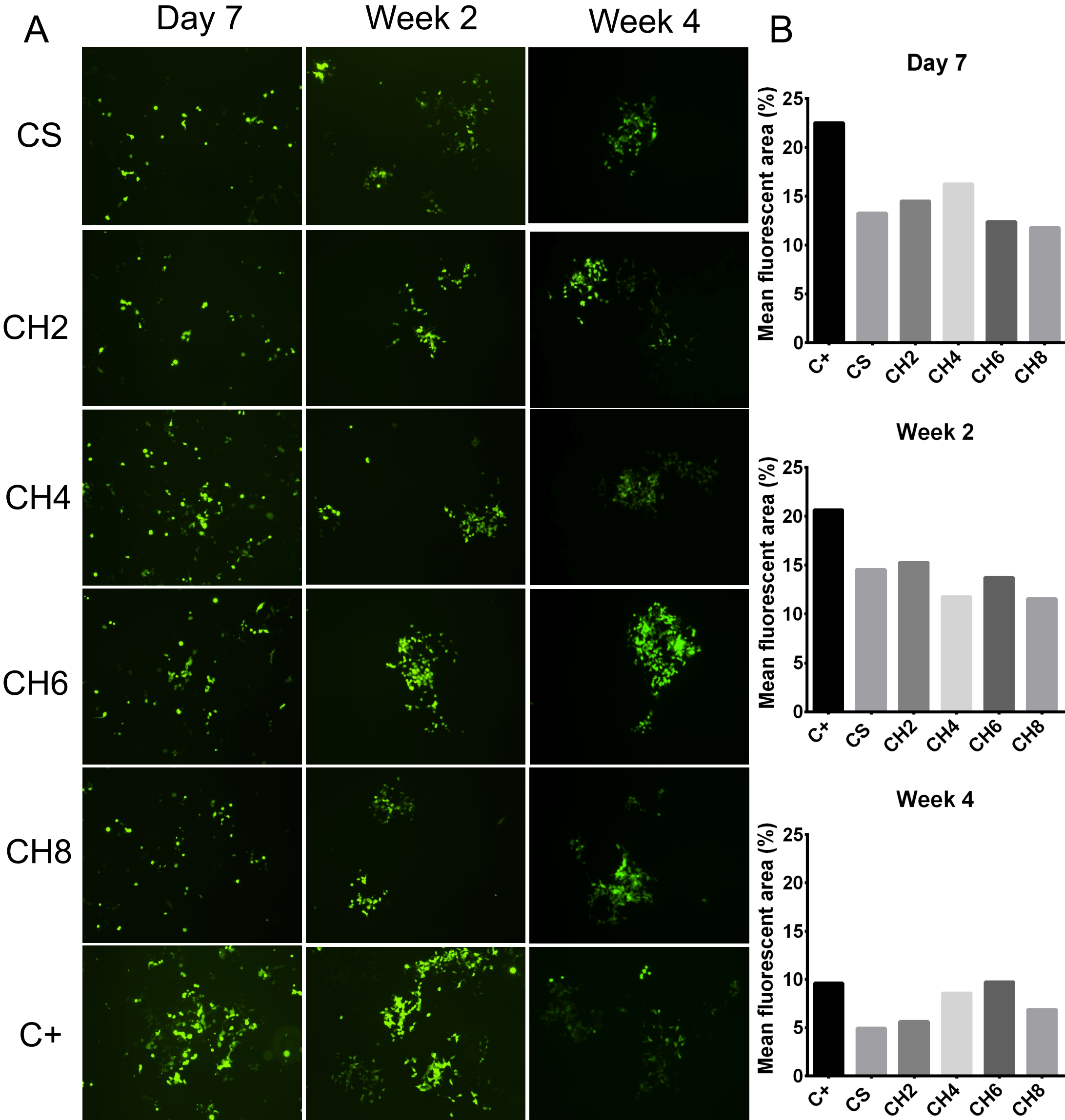


Figure 8.5 – GFP expression in HEK293 cells transfected with CS or CH polyplexes (prepared in sodium sulfate) over a period of several weeks. A) fluorescence microphotographs and B) mean fluorescent area of images in panel a) as quantified by ImageJ. Cells transfected with FuGENE HD were used as a positive control (Amp. 100x).

3.4. *In vivo* studies

The main goal of this study is to develop efficient retinal gene deliver vectors. In order to evaluate the performance of the developed formulations *in vivo* they were administered by subretinal injection to C57BL6 mice. Several different conditions were tested: mitotic and post-mitotic cells (defined by the age of the animals at time of injection: 5-7 days post birth cells are still mitotic; 1 month post birth cells are post-mitotic), polyplex formulation and sample collection time (2 or 4 weeks post injection). Firstly, polyplexes (CS N, CH2 N and CH2 H) were injected in pups (5-7 days post birth) and samples were analyzed for GFP expression 4 weeks post injection (Fig. 8.6). It was possible to observe transfected cells and GFP expression in eyes injected with all three formulations. As observed *in vitro*, formulations with HA showed a tendency to higher transfection although not statistically significant. Additionally, polyplexes (CH2 N and H) were also injected in adult mice (1 month old) and eyes were collected at two time points: 2 and 4 weeks post injection (Fig. 8.6). It was possible to observe transfected cells and GFP expression at both time points and with both formulations.

