Universidade de Aveiro Departamento de Engenharia Cerâmica e do Vidro
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Quitosano para vectorização de genes: do tráfego intracelular à expressão genética

Chitosan gene delivery: from intracellular trafficking to gene expression

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Biomédica - ramo Biomateriais, realizada sob a orientação científica da Dra. Ana Paula Pêgo, Investigadora Auxiliar do Instituto Engenharia Biomédica e co-orientação do Dr. Manuel Santos, Professor Associado do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT no âmbito do projecto
POCI/SAU-BMA/58170/2004

"Pedras no caminho?
Guardo todas.
Um dia vou construir um castelo."

Fernando Pessoa

o júri

presidente

Prof. Dr. Rui Nunes Correia
professor associado com agregação da Universidade de Aveiro (aposentado)

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agradecimentos

À Ana Paula Pêgo, em primeiro lugar pela oportunidade. Pelo optimismo, disponibilidade e apoio incansáveis. Pelo exemplo e orientação, pelo estímulo, pelo carinho e confiança. Muito, mas mesmo muito, obrigada ♥☺.
Ao Prof. Mário Barbosa, pelo acolhimento e apoio científico.
À Prof. Maria João Saraiva e Prof. Sérgio Simões pelo apoio científico.
Paula Sampaio, a pessoa responsável por aquilo que aprendi em confocal. O meu sincero obrigado.
À Maria Rosário Almeida e Elsa Leitão pela disponibilidade e apoio incansáveis no ensaio TNT, assim como na análise dos resultados.
À Cristina Barrias pela partilha de um marcador fluorescente e pelos conselhos para a preparação de amostras.
Isabel Amaral e Inês Gonçalves, obrigada pela ajuda na análise estatística.
À Cristina Ribeiro e Susana Sousa pelo vosso apoio nas análises de FT-IR.

NEURO TEAM foi um enorme prazer trabalhar convosco. Como o "team" é levado muito a sério, este trabalho é tão meu como vosso. Hugo: és um poço de ideias práticas e de experiência que gratuitamente partilhas. Obrigada pelos conselhos, pelas piadas☺, pelas discussões, pelo apoio generalizado: da microscopia, ao zetasizer. Carla, obrigada por partilhares a tua experiência, pelo teu esforço em me ensinares ser organizada, pelos desabafos, mas também pela boa onda e aquele humor!
A todos no INEB, pelo bom ambiente que paira nos laboratórios. Em especial à Sílvia ☼, pela disponibilidade e por aquele "Bom Dia Liliana" quando eu já estava "perdida" na escrita. Obrigada Pat pela tua eficiência a tratar das encomendas e as minhas dúvidas "técnicas"...e por tudo o resto! Dulce e Virgínia, obrigada e parabéns pela vossa eficiência. Ana Paula Filipe pelo carinho com que me recebeu. Sandrinha, Raquel ♫ Gonçalves, Meriem obrigada pelas vossas dicas. Manuela Brás, Eliana Vale, Susana Carrilho pelo apoio técnico. Prof. Fernando Jorge, Cristina Martins☺, Pedro Granja, Alis (a electroquímica tem menos de *salero* agora), Alejo, Sidónio, Rui, Sónia, Susana Guerreiro, Ana Lopes, Raquel Maia, Andreia, Judite, Keila... e a todos os que de alguma maneira (qualquer uma!) contribuíram para o sucesso deste trabalho – obrigada!
Aos que entretanto mudaram de rumo, mas ainda são parte desta cruzada: Ana Ribeiro, Andreia Cabral, Liliana Teixeira.
Suse, *grazie* por todos os "desenrasques", pela companhia e amizade.

Aos meus amigos: aos que estão longe, mas se fazem sempre sentir tão perto; aos que estão perto e são o meu refúgio e o meu "escape".
Toni obrigada pela paciência e por teres sempre aquela piada e aquele abraço.
À minha família (prrriiimas e tias!) pelo incentivo. À minha avó, pelas risadas que partilhámos.
À minha mana preferida, obrigada por me mimares tanto e pela tua infinita paciência para ouvires os meus desabafos sobre células, micoplasma, quitosano entre outras coisas "esquisitas".
Aos meus pais, porque a eles devo tudo o que sou. Pelo exemplo de luta, trabalho e de coragem que me fez levantar nos dias em que esta "batalha" se tornava demasiado difícil.

palavras-chave quitosano, biomateriais, nanopartículas, medicina regenerativa.

resumo

O quitosano é um policatião de origem natural que tem vindo a ser investigado como sistema não viral de vectorização de genes devido à sua biocompatibilidade e baixa toxicidade. No entanto, a sua baixa eficiência de transfecção tem dificultado o seu uso generalizado. Num estudo anterior mostrámos que a conjugação de resíduos de imidazol a cadeias de quitosano resulta numa melhoria da eficiência de transfecção do polímero. O principal objectivo deste estudo foi avaliar a aplicação de quitosano modificado com imidazol (CHimi) como vector para entrega de genes em medicina regenerativa, bem como encontrar novas vias para melhorar a eficiência.

A expressão genética mediada por CHimi com dois graus de substituição - 13% e 22% das aminas primárias do quitosano - foi avaliada em células 293T (células embrionárias humanas do epitélio do rim) por um período de 8 dias, usando o gene da β-Galactosidase (β-gal) como gene repórter. Os complexos de CHimi-DNA foram preparados numa razão molar de 18 entre aminas primárias e grupos fosfato. As células transfectadas com estes complexos apresentam um pico de actividade da β-gal às 72 horas pós-transfecção, verificando-se a expressão sustentada da proteína repórter durante todo o período de avaliação. Nestas condições a viabilidade celular não é comprometida. Quando se efectua um segundo tratamento das células com complexos à base de CHimi, a actividade de transfecção volta a aumentar, sem haver alterações na viabilidade celular. Verificou-se também que células transfectadas com estes vectores sobrevivem a um ciclo de congelação/descongelação, mantendo uma actividade de transfecção sustentada no tempo. Uma polietilenimina comercial (Escort V) foi usada como referência neste estudo. Apesar de os níveis de transfecção mediados por este vector serem duas ordens de grandeza mais elevados, a viabilidade celular decresce até aos 50% após cada tratamento.

De forma a investigar o processo de transfecção mediado por polímeros de CHimi, o tráfego intracelular destes complexos foi estudado por microscopia confocal de varrimento laser. Complexos de CHimi e DNA marcados com fluoróforos foram encontrados no citoplasma celular 2 horas depois da transfecção, sendo detectados até 48 horas pós-transfecção. Estes resultados podem em parte explicar a expressão sustentada de β-gal ao longo do tempo. Os complexos foram detectados no interior do núcleo 4 horas pós-transfecção. O DNA marcado com fluorescência não foi observado na forma livre em nenhum dos momentos analisados, enquanto que CHimi foi detectado num evento único no citoplasma. Num ensaio “cell-free” de transcrição/tradução *in vitro* não foi detectada a síntese de proteína quando o DNA estava complexado com CHimi, apesar de este ser expresso na ausência do polímero. Este conjunto de resultados sugere que, apesar dos complexos poderem ser encontrados no interior do núcleo rapidamente após a transfecção, a expressão genética parece depender da desintegração do complexo. O CHimi é um potencial candidato a vector para transporte de genes num cenário de regeneração. Este material medeia uma expressão proteica sustentada sem afectar a viabilidade celular. Com este sistema, as células toleram uma segunda adição de complexos, pelo que a administração repetida poderá ser potencialmente usada como estratégia para prolongar o efeito terapêutico de uma proteína de interesse. Em relação aos resultados de tráfego intracelular dos complexos, e considerando o perfil de expressão genética obtido, pode pôr-se a hipótese de que a expressão sustentada do gene resulta de um processo de libertação dependente do tempo. Neste sentido, ajustar a velocidade de degradação dos polímeros de CHimi pode ser usado como estratégia para melhorar o processo de expressão do gene tendo em vista o fim terapêutico pretendido.

keywords chitosan, biomaterials, nanoparticles, regenerative medicine.

abstract

Chitosan is a polycation of natural origin, emerging in the non-viral gene delivery vectors scene due to its biocompability and low cytotoxicity. However, its low transfection efficiency has hampered its wide application so far. We have previously shown that grafting imidazole moieties into the chitosan backbone results in improved transfection efficiency of this polymer. The main goal of this study was to assess the application of imidazole-grafted chitosan (CHimi) as gene delivery vector in a regenerative medicine scenario and to find avenues to further improve its efficiency.

Gene expression mediated by CHimi with two degrees of substitution - 13% and 22% of chitosan primary amines - was assessed in 293T cells for periods up to 8 days, using the β-Galactosidase (β-gal) gene as reporter gene. CHimi-DNA complexes were prepared at a primary amine to phosphate groups molar ratio of 18. Cells transfected with the CHimi-based complexes have a peak of β-gal activity 72 hours post-transfection and show a sustained β-gal production for 8 days. During this time period cell viability is not impaired. When a second treatment with CHimi-based complexes is performed, transfection activity increases, without changes on cell viability. Additionally, cells transfected with CHimi-based vectors are able to withstand a freeze/thawing cycle, maintaining a sustained transfection activity. A commercially available polyethylenimine (Escort V) was used as a reference. Though transfection levels are two orders of magnitude higher, cell viability decreases up to 50% after each treatment.

In order to investigate the transfection process mediated by CHimi-based vectors a study of the intracellular pathway of the complexes has been performed by confocal laser scanning microscopy. Complexes formed by fluorescently labeled CHimi and DNA were found inside the cell cytoplasm 2 hours after transfection and were detected up to 48 hours post-transfection. These results could explain in part the sustained gene expression over time. Complexes were detected inside cell nucleus since 4 hours post-transfection. Fluorescently labeled DNA in the free form was not observed at any of the time points analyzed. Free CHimi was detected in the cytoplasm in an atypical event. In a cell-free *in vitro* transcription/translation assay no protein production was detected when DNA was complexed with CHimi, though expressed when using plasmid DNA in the absence of CHimi. Taken together these results suggest that, though CHimi-based complexes can be detected inside cell nucleus promptly after transfection, gene expression is dependent on the complex disassembling.

CHimi is a potential candidate vector for gene delivery in a regenerative scenario. This material is able to mediate a sustained protein expression without impairing cell viability. In our system, cells can sustain another addition of the complexes suggesting that repeated administration could be used as a strategy to prolong the therapeutic effect. In view of the trafficking results and considering the gene expression profile, one can hypothesize that the observed sustained transgene expression is a time dependent release process. Thus, tuning the degradation rate of CHimi-based polymers could be a strategy to further improve the overall transgene expression process to fulfill the therapeutic end.