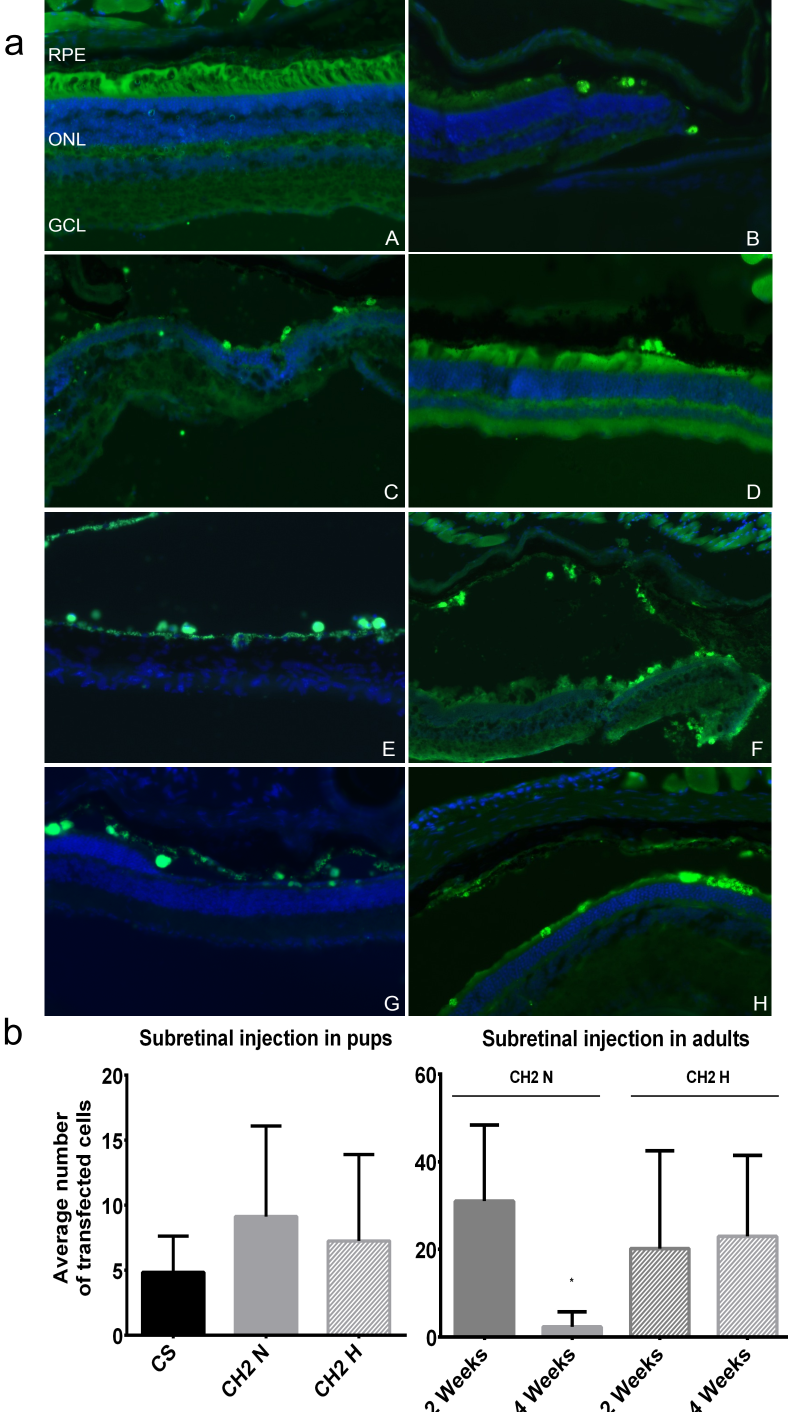


Figure 8.6 – Representative images of fluorescence microscopy of cryosections of injected and uninjected C57BL6 mice. a) A – D) one month after subretinal injection in pups, E and G) 2 weeks after subretinal injection in adults and F and H) 4 weeks after subretinal injection in adults. A) non-injected eye and eyes injected with B) CS N, C, G and H) CH2 N, D, E and F) CH2 H polyplexes.(Amplification 200x, RPE-retinal pigmented epithelium, ONL-Outer nuclear layer, GCL-Ganglion cell layer).
b) Average number of transfected cells per field quantified by manual counting of at least 12 images per condition. Statistical differences are indicated by the star (*) symbol, with * $P < 0.05$.

4. Discussion

4.1. Polyplex characterization

Previous positive results with the incorporation of hyaluronic acid led to the necessity of further tests and optimization of chitosan polyplexes. In this work chitosan polyplexes were compared to chitosan-hyaluronic acid polyplexes. Firstly, their physical characteristics were evaluated revealing differences in zeta potential between formulations prepared in sodium sulfate and water but not between chitosan and chitosan-hyaluronic acid polyplexes. This had been previously observed and described as related to the presence of the negative charges from sulfate anions, which reduce the overall surface charge (Chapter VI).

Secondly, their long-term stability was evaluated. Formulations prepared in sodium sulfate displayed a marked size and polydispersion increase noticeable from the beginning of the assay. The zeta potential of the polyplexes is one of the parameters used to estimate the stability of the polyplex dispersion. [14] It was also visible a decrease in surface charge so this reduced surface charge might have triggered aggregation of the polyplexes hence the large size and polydispersity. Also, polyplex stability is largely mediated by electrostatic interactions between the polycationic polymer and the polyanionic nucleic acid and can be influenced by the ionic strength of the surroundings. [15, 16] This could explain the differences observed in polyplexes prepared in sodium sulfate and water.

4.2. Cellular uptake and gene expression

A successful gene delivery strategy needs to overcome 3 main obstacles: cellular uptake, endo-lysosomal vesicles and nuclear entry. Considering cellular entry, there are several possible paths through which polyplexes can enter the cell. Chitosan polyplexes are thought to enter the cell via endocytosis in a process that may be dependent on polyplex charge. [17, 18] Since there were some differences in the surface charge of the developed polyplexes, differences in the cellular uptake were expected and hence

differences in the transfection efficiency were also expected. In fact, there seems to be a positive correlation between zeta potential and transfection efficacy with polyplexes with higher zeta potential exhibiting higher transfection efficiencies. Recent studies confirm this, using media acidification, to modulate and control the surface charge of chitosan polyplexes and showed a significant increase in cellular uptake and transfection. [19]

Also, formulations with the incorporation of HA into the polyplexes yielded better transfection results than chitosan alone, as expected. It is thought that this improvement is related to a destabilizing effect due to the anionic nature of HA along with increased cellular internalization. [12, 20] Recent studies showed improved cellular uptake and enhanced transfection in cancer cells overexpressing the CD44 receptor cells. [21] This along with the improved performance of chitosan in an acidic environment, *e.g.* tumors, suggests a therapeutic application for similar polyplexes in cancer gene delivery. [19]

Further studies to evaluate the involvement of HA receptors in cellular uptake, polyplex destabilization by HA and polyplex dissociation would broaden the understanding on chitosan-hyaluronic acid polyplexes mediated transfection and allow further optimizations.

Transfection and gene expression was also evaluated *in vivo* by subretinal injection to C57BL6 mice. GFP expression was detected in all conditions tested regardless of mice age at injection or collection time after injection. As observed *in vitro*, formulations with HA showed a tendency to higher transfection. However, due to the relatively low number of injected animals per condition (n=3) and high number of variables it would be necessary to expand these assays in order to gain statistical power.

Subretinal injection despite being the most effective way to target and deliver therapeutics to the retina is an invasive delivery method. [22] That can be observed by the retinal detachment still present 1 month after subretinal injection in the injected eyes. The use of an automatic microinjection pump where the injected volume and speed of injection is more controlled somewhat reduced the retinal detached (Fig. 6, injections in adult animals). However, less invasive alternative routes should be tested, namely intravitreal

injection and/or electroporation that have shown positive results targeting the retina. [23]

5. Conclusions

In this study, previously developed polyplexes were further optimized for retinal gene therapy. CS-HA polyplexes were characterized and evaluated for their *in vitro* transfection efficiency in two cell lines, HEK293T and ARPE-19. Our results are in agreement with previous studies and show that the incorporation of HA can greatly enhance CS-mediated transfection efficiency.

Gene expression was also evaluated both *in vitro* and *in vivo* one month after transfection showing prolonged expression with all tested formulations. These results are promising and further developments in the administration methods and targeting can further contribute to the advancement of non-viral retinal gene therapy.