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LIST OF ABBREVIATIONS

β-gal – β-Galactosidase
AIDS – Acquired immunodeficiency syndrome
ATP – Adenosine triphosphate
BCA – Bicinchoninic acid
BSA – Bovine serum albumine
CHimi – Imidazole-grafted chitosan
CHimi1 – Chitosan with a degree of substitution of 5%
CHimi2 – Chitosan with a degree of substitution of 13%
$CHimi2_{ROX}$ – CHimi2 fluorescently labeled with rhodamine
CHimi3 – Chitosan with a degree of substitution of 22%
CLSM – Confocal laser scanning microscopy
CMV – Cytomegalovirus
DA – Degree of N-acetylation
DAPI – 4'-6-Diamidino-2-phenylindole
DMEM – Dulbecco's Modified Eagle's Medium
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
DNA_{Cy5} – DNA Fluorescently labeled with Cy5
DS – Degree of substitution
E. Coli – Escherichia coli
EDC – 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
FT-IR – Fourier Transform Infrared Spectroscopy
GFP – Green fluorescent protein
GPC – Gel permeation chromatography
MES – 2-(N-morpholino)ethanesulfonic acid
Mw – Average weight molecular weight
NPC – Nuclear pore complex
N/P – Amine to phosphate molar ratio
NHS – N-hydroxysuccinimide
NLS – Nuclear localization sequences
ONP – o-nitrophenyl-β-pyranoside
ONPG – ortho-nitrophenyl-β-D-galactopyranoside
PBS – Phosphate buffered saline
PDL – Poly(D-lysine)
PEG – Poly(ethylene glycol)
PEI – Poly(ethylenimine)

PLL – Poly(L-lysine)
PAMAM – Polyamidoamine dendrimers
PNS – Peripheral nervous system
pTTR – Plasmid encoding for TTR protein
RNA – Ribonucleic acid
RNAi – RNA interference
ROX – 5(6)-Carboxy-X-rhodamine N-succinimidyl ester
SCID – Severe combined immunodeficiency
SD – Standard deviation
siRNA – Short interfering RNA
SV40 – Simian Virus 40
TTR – Transthyretin

CHAPTER I – INTRODUCTION

1. GENERAL INTRODUCTION

Conceptually, gene therapy appeared in the early seventies [1]. The idea rapidly spread out due to the enormous potential in a vast range of applications. In a broad sense, gene therapy attempts to provide to the cells of a patient the genetic information required for producing a protein that in turn will have a therapeutic effect, to correct or modulate a disease [2]. The premise is the use of genes as pro-drugs to induce *in vivo* the production of therapeutic proteins [3], using the "patient's own cells as mini-bioreactors" [4]. This approach circumvents limitations of direct recombinant protein administration like low bioavailability, systemic toxicity or high cost of manufacturing [3].

In a first stage, gene therapy was thought to be applied to monogenic diseases, like cystic fibrosis [5], but with the culmination of the Human Genome Project and the development of highly sensitive techniques, new targets have been identified [6]. Gene therapy is currently studied as strategy to treat, or modulate diverse health disorders, such as cancer [7], AIDS [8] cardiovascular [9] or neurological diseases [10]. Additionally, gene therapy has been applied in a regenerative medicine scenario as a mean to induce the production of proteins, such as growth factors, to promote the repair and/or functional recovery of an injured tissue or organ, either as a therapeutic tool by itself or in combination with cellular therapies and/or tissue engineering [11]. RNAi gene therapy is a recent approach and is particularly in fashion since the Nobel Prize in Medicine for RNAi research of Fire and Mello in 2006 [12]. RNAi strategy differs from classical gene therapy scheme once it introduces short interfering RNA (siRNA) in order to inhibit a specific protein expression, instead of promoting it. Although it induces a transient effect, RNAi is very promising in the gene therapy field [13], namely in cancer therapies [14]. Indeed, siRNA is being tested in ten of the ongoing gene therapy clinical trials [15].

The first registered gene therapy clinical trial dates from 1989 and in 2006 more than a thousand have been performed [15]. Very optimistic reports were published [16]. However, in 1999 the first drawback to gene therapy put a chill on the high expectations. A patient involved in a clinical trial for ornithine transcarbamylase enzyme deficiency died after multiple organ failure due to intrahepatic infusion of an adenoviral vector [4]. Since then, gene therapy has had cyclically its ups and downs. Promising results published in the first years after the beginning of a French trial for X-linked several combined immunodeficiency (SCID) initiated in 1999 – the so called "bubble boy" disease – brought back the optimism [17, 18]. However, up to now, four of the 10 patients involved in the trial developed leukemia [19] and one of them died in the past year [20]. This adverse event is consequence of the random insertion of the retrovirus vector in the proximity of an oncogene that leads to an aberrant expression of the "therapeutic protein" – IL- *2Rγ* – and malignant cell expansion [21]. Another setback in gene therapy clinical trials was reported very recently with a patient involved in a trial for arthritis. The causes are under investigation, but suspicions point to an adverse reaction caused by a second injection of the adeno-associated virus, used as gene vehicle [22]. This would be the first fatality in a trial not studying a life-threatening disease.

The recurrent setbacks limited gene therapy progress and concerns about viral vectors safety were highlighted. Viruses were the first choice for the transport of genes due to their high efficiency. They are naturally gene-delivery vehicles and as sophisticated products of evolution they are very skillful [23]. Therefore, viral vectors represent 70% of the ongoing gene therapy clinical trials [15]. Some of the viral vectors systems tested in clinical trials will be briefly described during this review.
The tribulations occurred in the trials, the potential oncogenicity and high immunogenicity after repeated administration led to a revaluation of the use of viral vectors for therapy [20]. Although several efforts are being made in order to increase viruses' safety [24], significant expectations shifted to non-viral vectors. So far, the non-viral approach is used in approximately 30% of the gene therapy clinical trials, but the relative percentage is increasing over time [15].
The primary challenge for the non-viral approach is delivery [25]. Non-viral gene delivery systems are comparatively less efficient, what hampers its wide application [26]. However, important attempts are being made in order to close the gap [27]. A broad literature has been published in the last few years with different strategies and formulations for increasing non-viral gene delivery efficiency and target cell populations. This will be reviewed as well. However, the strategy to get better non-viral gene delivery systems seems to depend on a more detailed knowledge about the way vectors interact with cells and which are the rate-limiting steps for delivery. The design of efficient and functional non-viral systems relies on the better understanding of the cell barriers and the mechanism involved in the intracellular trafficking of these gene carriers [26].

The present work has been performed in the framework of a project that aims at the development of safe and effective biomaterial-based delivery systems of therapeutic genes to promote neuroregeneration in the peripheral nervous system (PNS). It is proposed the use of chitosan in the design of novel polycations to serve as carriers of DNA for specific delivery of genes to PNS cell populations. The basic concept behind the approach is the modification of chitosan, a natural polymer with known low cytotoxicity, in order to develop a gene carrier with higher affinity for nervous system cells, improved trafficking into the cell and into the nucleus, as well as, in the cell cytoplasm. One of the strategies that were explored was the improvement of endosome escape potential of the vector. It was proved that the introduction of imidazole groups in the chitosan backbone increased the transfection efficiency of chitosan by enhancing the endosomal escape of the vectors [28]. The main goal of the present work was to further assess the application of these materials as gene delivery vectors for application in a regenerative medicine scenario.
In view of the above, the strategy that was followed was to study *in vitro* the intracellular mechanisms occurring in imidazole-grafted chitosan mediated transfection as a way to further understand the action of these vectors and, ultimately find new avenues to improve gene delivery efficiency.

2. GENE DELIVERY SYSTEMS

The greatest hurdle to gene therapy application is the development of non-toxic and efficacious gene delivery systems. The ideal gene delivery vector should assist DNA transport to the nucleus of the target cell, leading to appropriate transfection efficiency (normally defined as the percentage of treated cells that express the therapeutic gene or transgene) and suitable "therapeutic protein" production. Additionally, it should guarantee low toxicity and immunogenicity, be biodegradable and stable [29].

Gene delivery systems are usually divided in two categories: recombinant virus and non-viral vectors.

2.1. Viral gene delivery

The use of recombinant viruses as vehicles to transport genes was inspired in their capability to deliver their genetic material in host nuclei to initiate expression of its own genome using host machinery. For therapeutic purposes, the transgene can be assembled in the viral genome and viruses' innate mechanism of infection will assist the transgene transport into the cell nucleus [29].

To construct a virus-based vector for gene delivery applications, genes encoding viral components essential for propagation should be separated or removed to prevent reconstruction by recombination into productive viral particles. These genetic components are replaced by the therapeutic gene [24].

Adeno-, retro-, lenti-, adeno-associated or herpes simplex viruses are examples of some engineered viruses for gene delivery applications that have been tested in gene therapy clinical trails (see Table 1). These viruses can be divided in two categories: integrating and non-integrating. In integrating vectors as retrovirus and adeno-associated virus a long-life expression of the therapeutic gene is expected, whereas non-integrating virus (adenovirus or herpes simplex virus) trigger a transient expression.

Retrovirus vectors were the first being developed and used in clinical trials due to its relatively simple and effective design. They tend to establish a chronic infection but are unable to infect non-dividing cells [24]. Lentiviruses are part of the retrovirus family, but as they rely on active transport of their genetic material into cell nucleus, they are able to infect also non-dividing cells [24]. The wide tropism (range of cells that can be productively infected by a virus) of lentiviruses can difficult cell targeting, but the unspecific integration in host genome is the most important disadvantage of the viruses from the retroviruses family.

The main challenge in adenovirus-mediated infection is how to make expression persist [30]. These are non-integrating viruses, but they are very efficient in a large variety of tissues. Additionally, most adults had already been exposed to adenovirus what could compromise a gene therapy strategy due to pre-existing immunity [30]. Even though, adenovirus vectors are the most used vectors in ongoing gene therapy clinical trials [15], due to their great potential [31].

As the name suggests, adeno-associated virus require helper virus (as adenovirus) to mediate productive infection. These vectors carry limited size DNA inserts, but have wide tropism, being able to infect both dividing and non-dividing cells. Although adeno-associated viruses are generally classified as integrating viruses, these vectors can also promote episomal gene expression [24].
Herpes simplex based vectors can not integrate in host genome, but the virus persists after primary infection in state of latency. An important advantage of herpes simplex vectors is their capability to accommodate large DNA inserts. Herpes simplex vectors show wide tropism, but *in vivo* they are neurotropic [10] and therefore, particularly promising for neuronal gene delivery [32]. Even though, in the actual clinical trials scene they are mainly tested in the treatment of cancer diseases [15].