Acknowledgements

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Chapter IX

General discussion

Chapter IX - General discussion

1. Effects of polymer molecular weight on polyplex characteristics and transfection efficiency

The main goal of this study was to develop polyplexes with adequate properties for retinal gene delivery. As mentioned in Chapter I, there are several factors affecting polyplex characteristics and ultimately affecting transfection efficiency. Moreover, these factors tend to differ with the features of the polymer chosen for the delivery system. Studies described in Chapters III and IV were performed with commercial grade CS and allowed several optimizations and selection of conditions to be tested in the following work while using a cheaper source of the polymer. In the subsequent chapters - V, VI, VII and VIII - having as a goal *in vivo* applications, ultrapure CS with different MWs was used. As mentioned before, it was important to test different MW since it is one of the factors influencing polyplex size, surface charge, stability, DNA dissociation and more importantly transfection efficiency. [1-3] However, there are contradictory reports regarding MW contribution to CS-mediated transfection. Some studies support the use of high MW CS to attain higher gene transfer, while others support the use of lower MW CS. [4, 5] For example, according to Sato *et al.,* CS with MW between 15 and 52 kDa is optimal for gene delivery. [6]

Considering the polyplex formation process, MW can influence polyplex formation through the chain entanglement effect resulting in different polyplex compaction levels and consequently affecting polyplex size and stability. [7] Polyplex formation with lower MW CS has a smaller contribution from the chain entanglement because a larger number of polymer chains are necessary to achieve the same N:P ratio, which may not be energetically favorable to polyplex formation. On the other hand, longer chitosan chains can entangle free pDNA with greater ease after the initial electrostatic interactions have occurred. [1]

Regarding the effect of CS MW on polyplex size it is known that size and stability varies with chitosan MW and chitosan/DNA ratios. [8] But there are also some discrepancies in the literature; some studies show a decrease in polyplex size with the increase in CS MW, while others show no significant influence of MW on size. [2, 5] A possible explanation for some of these conflicting results could be

differences in the polyplex preparation method (*i.e.* ionic strength and polyelectrolyte concentrations) or the nature the nucleic acids used (linear or plasmidic, ssDNA or dsDNA). [5] Our results (Chapter V and VII) show increased polyplex size and surface charge with the use of higher MW CS. Moreover, contrary to the above stated hypothesis, the same trend was observed in our results even when different polyelectrolyte concentrations were used (0.2 or 1 mg/mL) and different N:P ratios (5:1 or 15:1). The effect of MW on surface charge is more consensual and, as reported in the literature we also noticed a trend regarding surface charge, which increased with CS MW. [2]

CS MW can affect polyplex stability, cellular uptake efficiency and dissociation upon endocytosis. [6] The effect on cellular uptake can be related to the above-mentioned effect on the surface charge of the polyplexes that facilitates electrostatic interactions with the negatively charged proteoglycans in cellular membranes while the effect on dissociation can be related to the chain entanglement effect. Our results also show that MW influenced polyplex stability with a clear trend towards polyplex destabilization with higher CS MW (Chapter VII and unpublished data in Chapter X, Fig. 10.S1 and 10.S2). This increased instability, associated with the larger size of the polyplexes, suggests a lower compaction of the polyplexes prepared with higher MW, which would in turn facilitate DNA dissociation. With a higher surface charge associated with an easier DNA dissociation both cellular uptake and DNA release would be facilitated and higher transfection efficiency would be expected. However, this was not the case and polyplexes with lower MW CS yielded better results (Chapters V and VII). A possible explanation is that smaller polymers can dissociate faster and since compaction is not as efficient due to the lack of participation of the chain entanglement effect short-term transfection is increased. [9]

Additionally, the size difference of CS polyplexes might have affected the internalization pathway. Studies have demonstrated that larger particles enter the cell in a slow manner and preferably through caveolae-mediated endocytosis or macro-pinocytosis whereas smaller particles enter the cell rapidly and preferably through clathrin-mediated endocytosis. [10, 11] Therefore larger polyplexes might have a slower progression once inside the cell, which associated to a slower dissociation rate can explain the lower transfection observed in our results 72 h post-transfection (Chapters III, V and VII). In fact when comparing GFP expression at later time points, for example in the long-term expression experiments described in

Chapter V, differences in GFP expression are not as obvious as the ones observed at the 72 h time point.

Cellular uptake is recognized as one of the major obstacles for a successful gene delivery strategy. Chitosan polyplexes are thought to enter the cell via endocytosis in a process that may be dependent on polyplex charge. [11, 12] As mentioned above, a higher surface charge can facilitate electrostatic interactions with the negatively charged proteoglycans in cellular membranes and hence promote polyplex internalization. [13] Our results (Chapters III, V, VI and VIII) show a positive correlation between polyplex surface charge and transfection efficiency and are in agreement with the literature.

2. Balance between stability and transfection efficiency

Polyplex stability is an important factor because it can influence therapeutical effectiveness by impacting DNA dissociation, polyplex degradation and polyplex shelf life. This is a critical aspect since a successful gene therapy vector needs to protect its load until it reaches the target and only unload it at the appropriate time. [14, 15] In our studies polyplex stability was evaluated in different ways either by analyzing DNA retention or size and surface charge measurements.

Firstly, polyplex stability at physiological conditions (pH, temperature and presence of serum) was assessed. DNA retention by the polyplex was evaluated by a gel shift assay after different incubation times, which is an indirect and qualitative way of assessing DNA binding. DNA binding affinity is determinant for CS ability to protect the DNA as well as polyplex stability. [5] All CS polyplexes tested remained stable and no signs of DNA release were observed (Chapters III, IV and VII). Some DNA release was only observed in polyplexes described in Chapter VI, prepared with the modified polymer $HASSNH_2$. In the presence of serum, we hypothesize that serum components establish electrostatic interactions with the polyplexes, destabilizing them, and causing the observed DNA release. Less stable polyplexes can be advantageous since several studies have shown a correlation between lower polyplex stability and higher transfection efficiency. [1, 2, 16, 17]. In our results (Chapter VI) less stable formulations showed higher transfection values as expected, based on the aforementioned relation between polyplex stability and transfection efficiency.

Additionally, polyplexes stability at physiological conditions was not affected by the plasmid size and number of plasmids used in the preparation. No free pDNA was detected whether when analyzing particles with pGFP and pCEP alone, or pGFP+pCEP and pCEP+PINT (unpublished data in Chapter X, Fig. 10.S3). Although no free pDNA was visible, there is a clear loss of signal intensity in the wells after 14 days at physiological conditions (Fig. 10.S3), indicating pDNA unloading or possible degradation. This is more noticeable in formulations containing CS213 (CS with higher MW), which is in agreement with the above-mentioned results that indicate a correlation between MW and polyplex stability.

Secondly, since transfection efficiency is related to particle stability, it is important to investigate not only the stability at physiological conditions but also the long-term stability at storage conditions. Long-term stability of unloaded and pDNA-loaded vectors was evaluated by measuring their size, polydispersity and zeta potential during incubation at 4 and 37ºC for extended periods of time. Results show that temperature plays an important role in particle stability as well as the MW of the polymer (Chapters V, VII and unpublished data in Chapter X, Fig. 10.S1 and 10.S2). This effect is especially noticeable in the decrease in zeta potential seen in particles kept at 37ºC. Positively charged polyplexes tend to aggregate as a function of incubation time and the aggregation rate is also dependent on charge and ionic strength of the medium. [18] Also, according to the Derjaguin–Landau–Verwey–Overbeek theory, a system will be stable in simple electrolyte solutions if the electrostatic repulsion between two particles is larger than their van der Waals attraction. [18] As the surface charge of the polyplexes decreases the repulsion between polyplexes is reduced and they start to aggregate, as observed by the increase in polyplex size and polydispersion.