Table 1: Examples of current gene therapy clinical trials using viral-vectors (information available at [15])

Virus-based vector	Disease	Category	Phase	Date Iniciated/approved	Administration route	Country	Total number
Adeno-associated virus	HIV infection	Infectious diseases	I	2003	intramuscular	Belgium	32
	Metastatic prostate cancer	Cancer diseases	III	2006	—	Netherlands	
	Cystic Fibrosis	Monogenic disease	I	2005	Intranasal	USA	
Adenovirus	Operable High grade glioma	Cancer diseases	I	2005	Intratumoral	Belgium	169
	Cystic Fibrosis	monogenic disease	I/II	—	Intrabronchial	France	
	Prostate cancer	Cancer diseases	III	2007	Intratumoral	USA	
	Head and neck cancer	Cancer diseases	II	1997	—	UK	
	End Stage Renal Disease	Cardiovascular diseases	III	2007	Perivascular collagen collar device	USA	
	Class II-IV stable angina	Cardiovascular diseases	II	2005	Intramyocardial	Belgium	
Herpes simplex virus	Colorectal cancer	Cancer diseases	II	2006	Intratumoral	USA	35
	Glioblastoma multiforme	Cancer diseases	I	2005	—	Germany	
	Glioblastoma	Cancer diseases	III	2006	Intratumoral	UK	
Retrovirus	Non-small cell lung cancer	Cancer diseases	I	—	—	China	146
	Severe combined immune deficiency due to adenosine aeaminase (ADA) deficiency	Monogenic disease	I/II	1999	*In vitro* - bone marrow transplantation	France	
	Metastatic melanoma	Cancer diseases	I/II	2003	Intravenous	Italy	
	HIV infection	Infectious diseases	I	1997	Intravenous	Australia	
	Malignant melanoma	Cancer diseases	I/II	—	*In vitro* - Subcutaneous	Scotland	
Lentivirus	HIV infection	Infectious diseases	I/II	2004	*In vitro* - Intravenous	USA	7
	CD 19+ Leukemia and Lymphoma	Cancer diseases	I	2006	*In vitro* - Intravenous	USA	

The high efficiency of viral vectors was the main impelling force for its widespread testing on clinical trials. Important developments were achieved over recent years [33], however the risk of insertional mutagenesis or immunological responses, and the problems that occurred in clinical trials held back viral gene transfer progress. While viral gene delivery research slowed down, non-viral gene delivery had a boost [34], towards the design of “artificial viruses” [35].

2.2. Non-viral gene delivery

Non-viral vectors were presented as an alternative to viral vectors to mediate the process of gene delivery. Nevertheless, despite the intensive research during the past decade, non-viral vectors still do not compare with viral vectors in terms of efficiency [36]. Therefore, the bulk of non-viral research is focused on improving vectors efficiency in the delivery of genes.

Non-viral gene delivery involves liposomal and polymeric vectors, as well as, direct DNA injection. The first case is a chemical approach in which lipids or polymers are used to complex and transport DNA into the cell. The direct DNA injection has been shown to lead to gene expression, but high levels of expression are limited to exposed tissues such as skin or muscle [37]. Naked DNA is unsuitable for systemic administration due to fast clearance by the mononuclear phagocyte system and degradation by serum nucleases that can eliminate DNA within 30 minutes [38]. Consequently, physical/mechanical methods are frequently applied in order to improve direct DNA delivery.

2.2.1. Physical/mechanical methods

Physical methods apply physical fields in order to compromise cell membrane integrity, inducing permeability to DNA molecules. Electroporation applies electric forces; sonication uses ultrasound waves; laser irradiation induces membrane permeability in the site of the beam impact by local thermal effect; magnetofection uses DNA-coated magnetic beads and an external magnetic field is applied in order to concentrate particles in the target cells [39]. Physical methods for gene transfer are normally well tolerated and no significant toxicity is reported [39]. However, its application seems to be limited to exposed or surgically exposed tissues [2] or for genetic modification *ex vivo*. The "physical approach" to gene delivery is relatively new and the poor efficiency or the lack of *in vivo* specificity, as well as the expensive equipment required, are issues still to be improved.
Microinjection and the gene gun are mechanical methods used to improve naked gene transfer. Microinjection uses the simpler approach: with a glass needle the genetic material is injected in the cell cytoplasm or inside the nucleus. Up to 100% of the recipient cells can be transfected with this method, but it is very meticulous and cells must be transfected one by one. Furthermore, this method is limited to *in vitro* or *ex vivo* applications [39]. With the gene gun, cells or tissues can be transfected by shooting accelerated gold particles coated with DNA. The major application of this method is DNA vaccination, where limited expression is needed in exposed tissues as skin and muscle [40]. The gene gun is being tested in a number of clinical trials [15], but the major barrier to its wide application is the shallow penetration of particles in biological tissues [41]. Even though, physical and mechanical methods have already demonstrated potential in gene transfer and whether independently, or in reasonable combination with other non-viral vectors, a place in gene delivery seems to be assured [39].

2.2.2. Liposomes

Cationic liposomes were introduced as gene delivery vectors by Felgner in 1987. He first used 2,3-bis(oleoyl)oxipropyl trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE) liposomes in order to assist DNA delivery into cells [42]. DOTMA is a cationic lipid with a hydrophobic moiety that ensures the assembly into bilayer vesicles and an amino head group to bind phosphate DNA groups (Figure 1). DOPE is a neutral lipid (Figure 2) typically called "helper"

lipid, because it increases gene delivery efficiency of cationic liposomes [43]. Cholesterol has been used as an alternative helper lipid [44]. The presence of DOPE in cationic liposomes formulations is thought to induce endosome destabilization, facilitating DNA escape from these vesicles and, ultimately, leading to an enhanced gene expression [45]. DOTMA/DOPE liposomes are nowadays commercially available as the transfection reagent, Lipofectin®.

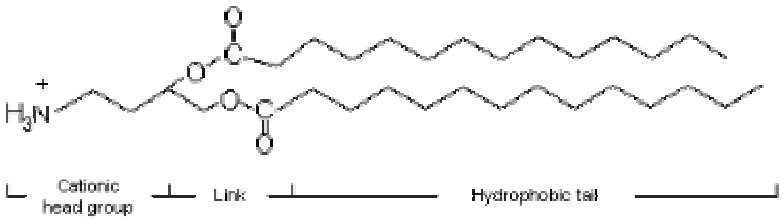

Figure 1: General structure of a cationic lipid.

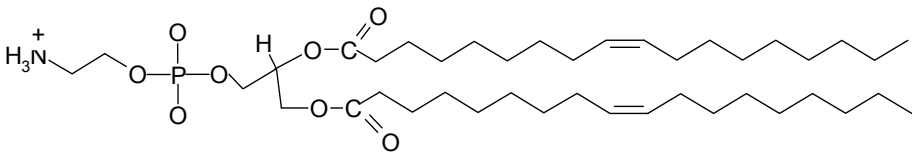

Figure 2: Example of a neutral lipid - DOPE.

In liposome-mediated gene delivery an electrostatic interaction is established between the permanently positive charge of the cationic lipid and the negatively charged DNA, making “transportable” condensed units – lipoplexes – able to be internalized by the cell. A positive charged lipoplex is essential to allow cell binding of the particles and subsequent endocytosis [46]. Based in Felgner strategy, several lipids were synthesized and used as liposomes for gene delivery (see Figure 3), such as DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), that efficiently protects DNA from serum DNases degradation [47] or multivalent cationic lipids (DOSPA – 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate – or DOGS – di-octadecylamidologycylspermine) that exhibit improved efficacy compared to monovalent lipids, but also higher toxicity [48].

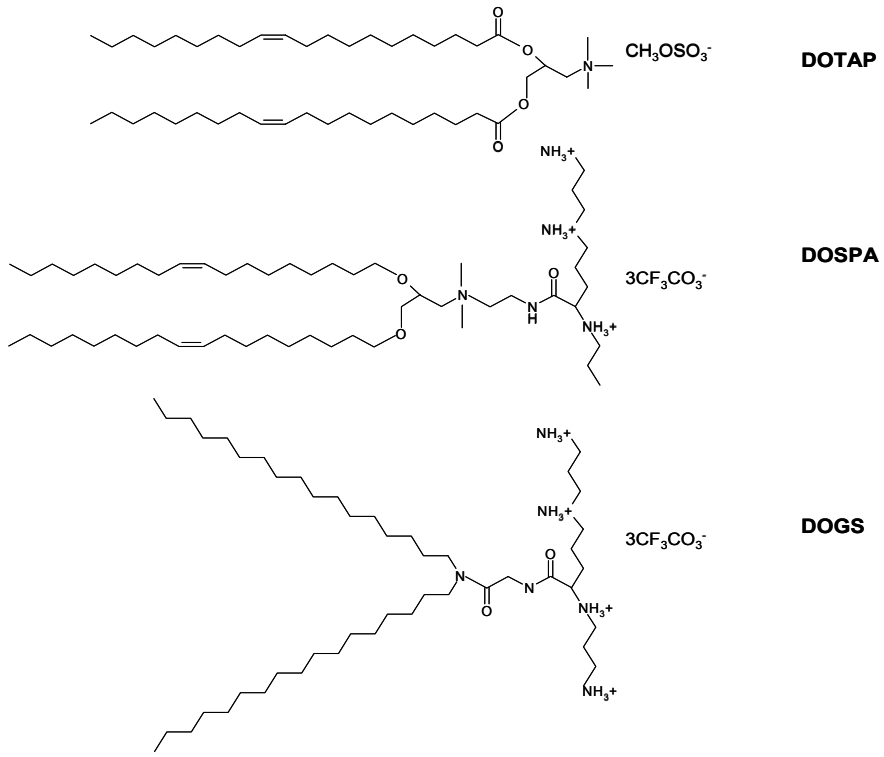

Figure 3: Example of other lipids used in gene delivery.

The clinical applications of cationic liposomes are mainly focused on cancer or cystic fibrosis [49] and represent about 8% of the gene therapy clinical trials [15]. However, cationic liposome-mediated gene delivery has some critical limitations, including the lack of reproducibility in the fabrication process, colloidal instability, significant toxicity after repeated administration [23], lack of targeting [40] and limited applicability by intravenous route due to impaired ability to go beyond the vasculature [49]. Aiming at circumvent these limitations, several authors are working on the improvement of biological properties of lipidic gene carriers. The incorporation of poly(ethylene glycol) (PEG) in the lipoplex structure intends to increase the particle circulating time and reduce unspecific interactions with serum components [44]. The binding of saccharides aims at enhancing gene delivery efficiency and also improve the lipoplex storage stability [44]. In a different approach, conjugation with transferrin was used to enhance gene expression by means of receptor-mediated endocytosis [50] and also to target transferrin-expressing tumor cells [51]. Formulations that expose cell-surface receptor binding peptides have also shown promising results on targeting upper airways epithelial cells [52].