Thirdly, considering long-term storage and the possibility of low temperature storage, the stability and performance of polyplexes after freeze-thaw cycles in the presence of cryoprotectants (glucose and sucrose) was assessed. Polyplex size remained stable after freeze-thaw following resuspension with cryoprotectants (Chapter VII and unpublished data in Chapter X, Fig. 10.S4 and 10.S5). The only noticeable differences were a slight tendency to higher polidispersion and lower zeta potential values. More importantly no differences were found in the transfection efficiency, meaning that polyplexes can be stored at -20ºC until administration maintaining their biological properties and can be re-stored and used multiples times.

Successful gene delivery requires a balance between polyplex stability, polymer buffering capacity and strength of polymer/DNA interactions. [8] Several studies have suggested that high stability and strong interactions between chitosan and DNA are the cause for the low transfection results. [1, 2, 19] One of the strategies to overcome this has been the incorporation of anionic polymers into the formulations that are thought to destabilize polyplexes and facilitate DNA release. [14, 17, 20-22] Anionic polymers, such as alginate and HA, have been used as coatings and for example have enhanced PEI-mediated gene delivery when used as a coating. [23-25] It has also been shown that improvements in transfection efficiency could be related to increased cellular internalization as HA containing polyplexes can be internalized via interactions with the membrane CD44 receptor. [26, 27] To determine if HA would have a role in promoting transfection, different formulations containing HA were produced and examined. Our long-term stability results (Chapters IV and VIII) revealed that HA containing polyplexes are indeed less stable than CS polyplexes, thus resulting in increased transfection efficacy. The observed improvement can result from the above-mentioned polyplex destabilization since the incorporation of HA can interfere with interactions between CS and pDNA and affect DNA binding, as well as the participation of the CD44 receptor in the internalization of the vectors. However it was not tested if this was due to enhanced internalization mediated by the HA- receptor.

Additionally, modifications to the polyplex formation process further enhanced transfection with HA containing polylexes (Chapter VIII). Elimination of the sodium sulfate contribution to the complexation process yielded more compacted polyplexes and also more positively charged. This resulted in increased transfection probably due to increased cellular uptake as mentioned above.

3. Effects of polymer modifications

Polymer modification is one of the strategies to solve or ameliorate a particular limitation of a polymer or polyplex formulation. As discussed in Chapter I polymers can be modified in different ways to address different problems. In the present work two polymers, CS and HA, were modified by thiolation. Modification with the addition of disulfide bonds has improved gene delivery and expressions when compared to unmodified polymers, as these can be cleaved intracellularly by

glutathione and thus promote a faster release of the genetic material. [28-34] Our results (Chapter III), however, showed that CS thiolation (Fig. 3.1, Chapter III) did not provide the intended enhancement in transfection efficiency. This unexpected lack of improvement may be explained by different factors: *i*) the increased bulkiness of the polymer caused by the incorporation of side groups in CS might have affected polyplex formation although the number of amines capable of electrostatic interaction was maintained; *ii*) the presence of residual tosylate counter-ions on CS-(AEDTP) polymer may have also hindered the chain entanglement process during polyplex formation. [35] This contributed for the formation of larger polyplexes that combined with a negative surface charge resulted in transfection values comparable to the un-modified CS. However, this apparent lack of improvement may be overcome by the replacement of mercaptoethylamine p-toluenessulfonate by its hydrochloride salt, since chloride ions do not present the same problems associated with tosylate.

HA was also modified with cystamine (Fig. 2.2, Chapter II) that, added not only amine groups to the polymer, but also disulfide bonds that could be cleaved in the presence of intercellular glutathione, thereby promoting a more rapid release of genetic material. [36] By modulating the amount of amine groups incorporated into the polymer a better balance between polymer buffering capacity and strength of polymer/DNA interactions was expected. [37] However, the extent of HA modification was not sufficient to yield polyplexes by itself and, when combined with CS, it did not improve transfection efficiency compared to unmodified HA.

4. Disparities between *in vitro* and *in vivo* data

Several studies have reported differences in *in vitro* and *in vivo* gene expression results. For example, Kiang *et al.*, showed that some formulations yielded better *in vivo* results than *in vitro.* [1] This brings forward the importance of selecting the most appropriate *in vitro* model for transfection studies when designing a gene delivery vector. The phagocytic nature of the RPE along with its capacity to produce and release trophic factors makes it an excellent target for non-viral gene therapy. [38, 39] The most established RPE cell line is the ARPE-19, which is a spontaneously derived human RPE cell line with normal karyology that forms polarized epithelial monolayers on porous filter supports. [39] These cells can exhibit morphological polarization when plated on laminin-coated filters and formation of

tight junctions occurs within 4 weeks. Therefore, it is considered that ARPE-19 cells share structural and functional properties characteristic of RPE cells *in vivo* and is valuable for *in vitro* physiological studies of RPE. [39]

Others have also reported great variations in polyplex transfection with different cell lines. In the present work HEK293 cells were used as control cell line since it has been widely used in transfection studies and most studies using CS polyplexes reported higher transfection results with this cell line. [1, 40-44] Additionally, two RPE cell lines were used: ARPE-19 and D407. ARPE-19 cell line was chosen as the one that more closely resembles RPE properties as described above and therefore used in most of the described studies. D407 cell line is also a spontaneously arising human cell line with epithelial morphology and the formation of a hexagonal cobblestone layer with intercellular junctions although they do not polarize as RPE does nor do they produce pigment. [45]

ARPE-19 cell line may not be a perfect *in vitro* model for transfection studies when targeting RPE cells since they display most morphological and biochemical indications of differentiated RPE cells only when cultures are allowed to grow to post-confluent densities. [39, 46] ARPE-19 cells in normal culture conditions are not polarized cells and they lack tight junctions that RPE cells display *in vivo*. This discrepancy in the characteristics of the cell lines with the normal cells *in vivo* can lead to a miscalculation of transfection efficacy *in vivo*. It has been previously shown by Puras *et al.*, that despite not being able to effectively transfect ARPE-19 cells *in vitro* they were still able to effectively transfect rat RPE by subretinal injection. [47] Indeed all the developed polyplex formulations displayed low transfection results in ARPE-19 cells despite the improvements obtained in HEK293 cells, especially with HA containing polyplexes (Chapters IV and VIII). And more importantly, *in vivo* transfection results seem to be closer related to HEK293 results than ARPE-19 *in vitro* results (Chapters VII and VIII) in terms of formulation effectiveness. It might be beneficial to perform transfection assays in filter-cultured ARPE-19 cells since they would resemble more closely native RPE cells and would allow a more accurate estimate of *in vivo* transfection efficacy.

5. Retinal gene delivery

Retinal gene therapy has seen great advances mainly through the use of AAV vectors. These have been used in gene therapy clinical trials showing both safety and efficacy. [48-50] The main limitation of this virus considering ocular applications is its limited packaging capacity since the size of several disease-causing gene exceeds its packaging limit. As more genes are identified as the cause of retinal diseases the need for delivery vehicles that can deliver larger genes also grows. Several large genes responsible for retinal disorders have been identified such as CEP290 (7.5 kb), ABCA4 (6.8 kb), MYO7A (6.7 kb), CDH23 (10 kb), USH2A (15.6 kb) and GPR98 (18.9 kb). [51]

Viral alternatives to AVV include the use of lentivirus or helper-dependent adenovirus, which can deliver 8 and 36 kb, respectively. However, these raise questions regarding random integration and insertion mutagenesis as well as immunological complications. Another strategy is the use of the AAV dual vector approach, where the transgene is split into 2 complementary vectors that are trans-spliced in the target cell. However, this strategy has had limited success. [52, 53]

Non-viral vectors, on the other hand, are biocompatible with reduced immunogenicity and show a great potential to deliver large genes. [54] In chapter V, we showed that our vectors could complex and deliver a large gene, CEP290, which is over the packaging limit of AAV vectors. In this work we showed that CS-mediated delivery could be an alternative to the use of AAVs and in combination with integrase, CEP290 over-expression could be detected several weeks post-transfection. These results are very encouraging since to date there are no effective gene therapies for the treatment of diseases caused by mutations on the CEP290 gene. [51] Also, to the best our knowledge, this is the first report of CS-mediated CEP290 delivery.

The degenerative nature of most retinal disorders creates a limited therapeutic window where gene therapy can be effective. [51] Therefore, the combination of early diagnosis and effective gene therapy strategies with persistent gene expression are critical. From the several strategies currently in development regarding sustained transgene expression we focused on the use of phage phiC31 integrase. This site-specific recombinase can promote safe integration and long-term

expression and from our results it can increase levels of expression for prolonged periods of time (Chapter V). The use of integrase *in vivo* would be a great advantage since it would prevent multiple administrations that despite technological advancements are not without associated risks, as discussed in Chapter I. Currently the best ways to target the retina *in vivo* are by intravitreal or subretinal injection that are both clinically viable administration routes. This, allied with the targeting of RPE as cells that can secrete transgene products to the neuronal retina, as well as choroid vasculature, shows great promise to retinal-targeted therapies. [47, 55]

Our proof of principle *in vivo* studies show that is possible to observe GFP expression 6 months following subretinal injection, even without the use of integrase (Chapter VII). Additionally, preliminary results indicate that with the co-encapsulation of the integrase plasmid, transgene expression can be extended up to at least one year (unpublished data in Chapter X, Fig. 10.S6). Few studies have focused on retinal CS gene delivery and so far studies only report gene expression 72 h or 2 weeks post-injection. [47, 55, 56] To the best of our knowledge, this is the first study where transgene expression is shown months after administration in mice. Although transfection efficiency needs to be further improved, this set of results indicates that CS is a good candidate as a non-viral retinal gene delivery system.

In the present work important issues that are currently a problem for the success of gene therapy were approached:

i) Low transfection efficiency associated with vector high stability: CSHA and CH polyplexes showed higher transfection associated with polyplexes with lower stability and probably better DNA release;

ii) Short-term/transient transgene expression: co-complexation of integrase plasmid in the polyplexes increases transgene expression and allowed sustained transgene expression for several months;

iii) Limited transgene packing capacity: complexation of large genes such as CEP290 did not affect polyplex properties and allowed for efficient transfection, and

iv) Limited *in vivo* transfection: non-viral mediated delivery allowed retinal transgene expression for extended periods of time.

Altogether, the non-viral retinal gene therapy described in the present work meets several important features for it to be a clinical alternative to viral gene therapy.

6. Future perspectives

Despite having all the aforementioned polymer design strategies and tools it still remains a challenge to integrate different functionalities into one single vector. A delicate balance between structural and chemical characteristics must be achieved for optimal gene delivery, especially since properties designed to overcome one barrier can directly hinder the ability to overcome other barriers. Efforts to achieve the perfect balance between stability, targeting and persistence of expression are needed.

Polymer modification has been able to ameliorate some of the drawbacks associated with polymer-based gene delivery. However, the extent of polymer modification can dramatically alter the original properties of the polymer. [57] So it is critical to understand which percentage of modification will be the most advantageous in order to preserve some of the desired qualities of the polymer (*e.g.* low cytotoxicity, interaction with specific receptors) and at the same time acquire new or improved properties (*e.g.* buffering capacity, DNA dissociation). In this study the tested modifications or the degree of modification tested did not yield the desired improvement in transfection efficiency. Since the degree of HA substitution was low (~10%) in the future it would be important to test higher modification degrees to try to modulate the polymer behavior and achieve higher transfection efficiencies. As mentioned before, in the case of CS it would be interesting to replicate the substitution reaction using a smaller counter ion that would not interfere with polyplex formation.

Persistence of gene expression is one of the great obstacles gene therapy needs to overcome. Repeated administrations are not always possible due to adverse effects related to the delivery procedure; especially in the eye, where degenerative diseases may leave tissues too fragile to withstand repeated administrations. [58] So it is important to develop systems capable of sustaining prolonged gene expression at therapeutic levels. The phiC31 integrase system has shown great promise [59] and from our preliminary *in vivo* results it promotes continuous gene expression even one year after injection (unpublished data in Chapter X, Fig. 10.S6). Therefore it would be interesting to combine the use of our best performing polyplexes, CH2 or CH4, with the co-complexation of the integrase system and test them *in vivo*.

To further clarify the reason for the improved performance of HA containing polyplexes it would be valuable to study if the CD44 receptor has a role in the uptake of CSHA or CH polyplexes. Firstly, the presence of the specific receptor should be confirmed in the cell lines used in transfection assays (HEK293 and ARPE-19) by immunocytochemistry. Then, to evaluate if the interaction between HA and CD44 receptor occurs during polyplex uptake, several transfection conditions would be tested using previously labeled fluorescent polyplexes.

It would also be important to evaluate the role of HA on the polyplex dissociation process. This could be done through fluorescence resonance energy transfer (FRET) experiments. By labeling both polymer and DNA with specific fluorophores it would be possible to measure how fast polyplexes dissociate and also polyplex intracellular localization upon dissociation. [60]

Moreover, it would be interesting to investigate the role of HA and its receptors in the pathogenesis of diabetic retinopathy. Studies have demonstrated that in the context of diabetic retinopathy polyplexes containing high MW HA can have an anti-angiogenic effect while their degradation products can have the reverse effect. Neovascularization is thought to result from cellular damage due to prolonged exposure to high glucose levels and advanced glycation end-products. Protein exposure to these factors can cause HA degradation into oligo-HA that has an angiogenic effect. [61] So, it would be of interest to evaluate the effect of HA containing polyplexes in the progression of diabetic retinopathy in a mouse model of the disease, such as the Ins2Akita mice.