2.2.3. Cationic polymers

As cationic lipids, polymers bearing groups which are protonated at physiological pH have been used as gene carriers. Cationic polymers can establish electrostatic interactions with DNA and form condensed particles – polyplexes – shielding DNA from nucleases activity.

The use of polymers shows important advantages for gene delivery. Polymers can be specifically tailored to a proposed application by choosing the appropriate molecular weight, controlling physicochemical properties and coupling cell specific target moieties [53].
Both synthetic and natural polymers have already been tested as gene carriers and will be reviewed in the next sections.

2.2.3.1. Synthetic polymers

Synthetic polymers as “off-the-shelf” materials form the basis for much of the non-viral gene delivery literature. However, these polymers face significant problems such as toxicity or limited biodegradability. Three of the major synthetic polymers used as gene carriers are described in the following paragraphs.

2.2.3.1.1. Poly(L-lysine)

Poly(L-lysine) (PLL) (Figure 4) was one of the first cationic polymers to be intensively studied as gene carrier. A large variety of molecular weights [54] and conjugation with specific ligands such as folate [55] or histidine [56] have already been tested. With an optimum ratio between PLL amino groups and DNA phosphate groups, small complexes can be produced, capable to enter the cell. However, these complexes tend to aggregate [2] and accumulate in the endosome. The end result is a rather high toxicity [57], also attributed to the low degradation rate of PLL [58].
Although several efforts has been made to overcome PLL-based vectors low transfection efficiency and cytotoxicity [57], in the actual scene of gene delivery it looks unlikely that PLL-based polyplexes will find some clinical application [23]. Its low transfection efficiency is attributed to the poor escape from endosomal vesicles, as will be described in more detail afterwards.

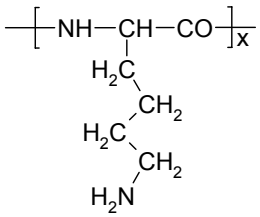

Figure 4: Poly(L-lysine) (PLL) chemical structure.

2.2.3.1.2. Poly(ethylenimine)

In 1995, Boussif and colleagues [59] introduced poly(ethylenimine) (PEI) as a potential gene carrier. Transfection experiments with this synthetic polymer were promising from the beginning, and PEI became the gold standard in non-viral gene delivery [60].

PEI can occur as branched (bPEI) or linear (lPEI) morphological isomers, depending on the linkage between the repeating ethylenimine units (Figure 5). The branched isoform contains primary, secondary and tertiary amines, each with the potential to be protonated. This results in a high positive charge density polymer, which can effectively buffer a wide pH range [61]. Moreover, bPEI can condense DNA in small complexes, protecting it from serum nucleases degradation [47]. However, the high density of positive charges that confers particular properties to bPEI results in a rather high toxicity, which is one of the major limiting factors for its *in vivo* use [53].

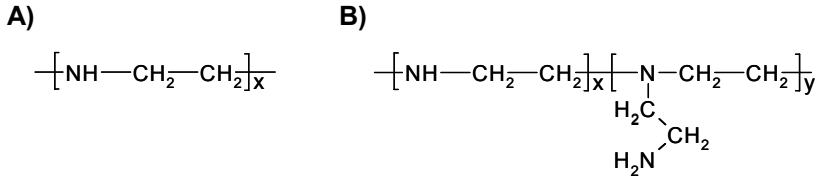

Figure 5: Chemical structure of poly(ethylenimine) (PEI). A) linear; B) branched

The linear isoform of PEI was introduced in gene delivery studies more recently and promising results were published. Some authors reported improved transfection efficiency and less toxic effects on lPEI-mediated gene delivery, comparing to bPEI [62, 63]. However, for these materials – lPEI and bPEI – cytotoxicity and transfection activity seem to be dependent on the molecular weight [62, 64], the degree of branching of the polymer [63] as well as some experimental conditions, like DNA compactation [59] or the cell type studied [65].

Despite the superior transfection efficiency mediated by PEI-based vectors, the high positive charge hampers cell targeting on account of several unspecific interactions. To overcome this lack of targeting ability of PEI-based vectors some authors are working to achieve specificity by conjugation with specific ligands like transferrin (in order to target tumors) [66] or epidermal growth factor (to enhance uptake by epithelial cells) while masking the charge surface in PEG-shielded particles [67].

One auspicious strategy recently developed is to make PEI-based vectors biodegradable as mean to reduce the toxicity, while maintaining the high transfection efficiency. Possible strategies are crosslinking low molecular weight PEIs [68] or to link PEI with β-cyclodextrin [69]. The degradation products are thought to be easier eliminated by the cell [69]

2.2.3.1.3. Dendrimers

Chemistry similar to that described for PEI could be found in dendrimers. Starburst® polyamidoamine dendrimers (PAMAM) are synthetic and highly branched, spherical polymers with a large number of amines in the perimeter of the molecule (Figure 6). The size and surface charge are controlled by varying the number of synthetic steps (“generations”) and cytotoxicity seems to be dependent on these parameters [70]. Dendritic polymers gained popularity because of their versatility and simplicity in transfection [40]. These cationic polymers mediate relatively high

transfection efficiency in a wide variety of cell lines, including primary cells that are usually more difficult to transfect [71]. Introducing arginine residues to the dendritic surfaces increases gene delivery efficiency, comparing with that of native PAMAM [72].

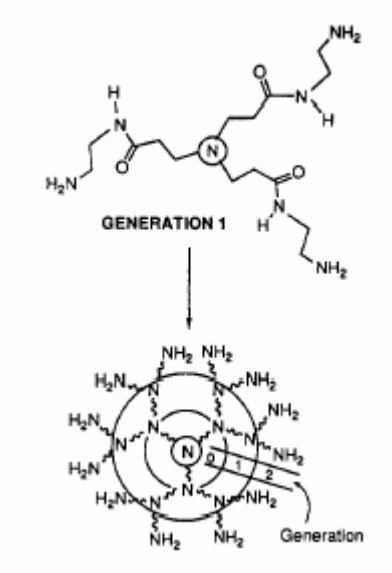

Figure 6: Synthesis scheme of PAMAM cascade dendrimers (adapted from [70]).

Recent advances on dendrimers research include a better understanding on the role of dendrimers chemistry *in vivo* and the development of biodegradable chemistries (see [73] for a review).
There is no agreement regarding dendrimers toxicity. Several authors did not ascribe toxicity to PAMAM [71, 74], but some damaging effect seems to occur, especially in the case of dendrimers with higher degrees of positive charges on the surface, like the ones resulting from the grafting of arginine moieties [72]. Issues related to the toxicity associated with the positive charge of dendrimers must be solved to introduce such systems in the clinic [73].

2.2.3.2. Natural polymers

There are only a small number of cationic polymers of natural origin available. Nevertheless, natural polymers present striking properties for gene delivery applications such as biocompability and minimal cytotoxicity. Chitosan is one of the most studied naturally derived polymeric gene carriers.

2.2.3.2.1. Chitosan

Chitosan is an aminopolysaccharide poly [β-(1–4)-2-amino-2-deoxy-D-glucopyranose] comprised of N-acetyl glucosamine and glucosamine units (Figure 7). This polymer is biodegradable, biocompatible, cheap, non-toxic and tightly condenses DNA, forming polyelectrolyte complexes

suitable for gene delivery [75]. Reduced amounts of chitosan can be found in some fungi. However chitosan major source is obtained by alkaline deacetylation of quitin, naturally occurring in crustacean shells, squids or insects.

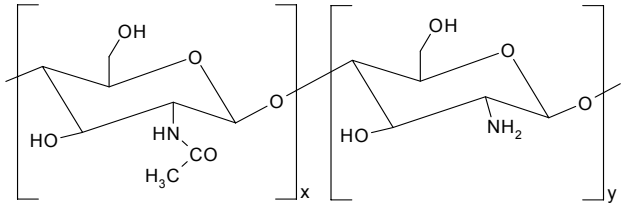

Figure 7: Chemical structure of chitosan constituted by x) repeating units of N-acetyl-D-glucosamine and y) repeating units of D-glucosamine.

Chitin can be distinguished from its deacetylated derivative chitosan by the latter solubility in dilute aqueous acid solutions. Solubility of chitosan depends on its degree of acetylation (DA) defined as the molar percentage of acetyl units per mol of chitosan [76]. β-(1–4) linkages between D-glucosamine residues of chitosan are specifically cleaved by chitosanase, produced by some bacteria. However, chitosan can also be hydrolyzed by several other enzymes such as lysozyme, pectinase, cellulases, hemicellulases, lipases, and amylases, among others [77]. The *in vivo* degradation of chitosan was soon taken as an interesting property for several applications, because biodegradability is critically related to safety issues [78].

Mainly due to its high biocompatibility, chitosan has been proposed as an alternative to other non-viral vectors, however the transfection activity is rather low comparing with commercial available liposomes [79-81] or PEI [82, 83]. Nevertheless, there is a broad interest on chitosan-based vectors, and several efforts are being made towards improving chitosan transfection efficiency. Many strategies have been tested as hydrophobic modification with 5β-cholanic acid [84], chitosan thiolation [85], grafting with PEI to increase transfection while maintaining the low toxicity [82] or binding specific ligands, like transferrin [81], lactose [79] or folate [86] to enhance cell uptake. The transfection experiments with chitosan are strongly influenced by the DA [80] and molecular weight of the polymer, as well as by the stoichiometry of the chitosan–DNA complex, serum concentration and pH of the transfection medium [87].

2.2.3.2.2. Imidazole-grafted chitosan

Grafting imidazole moieties to a polymeric backbone has been first proposed for PLL as a mean to improve the transfection efficiency of these vectors [56]. It was suggested that due to its pKa (~6) imidazole could increase the polymer buffering capacity in the endosomal/lysosomal pH range, improving vectors escape from endosomal vesicles. Furthermore, some authors proposed that once imidazole is part of histidine aminoacid it could induce better biocompatibility of these systems [88].

In a previous study, we showed that grafting imidazole moieties to a chitosan backbone (Figure 8) results in the improvement of the transfection efficiency mediated by this polymer [28]. Three degrees of substitution with imidazole were studied: 5% (CHimi1), 13% (CHimi2) and 22% (CHimi3), at various N/P molar ratios (ratio between primary amine groups of chitosan to phosphate groups of DNA). Transfection efficiency of imidazole-grafted chitosan (CHimi) was tested in 293T cells. It was shown that the transfection efficiency increases up to an N/P molar ratio of 18, being higher in CHimi2 and CHimi3 mediated transfection. In terms of particle size, zeta potential or DNA protection there is no significant differences between imidazole-grafted and the unmodified chitosan at the higher N/P molar ratios, suggesting that the increase in transfection efficiency is caused by the incorporation of imidazole moieties. Additionally, no toxic effects were attributed to CHimi based polymers [28]. Therefore, as CHimi-mediated transfection shows great potential for gene delivery, a more detailed study on the mechanisms underlying the gene delivery promoted by these new vectors is required.