Ongoing efforts to improve non-viral gene delivery include the use of nuclear localization signals (NLS) that can mediate nuclear intake, therefore improving transfection. Despite encouraging results most studies uses peptides derived from viruses (*e.g.* SV40) that can lead to increased immunological reactions by the host tissue. For this reason, an endogenous NLS assisted method could prove to be a way of ameliorating nuclear translocation without compromising the fairly low immunological profile of non-viral vectors. One of the possibilities is the use of Insulin-like Growth Factor Binding Proteins (IGFBPs), namely IGFBP-3 and IGFBP-5. It has been described that both of them contain NLS-like sequences within the C-terminal domain that are necessary and sufficient for their nuclear accumulation in a process mediated by importin β. [62] Also, IGFBP-3 was shown to interact directly with nuclear retinoid receptors. [63] This is of particular importance since it has been

described a specific retinal endocytosis mechanism, in retinal pigment epithelial cell lines, mediated by IGFBP-3 and IGFBP-5. [64] It would be interesting to combine the use of these NLS with our CS vectors to determine which of the two, aforementioned, 18-amino-acid-domains is most suitable for retinal gene delivery and promotes higher transfection efficiencies. Ongoing work has included co-complexation and co-administration of the NLS with CS polyplexes and their effect on transfection efficiency is being evaluated.

Given the promising results *in vitro,* it would be important to evaluate *in vivo* the delivery of CEP290 using the developed vectors in animal models of disease. For example, using the rd16 mouse as a model of CEP290-induced retinal degeneration, it would be possible to evaluate not only *in vivo* delivery efficacy but more importantly functional rescue of the disease phenotype. Preliminary experiments in C57BL6 mice have shown that our vectors can deliver this large gene *in vivo.* Nonetheless, these results indicate that our vectors could deliver the transgene *in vivo* and possibly rescue or prevent further degeneration.

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Chapter X

Supplementary data

Supplementary data

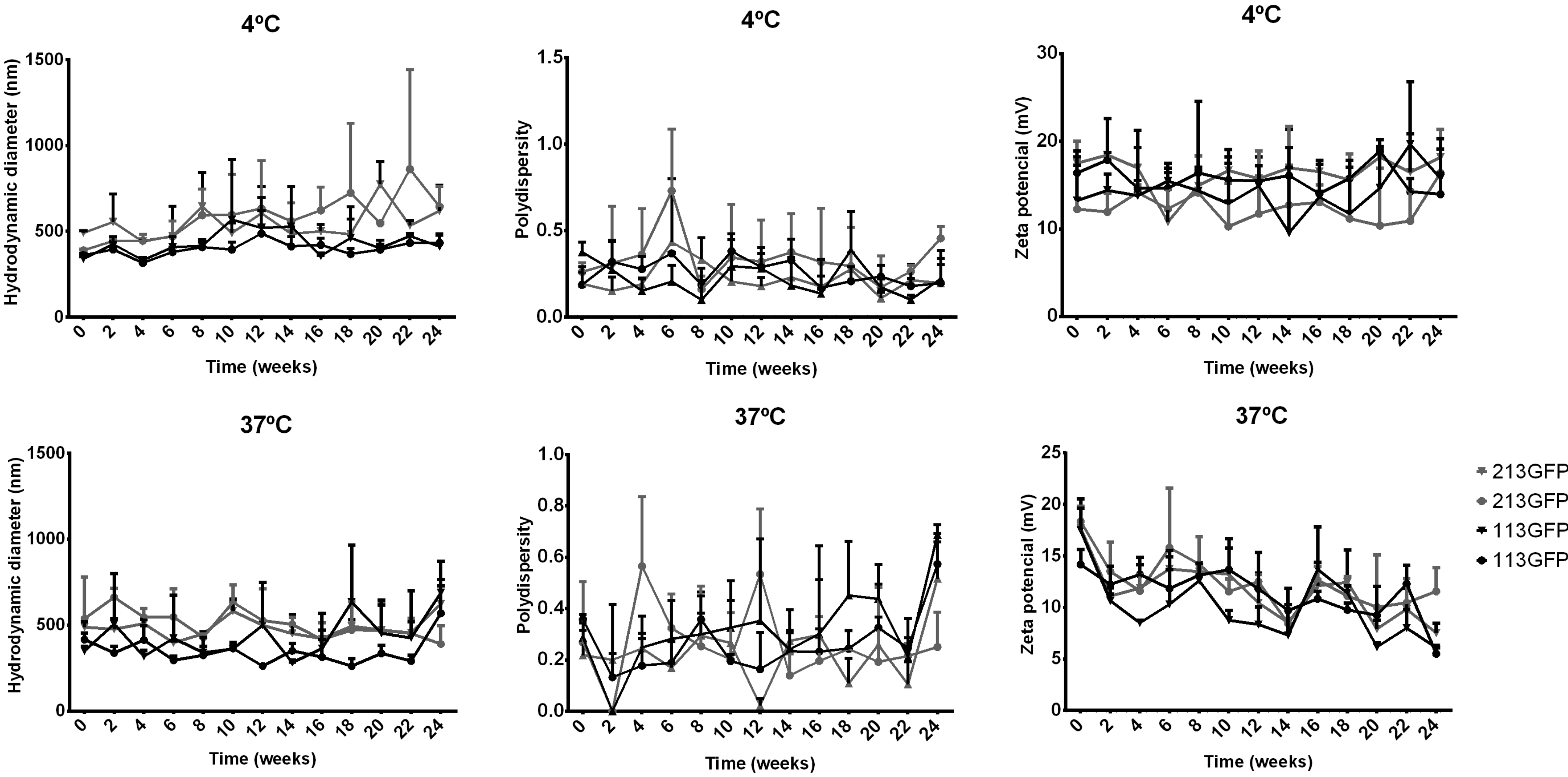

Figure 10.S1 -– Polyplex stability at 4 and 37ºC throughout time; size, polydispersity and zeta potential measurements over time for polyplexes with GFP coding plasmid.