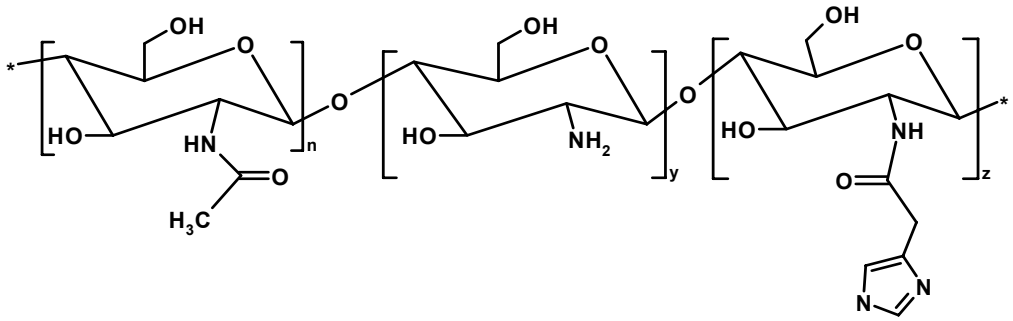

Figure 8: Chemical structure of imidazole-grafted chitosan constituted by (n) units of N-acetylated monomer, (y) units of the deacetylated monomer and (z) units of the imidazole-grafted monomer.

From mechanical strategies to tailored polymers, several efforts are being made in order to create efficient strategies to deliver DNA into the cell nucleus. However, non-viral gene delivery vectors have still a long way to go in order to get closer to viruses efficiency. The first approach to improve transfection efficiency was essentially focused on testing different vector compositions. Some of these strategies were reviewed in the previous paragraphs. More recently, many authors turned their attention to the understanding of the molecular mechanisms underlying transfection and the interactions of non-viral vectors with cells. The premise is that the future non-viral systems design will probably benefit from the attained *know-how* in such studies.

3. INTRACELLULAR TRAFFICKING

As discussed in the previous paragraphs, liposomes and cationic polymers are currently being explored as carriers to transport genes into the nucleus of cells. In this process they must overcome several biological barriers toward an efficient non-viral gene delivery (Figure 9). These barriers include binding to the cell surface and cross the membrane, escaping lysosomal degradation, and get into the nucleus [89]. The understanding of these barriers is critical for the development of more effective gene vehicles [90].

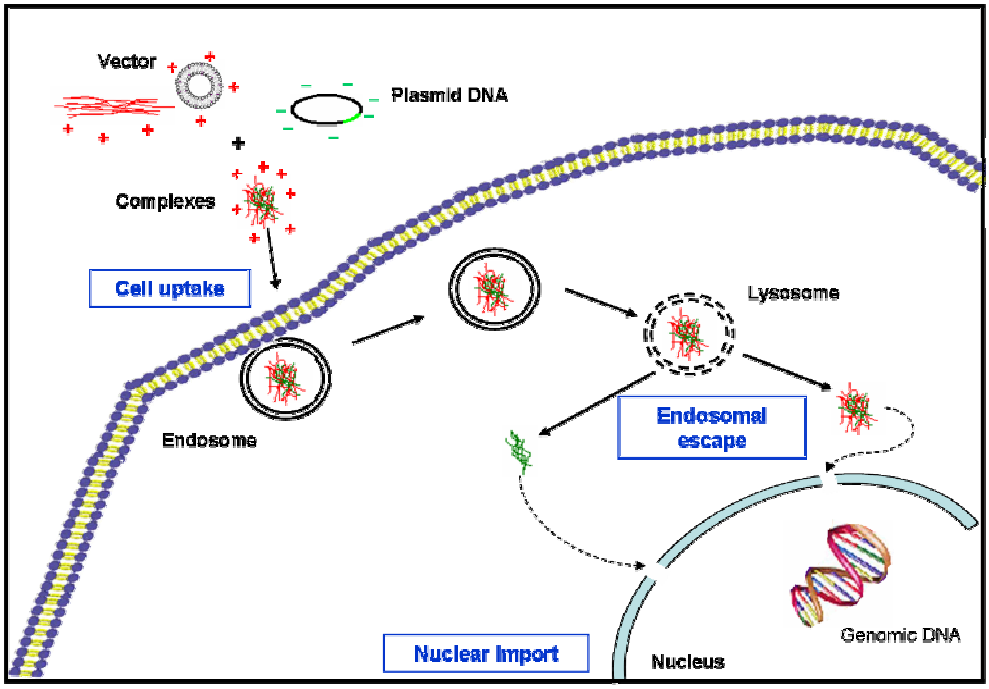

Figure 9: Biological barriers to gene delivery.

3.1. Cellular uptake

The cell membrane is the first barrier for non-viral gene delivery. In the absence of a carrier molecule, the highly anionic nature of DNA hinders its passive diffusion through the hydrophobic cell membrane. Association of DNA with cationic lipids or polymers greatly reduces the net negative charge of the particles, increasing the cellular uptake [36]. Even though, most lipoplexes and polyplexes cannot readily cross the cell membrane due to their large size and, in case of polyplexes, also due to the hydrophilic nature. In a first stage, DNA-carrier complexes interact with glycosaminoglycans or specific cell receptors on cell membrane and are thereafter internalized into endosomal vesicles [91]. Endocytosis has been established as the main mechanism for non-viral vectors internalization [26].

Complex physico-chemical properties, as size [92, 93], or the surface charge density determine the interactions with glycosaminoglycans presented on the cell surface. Ultimately, these properties are critical in the uptake process as well as in the overall transfection efficiency [87]. Several studies have been performed in order to correlate physico-chemical properties and polyplexes uptake. Some authors established a size requirement below 100 nm [94] to allow endocytosis of DNA-polymer complexes. However, this is not a well defined boundary, since nanoparticles with a mean size of 150-300 nm are also assumed as suitable for gene delivery [95]. Conversely, some authors suggested that particle size is not a restriction for cellular uptake [87], since Ishii and co-workers obtained good transfection results with 5-8 μm chitosan-DNA complexes [96]. It is important to note that it could be difficult to establish a maximum size for complexes uptake by cells because DNA-polymer suspensions are often polydisperse suspensions. Furthermore, some issues about endocytosis are yet to be fully understood [97] what could explain this apparent lack of agreement.

3.1.1. Endocytic pathways

The three main endocytic pathways discussed in this review are clathrin-mediated endocytosis, caveolar-mediated endocytosis and macropinocytosis (Figure 10).

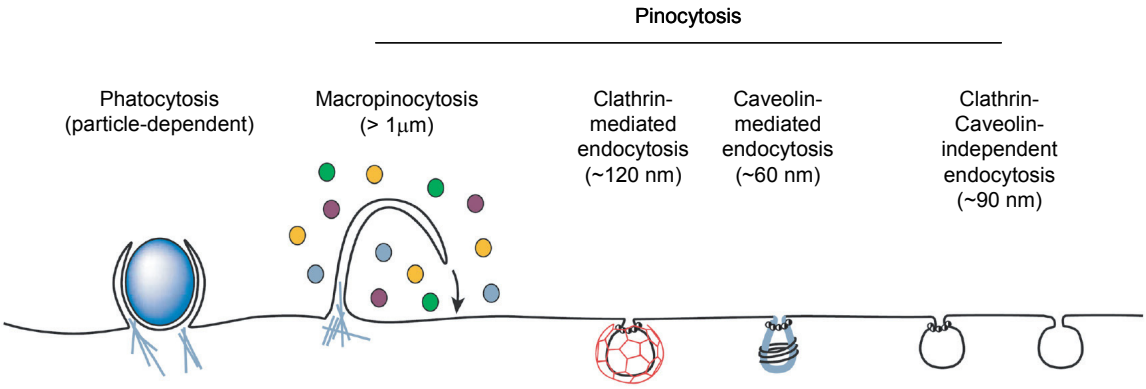

Figure 10: Multiple portals of entry in mammalian cells (adapted from [98]).

Clathrin-mediated endocytosis is the best characterized and the most common pathway. It occurs constitutively in all mammalian cells being responsible for the uptake of several essential nutrients. For a long period it was referred as “receptor-mediated endocytosis”, but is now clear that the title was unsuitably applied, because other pinocytic pathways also involve receptor-ligand interactions [98, 99]. In clathrin-mediated endocytosis the internalized material follows an endolysosomal pathway, pursuing from early to late endosomes, and ultimately ending in the lysosomal compartment. The early endosome represents the first stage to which clathrin-coated vesicles deliver their content. The early endosomes traverse to the perinuclear cytoplasm on microtubules tracks, where they fuse with late endosomes or lysosomes. Along this route, the pH drops from 6.0-

6.8 in early endosomes to approximately 5 in lysosomes [100]. This pH reduction combined with high concentration of lysosomal enzymes aims at degrading the internalized material [100].

In caveolar-mediated endocytosis, particles are internalized in primary endocytic vesicles that fuse, to larger, more complex tubular membrane organelles – "caveosomes" – which maintain a neutral pH [101]. These organelles are transported through microtubules to the perinuclear region and their content is delivered in the endoplasmatic reticulum [101]. It has been described that the Simian Virus 40 (SV40) can activate a signaling cascade to activate caveolae-localized surface receptors and trigger its own uptake [101]. Calveolar-mediated endocytosis of DNA-carrier complexes is still extremely unexplored and some authors believe that it is unlikely to significantly contribute to constitutive endocytosis, because caveolar vesicles are slowly internalized [26] and generally considered small in size [26, 98]. However, it is important to notice that caveolae constitutes 10-20% of cell surface in endothelial cells [98], where this endocytic pathway could be a relevant portal to enter into the cell [26].

Macropinocytosis has been recognized as an alternative endocytic pathway for some time, but its relevance remained elusive in many cellular processes [102], as in polyplexes uptake. Macropinosomes are formed by actin-driven ruffling of plasma membrane which is dependent on membrane cholesterol content [103]. The fusion between macropinosomes and lysosomes occurs only in macrophages [102]. However, some reports suggest that in non-phagocytic cells macropinosomes can also acidify utilizing a vacuolar-type H^+-ATPase and electrogenic Cl^- shunt mechanism, similar to the one described for endosomes [104]. Furthermore, it was reported that the pH reduction is slighter than in late endosomes or lysosomes [92]. Macropinocytosis has been taken into account in gene delivery studies very recently [92, 93, 104], and its specificities may compromise some endosomal escape strategies, as will be pointed out in the section 3.2.

A recent study from Rejman and co-workers with latex beads intended to differentiate the endocytic pathway of particles according to their diameter. They found out that particles up to 200 nm are internalized in clathrin-coated pits, whereas bigger particles enter non-phagocytic cells by macropinocytosis or caveolar mediated endocytosis [93]. This was a very surprising result because caveolar vesicles typically appear as rounded plasma membrane invaginations of 50±80 nm [98, 101]. However the most striking result of this study is to point out macropinocytosis as an alternative pathway to mediate bigger particles uptake in non-phagocytic cells. This issue can help to explain Ishii and colleagues results [96] and challenge the concept that the optimal vector should form small complexes with plasmid DNA [88, 94, 95].
Another remarkable point that should be considered when analyzing endocytosis and its role on gene delivery is the cell cycle. The membrane tension varies in the different phases of the cell cycle. During mitosis, it significantly increases, inhibiting deformation required for invagination [105] and therefore limiting the internalization of particles by endocytic pathways.

3.1.2. Receptor-mediated endocytosis to enhance uptake

Grafting specific ligands to target a specific cell population can also improve transfection efficiency by promoting cellular uptake via receptor-mediated endocytosis. Transferrin-conjugated polymers are thought to be internalized via transferrin receptor that is overexpressed in cells with high metabolic activity. Mao and colleagues developed three schemes to conjugate transferrin with chitosan vectors and they achieved a three-fold increase in transfection in human embryonic kidney 293 cells [81]. In contrast, Simões and colleagues showed that transferrin-conjugated lipoplexes may enter cells even in the presence of free transferrin, what suggests that conjugation of transferrin is not increasing complexes uptake [50]. The authors proposed that the large size of the conjugate can hinder the interaction ligand-receptor, impairing endocytosis via transferrin-receptor. Other ligands such as integrins [106], sacharides [79] or growth factors [107] have also potential to enhance gene delivery efficiency by means of receptor-mediated endocytosis. However, the success of a targeting strategy depends on the conjugation chemistry, the length of spacer between ligand and complex, the ligand-receptor binding strength, and the number of targeting ligands per complex [23]. Additionally, targeting can also be hampered in high positive charge density particles due to unspecific interactions [66].
Sometimes, conjugation of specific ligands gets irreproducible results, so the efficient cell-specific targeting requires careful optimization of the various parameters that affect cell-surface binding [23]. When the strategy is to promote receptor-mediated endocytosis it is important to keep in mind that the success of targeting and the transfection upgrading will primordially depend on the finite number of receptors available on cell surface for binding and internalization [36].

3.2. Endosomal escape

For long time authors believed that all the endocyted complexes followed the endolysosomal pathway, but with recent considerations about macropinocytosis and caveolar-mediated endocytosis [92, 93] new concerns arose. Even though, after cell uptake via any endocytic pathway, the complexes are limited to endocytic vesicles with no access to the cytosol or to the nucleus. The escape from this vesicles is critical for efficient transfection [26], since cellular uptake does not seem to be the limiting step for either polyplexes or lipoplexes-mediated gene delivery [91]. Therefore, several authors are focused on developing strategies to promote endosomal escape of the complexes and they are mainly taken into consideration the endolysosomal pathway.

3.2.1. The flip-flop mechanism

There are some proposed mechanisms for endosomal escape in lipidic carriers. The most accepted is probably the flip-flop model proposed by Xu and co-workers [108]. They propounded

that, after being internalized by the cell, lipid-DNA complexes initiate destabilization of the endosome membrane what results in flip-flop of anionic lipids on the cytoplasmatic face of the membrane. The anionic lipids laterally diffuse into the complex and form charge-neutralized pairs with the cationic lipids. Ultimately, the membrane is destabilized and the DNA released into the cytoplasm [108].

Alternative theories suggest that DNA can be released from the lipoplex due to charge neutralization with anionic macromolecules, or membrane destabilization by pH-sensitive lipids like DOPE [109].

3.2.2. The proton sponge effect

For polyplexes the endosomal escape discussion is mainly focused on works based on the gold standard bPEI. The high transfection efficiency of this polymer is attributed to its buffering capacity within the physiological pH range. According with the so-called “proton-sponge” hypothesis first proposed by Behr [110], cationic polymers with the ability to buffer endosomal pH induce osmotic swelling of endosomes due to excessive proton and chloride accumulation with secondary water movement (see Figure 11). Buffering capacity in the endosomal pH range has also been proven in poliamidoamine dendrimers. In contrast, no chloride accumulation was detected with PLL [104]. The amino groups of PLL have a pKa around 10 and are all protonated at physiological pH what explains the lack of buffering capacity of this polymer. PLL-DNA complexes are sequestered for several hours in endosomal vesicles what makes endosomal escape the main limiting factor in PLL-driven gene delivery [56].

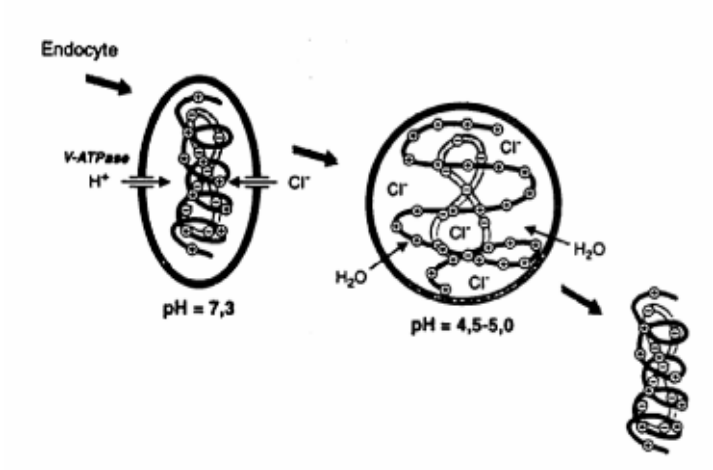

Figure 11: The proton sponge effect (adapted from [111]).

Chitosan has limited buffering capacity in the endosomal acidic pH range 4.5-5.5 [81, 112]. The pKa of chitosan primary amino groups is around 6.3, what limits its buffering capacity in more acidic pH [112]. Studies with bafilomycin A1 – a specific endosomal proton pump inhibitor - report no influence in chitosan mediated transfection, suggesting that this polymer could not induce a proton sponge effect [83]. According to this concept and because the cellular toxicity and side

effects of lysosomotropic agents like chloroquine make it impractical for *in vivo* gene delivery some authors are focused in designing new vectors with buffering capacity [88].

Despite the widespread acceptance of the proton sponge hypothesis, there are some reports that challenge this theory. Godbey and co-workers found no lysosomal involvement in PEI-DNA complexes routing to the nucleus [113]. Their results are based on confocal images through time of fluorescent-labeled lysosomes and complexes. PEI-DNA complexes have been found in the nucleus 4 hours post-transfection [114] and no changes were detected in lysosomal pH [113]. However, in Behr proton sponge hypothesis, PEI-induced osmotic rupture occurs in endosomes, previous to fusion with lysosomes, as explained by Akinc and colleagues, who further confirmed Behr's theory [115]. Also in disagreement with Godbey and colleagues, fluorescent and electron microscopy results obtained by Bieber demonstrate that PEI-DNA complexes accumulate in lysosomal compartment [116]. Nevertheless they also challenge the endolysosomal swelling and suggest that PEI-DNA complexes could induce enough local membrane damage to trigger the release of the complexes into the cytoplasm [116]. In accordance with both theories, some research with linear PEI (lPEI) showed that smaller complexes could escape rapidly from lysosomes via proton sponge mechanism, whereas larger complexes reach the cytoplasm via membrane damage [62]. To all this discussion we can add the recent report of Rejman and colleagues [117] that suggests that PEI-mediated transfection occurs after caveolar-mediated endocytosis of PEI-DNA complexes. This report indirectly confronted the proton sponge effect and its relevance for PEI transfection efficiency, because caveolar vesicles have neutral pH and do not fuse with late endosomes [101].
It is now clear that there is a broad lack of accordance in literature about cell transfection mechanisms. A clear explanation can only be achieved with standardized protocols as well as a deeper knowledge about specific features of each endocytic pathway and the specificity of each inhibitor recurrently used in this kind of studies.

3.2.3. Imidazole containing polymers

As referred in section 2.2.3.2.2, imidazole group of histidine has a pKa around 6.0 and turns cationic in a slightly acidic medium (like in endosomal vesicles). Therefore, it owns some buffering capacity in the endosomal pH range and could induce vesicular escape of complexes based on imidazole containing polymers by the "proton sponge" mechanism [53]. Midoux and co-workers describe significant improvement in PLL-mediated transfection efficiency after grafting histidine to the polymer [56]. Complexes based on histidylated PLL were smaller, had lower surface charge, reducing non-specific interactions [118] and enhanced cell viability [119].
The homopolymer of histidine has also been tested for gene delivery. However, since polyhistidine is insoluble in aqueous solution at pH> 6, it was conjugated with gluconic acid to impart solubility at physiologic pH [88]. This conjugated polymer showed moderate transfection ability; still, the study

confirms the advantage of imidazole containing polymers as an initial basis for construction of biocompatible gene delivery vehicles [88].

A similar approach is found with urocanic acid-grafted polymers. Urocanic acid, bearing an imidazole ring, was grafted to water soluble chitosan as mean to improve endossomal escape of chitosan-based complexes through the proton sponge mechanism [120]. As previously referred, chitosan amino groups have a pKa value around 6.3, but very limited buffering capacity is found [81, 112]. On the other hand, imidazole ring of urocanic acid has a pKa of 6.9. This slight difference could help urocanic acid-modified complexes to protonate in endosome in a faster and tighter way, probably leading to proton sponge effect [120]. Modified complexes shown stronger compaction, smaller size and improved transfection results, while cell viability is maintained [120].
We have recently proved that grafting imidazole moieties to a chitosan backbone results in improved transfection efficiency [28]. This improvement seems to be consequence of the polymer buffering capacity, since transfection activity is significantly reduced in the presence of bafilomycin A1 (unpublished data). Our own results further corroborate that the incorporation of imidazole moieties represents a promising strategy to enhance polymers transfection efficiency while maintaining cell viability [53].