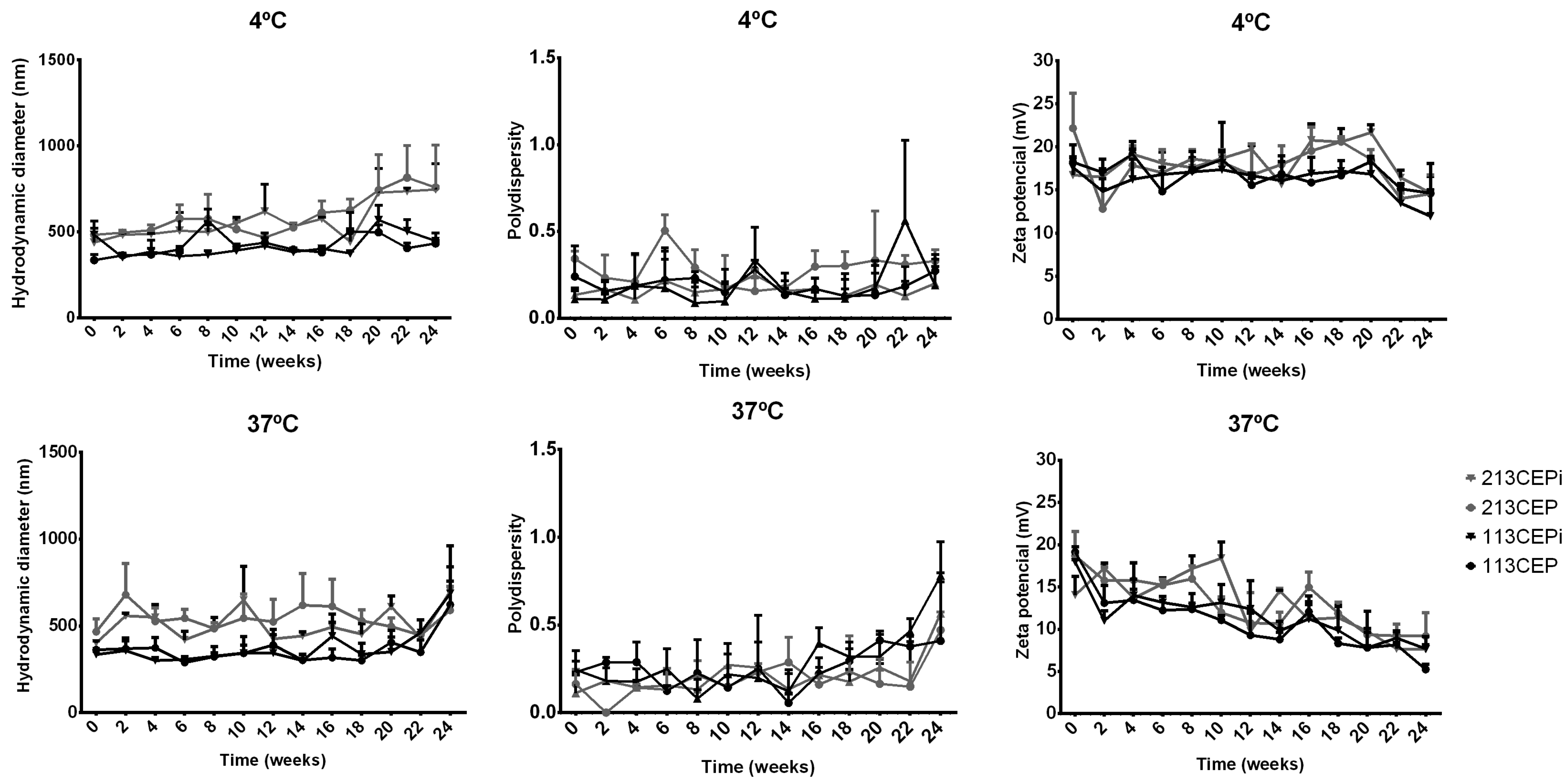

Figure 10.S2 – Polyplex stability at 4 and 37ºC throughout time; size, polydispersity and zeta potential measurements over time for polyplexes with CEP290 coding plasmid.

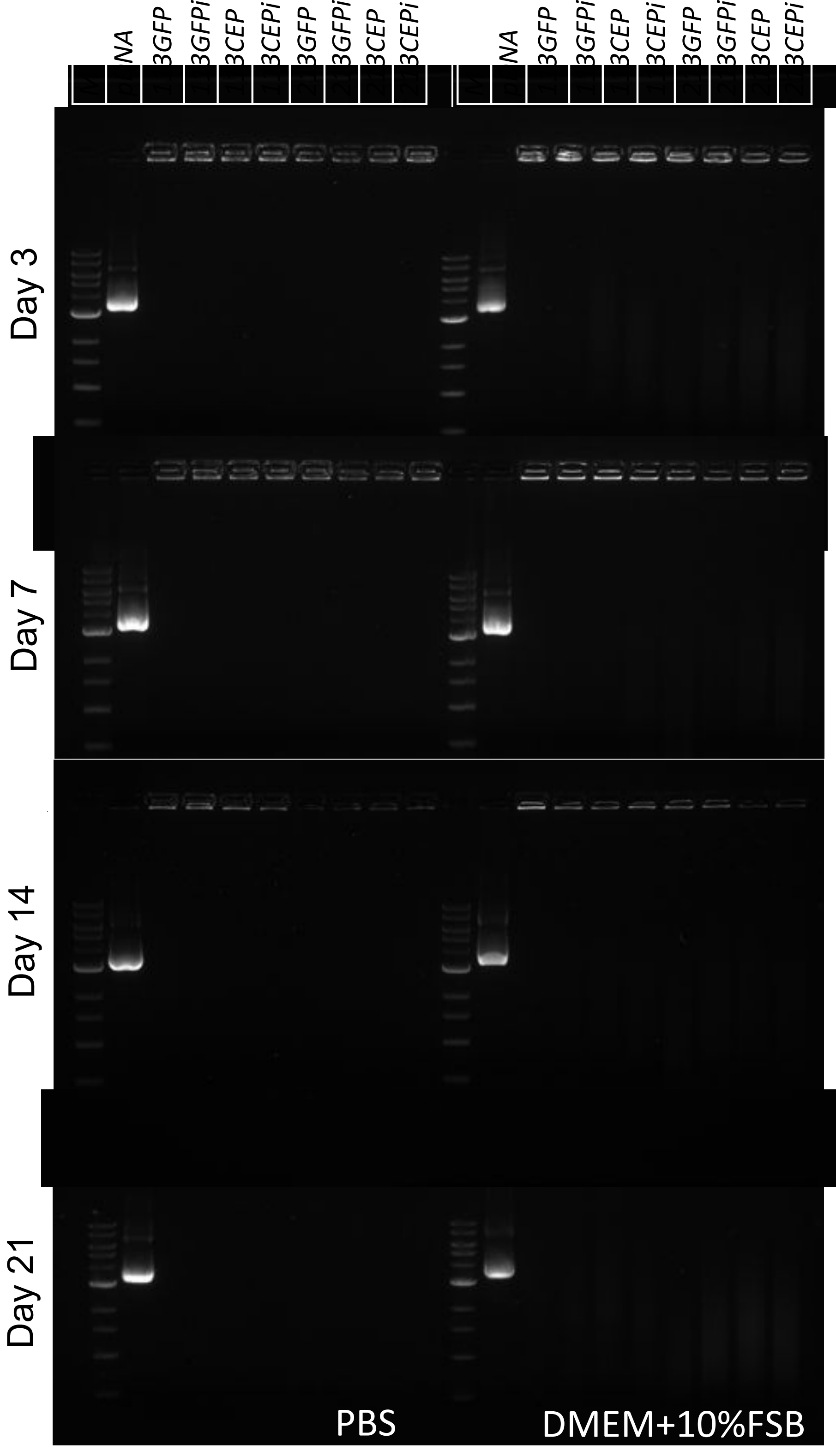

Figure 10.S3 - DNA complexation in CS polyplexes was analyzed by 1% agarose gel electrophoresis and DNA visualized with GreenSafe Premium after incubation with PBS or DMEM with 10% FBS.

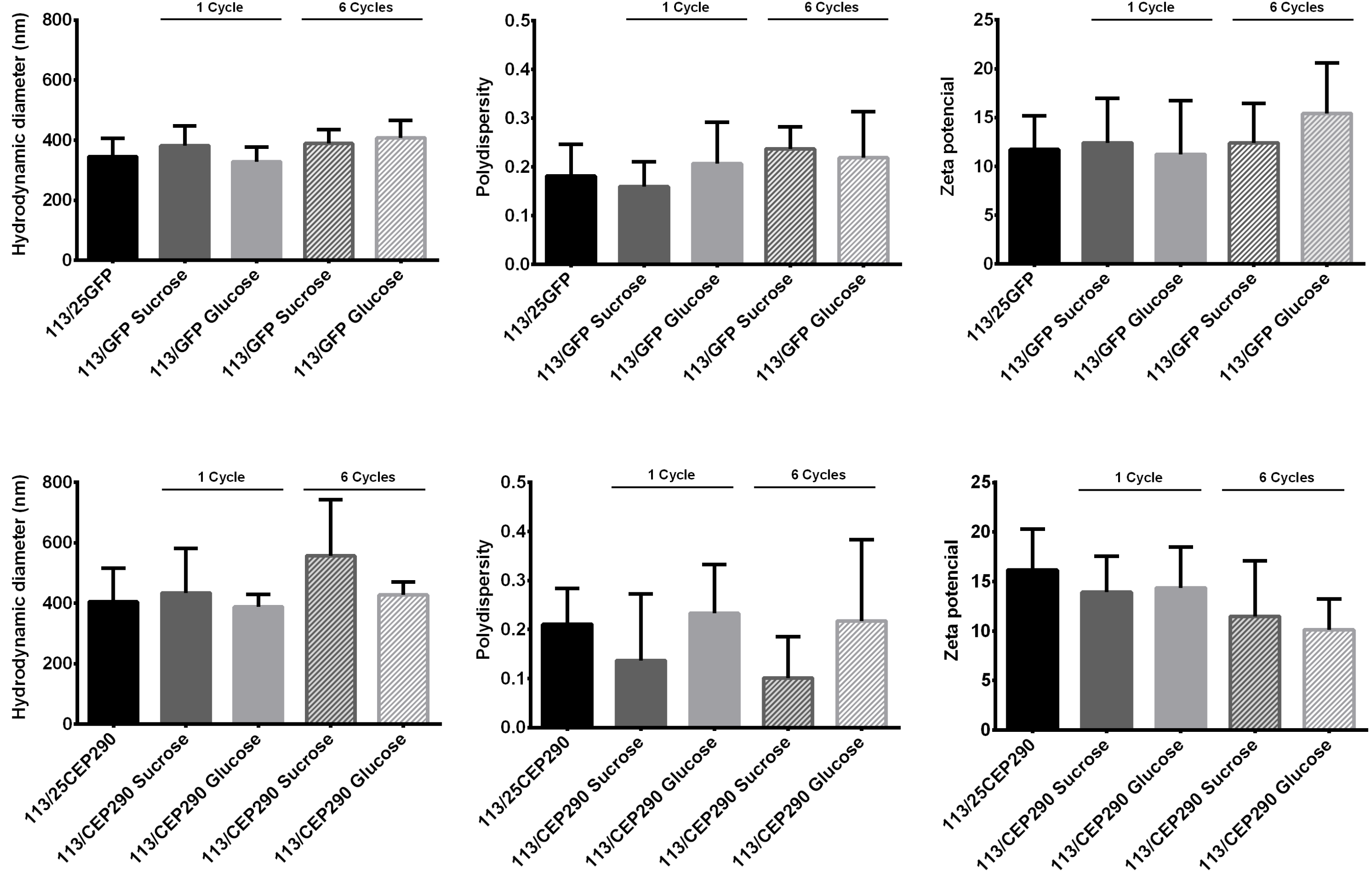

Figure 10.S4 – Polyplex stability evaluated by measuring their size, poydispersity and zeta potential after treatment with cryoprotectants (glucose or sucrose) and different freeze-thaw cycles. No statistical differences to the original formulation (113/GFP or 113/CEP290) were found.

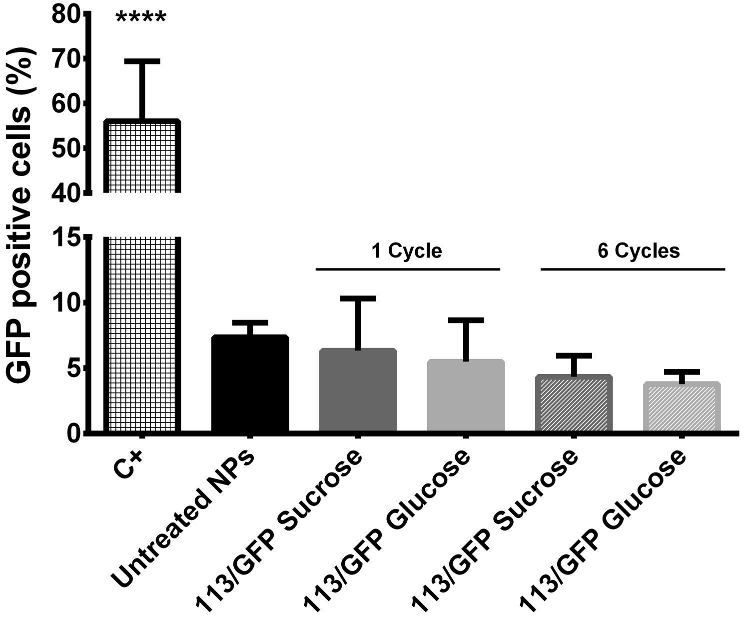

Figure 10.S5 - Transfection efficiency 72h post-transfection as percentage of GFP positive cells. 113/GFP polyplexes treated with cryoprotectants prior to transfection of HEK293 cells. As a positive control (C+) FuGENE HD transfected cells were used. Statistical differences are indicated by the star (*) symbol, with **** P < 0.0001.

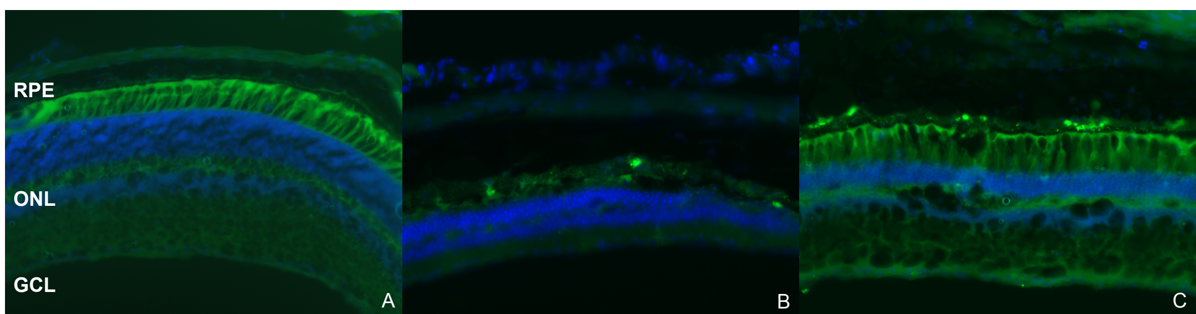

Figure 10.S6 - Representative images of fluorescence microscopy of cryosections of injected and uninjected C57BL6 mice. A) uninjected control eye, B) C1GFP injected eye and C) C1GFPi injected eye 12 months after subretinal injection (Amplification 200x, RPE-retinal pigmented epithelium, ONL-Outer nuclear layer, GCL-Ganglion cell layer.