3.2.4. Fusogenic peptides

Other strategies are being developed in order to overcome the endosome bottleneck to gene delivery. One important approach is adapted from viruses that use short amino acid sequences with fusogenic properties. It was revealed that these sequences have particular features. There is a conserved hydrophobic side chain with several residues of glutamic acid. The carboxylic groups in the side chain of this amino acid protonate when the pH drops and the conformation of the peptide changes from random coil to α–helix. The helix is amphipatic with the hydrophobic residues on one side what can disturb the lysosomal membrane leading to vesicle content release [121]. However, the use of protein structures like fusogenic peptides has important disadvantages such as low stability of peptides, high costs for peptide synthesis and immunogenic potential of these structures [53].

3.3. Nuclear import

Nuclear entrance has been presented as the Achilles' heel of non-viral gene delivery [122]. After escaping from endosomal vesicles, DNA should transverse the cell cytoplasm to the proximity of cell nucleus and, ultimately, get in. Indeed, in most cell types a fundamental limitation to gene expression in currently used non-viral systems seems to be the inability of DNA to migrate from the cytoplasm to the nucleus [123].

3.3.1. From the cytoplasm to the nucleus proximity

The cell cytoplasm is composed of a network of microfilament and microtubule systems and a variety of subcellular organelles bathing in cytosol. In addition, cytosol has a high protein concentration, resulting in a significant molecular crowding which limits the diffusion of molecules, such as polyplexes [60]. Hence, after endosomal escape, is not expected that cationic polyplexes or even DNA can diffuse freely. Furthermore, low mobility in the cytoplasm makes plasmid DNA an easy target to cytoplasmatic nucleases [53], limiting its availability.

Very little data is published on transport of nucleic acid through the cytoplasm to within proximity of the nuclear pore. However, some studies reported the importance of DNA size on diffusion. DNA of 100 bp could achieve high mobility and enhanced transfection efficiency, whereas for larger fragments (>1000 bp) the diffusion is remarkably reduced [124], suggesting the existence of transport mechanisms other than diffusion. Polymer-DNA complexes transport seems also to be an energy dependent process. Suh and colleagues showed that bPEI-DNA complexes are transported into the perinulear region by an energy-dependent mechanism [122]. This mechanism could be only part of cationic complexes transport, but there is evidence on microtubule network involvement [122] and association to motor proteins such as dynein [125].

3.3.2. Complex disassembling

Concerning the nuclear import, one question that needs to be addressed is whether the DNA disassembles from the liposome or polymer prior to or after nuclear entry. For liposome-mediated gene delivery Xu and colleagues postulate that during transfection, DNA is released from the lipid in the cytoplasm and is then trafficked uncoated by an inefficient mechanism into the nucleus [108]. The disassembling of DNA from the lipid prior to entering the nucleus was thought to be critical for the ultimate expression of the gene, because when lipid-DNA complexes were directly injected in the nucleus the transgene expression was reduced [126]. In this way, the incorporation of lipids such as DOPE or cholesterol, which favors the DNA release from lipid formulations, is common strategy to enhance gene delivery [36]. However, the opposite theory has also been proposed by Cornellis and colleagues, who recently showed that lipoplexes microinjected into the nucleus can also promote gene expression and that the cytoplasmatic dissociation of DNA from the lipoplex is not the limiting step for lipofection [127].

The intracellular mechanisms occurring after polymeric vectors-mediated transfection has been slightly explored and the published studies are mostly focused on PEI or PLL-based complexes. Pollard and co-workers proposed that cationic polymers (bPEI and PLL) could promote gene accessibility to the nucleus without complex disassembling [126].

Nuclear localization of bPEI was demonstrated by fluorescence labeling in immortalized endothelial cells [114]. bPEI enters the cell nucleus alone or assembled with DNA [114] by diffusion, rather than by some active transport mechanism [128]. It was suggested that, through interaction with

anionic lipids from the cell or endosome membrane, complexes are covered by a lipidic coat that promotes fusion with nuclear envelope and DNA is released inside nuclei [114]. The complex disassembling is point of disagreement and some authors proposed that DNA is released by competition with genomic DNA [126], or rRNA highly concentrated in the nucleolus [129]; whereas other reports support that there is no DNA release, since transcription occurs efficiently, even with DNA tightly complexed with the polymer [116].
As discussed in section 2.2.3.1.2, linear PEI (lPEI) shares the chemical formula with branched PEI (bPEI) but the different structure confers remarkable differences in intracellular trafficking. lPEI complexes disassemble in cytoplasm which is correlated with the earlier detectable gene expression relative to bPEI [130]. However, to low molecular weight lPEI polymer, alternative mechanisms had been revealed by confocal laser scanning microscopy. lPEI-based complexes can reach the nucleus intact; some of these polyplexes may dissociate inside the nuclei, but disassembling can also occur in the cytosol and, in that conditions, DNA or polymer reach the nucleus on their own [62].

Experiments to determine transfection mechanism of PLL complexes are quite difficult since these polyplexes are mostly entrapped in the endosomal vesicles and no nuclear localization is detected except after lysosome destabilization induced by agents such as chloroquine. Even though, in long incubation experiments *in vitro*, the dissociation of DNA and the polymer can be proved [130].

In chitosan-mediated transfection no significant dissociation of the complexes in the cytoplasm was detected [87]. Chitosan can be found inside the cell nucleus [96], where the disassembling is supposed to occur [87]. It was also proposed that the presence of the polymer in the nucleus does not interfere with the gene expression [87], however there are no evidences attesting these theories.

3.3.3. The nuclear membrane

The nuclear envelope is the final obstruction to plasmid DNA entry into the nucleus. The transport of macromolecules through the nuclear membrane is crucial for the metabolism of eukaryotic cells [109]. In consequence, there are specific mechanisms to allow and control substances access into the nucleus. The nuclear envelope is a double membrane perforated by nuclear pore complexes (NPC). The NPC allows passive diffusion of molecules up to 50 kDa. Larger molecules should contain specific sequences – nuclear localization sequences (NLS) – that are able to mediate nuclear import and transverse the nuclear pore in an energy-dependent manner [60]. The size scale of the NPC make it unlikely transporter for DNA, but some studies report DNA entry in intact nuclei [131] and in reconstituted nuclei extracted from *Xenopus laevis* eggs [132].
Besides passive diffusion and energy-dependent transport, a third process can allow particles to get into the nucleus. When cells undergo mitosis, the nuclear envelope breaks down and the

permeability barrier is lost. Thus, if plasmids or complexes are present in cytoplasm, they can enter the nucleus when the envelope is disrupted during this stage of the cell cycle [133]. This aspect explains why dividing cells are generally considered to be easier to transfect than non-dividing, differentiated cells [134]. During interphase, in non-dividing or growth-arrested cells, the only way to enter into the nucleus is through the NPC. Therefore, mainstream of the research for improving DNA nuclear import is based on the use of NLS-containing peptides or proteins. However, there is no consensus on the positive effects of inclusion of these peptides so far and the transfection results in these cases are not easily achieved with almost any method [133].

3.3.4. The effect of cell cycle on nuclear uptake

The nuclear import makes cell cycle a critical point in gene delivery research. Several authors are concerned about the influence of cell cycle in transfection efficiency and different results were obtained depending on the vector employed or the cell type.

Some viruses, such as adenoviruses are able to infect cells independently of their cell cycle phase [135], since the cytoplasmic movement of viral DNA towards the nucleus is facilitated by the interaction of viral proteins with the microtubular network [131]. In contrast, retroviruses-based vectors are unable to infect non-dividing cells and their nuclear uptake is dependent on nuclear membrane breakdown during mitosis [136]. In non-viral vectors is generally accepted that transfection is cell cycle dependent, because cationic liposomes or polymers lack specific sequences that can drive nuclear uptake. However, it was reported that cationic lipid-mediated DNA transfer into cell nucleus is not completely abolished in non-dividing cells [123]. Moreover, the nuclear membrane breakdown seems not to be absolute requirement in bPEI-mediated transfection too [126]. It is important to note that most studies only evaluate the role of nuclear membrane breakdown, ignoring that several cell functions are cell cycle dependent. Apart from nuclear membrane breakdown, during cell cycle intracellular kinetics are variable: membrane tension is irregular and consequently, endocytosis can vary [105]; the size of nuclear pores change according to cellular activity, as well as the intracellular electrical potential that is more negative near mitosis [134]. All these aspects of cell behavior should be considered when one aims at evaluating the cell cycle effect on transfection experiments.

3.4. Beyond delivery

In a general sense, the nuclear membrane is considered the last barrier for gene delivery and the transport of genes into the nucleus the ultimate propose. However, an efficient delivery could not result in a high gene expression. Pollard and co-workers showed that the levels of DNA transferred into the nucleus and those of gene expression do not correlate [126]. This work highlights the importance of taken into account some parameters that could influence DNA transcription and

translation in gene delivery studies. One effect could be simply saturation of gene expression, which has been observed when excess amounts of plasmids are delivered [36]. Additionally, DNA fragments of all sizes are nearly immobile in the nucleus, probably due to extensive binding to nuclear components what can also limit gene expression [124]. Furthermore, polymer carriers detected inside nuclei [87, 114] can impact the functionality of delivered DNA. Cellular transcription is known to be regulated by natural polycations, including histones and polyamines. Thus, one would expect similar “regulatory activity” mediated by cationic carrier systems in cells [36]. The interaction between cationic vectors and cell components can have a role in gene expression and also in cell behavior.

In view of the above, to study the intracellular mechanisms occurring during transfection is of key importance to pinpoint the bottlenecks towards gene expression for each gene delivery system. Moreover, this study could provide important cues to the design of new vectors with a proper gene expression profile prospecting a specific clinical application.
The main goal of the project in which this work is integrated is to develop an efficient and safe vector to be applied in neuroregeneration. After a previous study on the transfection efficiency evaluation of this new material, the present work aims at testing its overall potential as gene delivery vector for a regenerative medicine application.

CHAPTER II – AIM OF THE THESIS

In regenerative medicine one aims at the development and manipulation of laboratory-grown molecules, cells, tissues, or organs to repair, replace or support the function of defective or injured body parts. Gene delivery has been proposed as powerful tool in this field either as a therapeutic strategy by itself or in combination with cellular therapies and/or tissue engineering [11]. The premise is deliver genes to cells in order to trigger *in loci* the production of therapeutic proteins and provide the correct signals to promote/modulate regeneration. Genes encoding for growth factors are of particular interest for these applications, due to their relevance in the regeneration process. However, the expression of these molecules should be strictly tuned in order to avoid over-production and abnormal cell behavior. Therefore, a gene carrier to be applied on a regenerative medicine strategy should guarantee not only non-toxicity and biodegradability, but also a sustained delivery limited to the healing process time period.

Natural polymers are very popular for regenerative medicine applications [137], due to their low toxicity and potential biodegradability. Chitosan is one of the few polycations of natural origin and it has been described as a promising gene carrier [75]. However, its low transfection efficiency hampered so far its widespread application.

We have previously shown that grafting imidazole moieties to chitosan backbone is an advantageous strategy to increase transfection efficiency of chitosan-based vectors [28]. The polymers with higher degrees of substitution with imidazole moieties (13% - CHimi2 and 22% - CHimi3) promote improved transgene expression. Additionally, the chemical modification of chitosan does not trigger any cytotoxic effect, making CHimi-based vectors a potential gene carrier for regenerative medicine applications.

The aim of this thesis is to further characterize CHimi-mediated transfection as means to assess if this vector fulfills the requirements to be applied in a regenerative medicine approach. A detailed study on gene expression mediated by CHimi-based vectors (CHimi2 and CHimi3) has been performed, in which the protein production profile after transfection was assessed over time. The possibility of a second treatment of the cultures with CHimi-based complexes was also investigated, mimicking a repeated administration of the vectors. The feasibility to constitute a frozen stock of transfected cells was also evaluated due to the potential interest when prospecting an *ex vivo* or tissue engineering application. The monitoring of cell viability as a function of time and treatment was also performed as this is a key parameter envisaging a regenerative therapeutic end.

Due to the relevance of intracellular trafficking studies to attain know-how to develop effective and functional gene delivery systems [90], it was also purpose of the present work to follow the intracellular pathway of CHimi-based complexes in the transfection process. This study aims to detail the intracellular events underlying CHimi-mediated transfection in order to find new ways of improving the system.

CHAPTER III – MATERIALS AND METHODS

1. MATERIALS

Unless mentioned otherwise, all reagents were obtained from Sigma-Aldrich and were of analytical grade.

1.1. Imidazole-grafted chitosan

Imidazole-grafted chitosan was obtained by EDC/NHS (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride – EDC; N-hydroxysuccinimide – NHS) mediated amidation of technical grade chitosan (Chimarin™) from Medicarb (Sweden). Chitosan had endotoxin levels below 0.1 EU/ml, which are below the minimal detection level of the Limulus Amebocyte Lysate test used (0.1 EU/ml, Cambrex). This enables its use as biomaterial, in view of the endotoxin levels recommended for implantable devices (below 5 EU/kg of body weight) [138]. The starting material had an average weight molecular weight (Mw) of $1.2x10^5$, as determined by gel permeation chromatography (GPC, in 0.5 M CH_3COOH – 0.2 M $NaCH_3COO$ at room temperature) [139, 140], and a degree of N-acetylation (DA) of 16% (Fourier Transform Infrared Spectroscopy – FT-IR) [141]. Two degrees of substitution (DS=mol of imidazole moieties per mol of free amines of chitosan) were obtained by adding different amounts of imidazole-4-acetic acid sodium salt to a chitosan EDC/NHS mixture: 13% (CHimi2) and 22% (CHimi3) (determined by FT-IR). The reaction was carried out in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) at room temperature for 24 hours. The final imidazole-grafted polymers were obtained after precipitation with ammonia:water (3:7) solution followed by several washes with deionized water until the pH of the the supernatant reaches water pH.

1.2. Plasmid DNA

pCMV-GFP encoding for the green fluorescent protein (GFP) (7424 bp, a kind offer of Luigi Naldini, San Raffaele University, Milan, Italy) and pCMV-Sport-βgal (Invitrogen) encoding for the β-galactosidase (7853 bp) were used as reporter genes for transfection (Figure 12). Plasmids were amplified in DH5α Escherichia coli (*E. coli*) and isolated using GENELUTE™ high performance endotoxin-free plasmid maxiprep kit (Sigma) according to manufacturer instructions, followed by ethanol precipitation. Concentration and purity were assessed spectrophotometrically (λ=260 nm and 280 nm). Plasmid purity, calculated as the ratio Abs260/Abs280, was higher than 1.7. Plasmids integrity was evaluated by electrophoresis on 1% agarose gel after digestion (37 °C, 1 hour) with the restriction enzymes BamHI and SalI (Fermentas). SalI was used to linearize pCMV-Sport-βgal plasmid and both BamHI and SalI were applied in pCMV-GFP digestion.

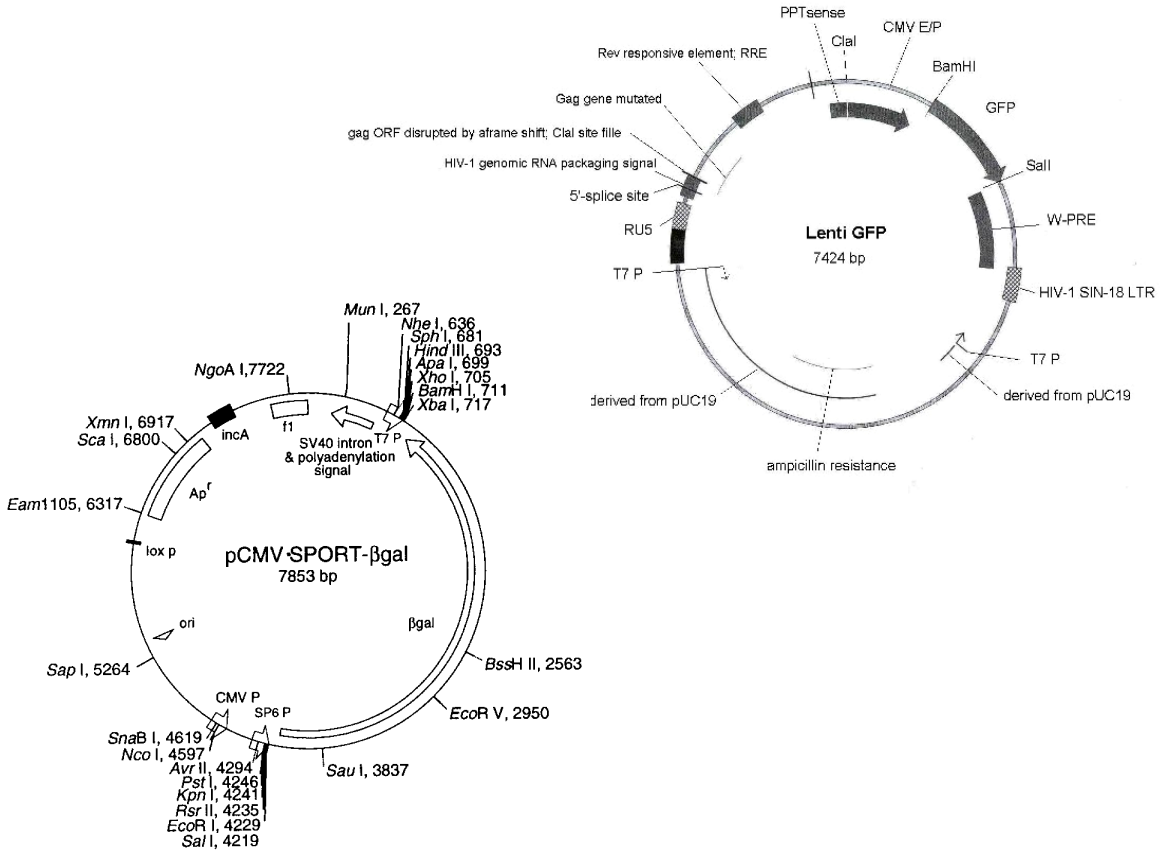

Figure 12: Schematic drawing of the pCMV-SPORT-βgal and pCMV-GFP constructs with the respective restriction sites.

A pET-3a based plasmid encoding for transthyretin (TTR) (4.9 Kbp, kind offer of Maria João Saraiva, Molecular Neurobiology Division, Instituto de Biologia Molecular Celular, Porto, Portugal) was used in the *in vitro* transcription/translation assay.

2. METHODS

2.1. General procedures

2.1.1. Cell culture

293T Human Embryonic Kidney endothelial cells (a kind offer from Simone Niclou - Netherlands Institute for Brain Research, Amsterdam, Netherlands) were cultured under standard conditions (humidified incubator, 37 °C and 5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4500 mg/l, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin (10.000 units/ml penicillin and 10.000 μg/ml streptomycin, Gibco).

For cell culture maintenance, cells were harvested twice a week by trypsinization. In brief, cultures were rinsed with pre-warmed (37 °C) phosphate buffered saline (PBS) and subsequently incubated with a trypsin solution (0.25% (w/v) trypsin, 0.1% (w/v) glucose and 0.05% (w/v) ethylenediaminetetraacetic acid, in PBS) for 5 minutes at 37 °C. Afterwards, cells were resuspended in supplemented medium and $1.33x10^4$ cells/cm^2. Cells were routinely tested for mycoplasma contamination by PCR and only negative cultures were used.

2.1.2. Polymer stock solution preparation

Polymer stock solutions were prepared at a final concentration of 0.1% (w/v). Briefly, 10 mg of CHimi-based polymer was dissolved overnight in 4 ml of 1% (v/v) acetic acid solution. Afterwards, 4 ml of sodium acetate buffer solution (5 mM, pH 5.5) was added. The pH was adjusted to 5.5 with a 1 M sodium hydroxide (NaOH) solution and the volume was completed to 10 ml with sodium acetate buffer (5 mM, pH 5.5).
All the solutions used in the preparation of the polymer stock solutions were sterilized by filtration (0.22 μm filters, TPP). CHimi solutions did not allow filtration due to its high viscosity.

2.1.3. Complex preparation

CHimi-DNA complexes were prepared in a molar ratio between primary amine groups of CHimi to phosphate groups of DNA (N/P) of 18. Similar volumes of plasmid DNA (sodium sulphate, 25 mM) and CHimi (in sodium acetate buffer 5 mM, pH 5.5) solutions were heated at 55 °C for 10 minutes. Subsequently, the DNA solution was added drop-wise to the polymer solution while vortexing the latter. Complexes were let to assemble and stabilize for 15 minutes at room temperature prior to further use.

2.1.4. Transfection

293T cells (passage 8-14) were seeded 24 hours before transfection at a cell density of $2.7x10^4$ viable cells/cm^2 on poly(D-lysine) (PDL, Sigma) coated 24-well tissue culture plates (Greiner bio-one). For PDL coating of the wells, 50 μl of a 0.1 mg/ml PDL aqueous solution was placed in each well and pipetted up and down to cover the entire well surface. Subsequently, wells were left to dry at room temperature and, finally, exposed to UV light for 20 minutes. Cell viability was assessed by means of the trypan blue exclusion test. This dye is incorporated only in non-viable/dead cells that become stained in blue. Briefly, after trypsinization, the cell suspension was incubated with trypan

blue solution (0.4% (w/v), in PBS) for 2 minutes. Subsequently, viable cells were counted in an improved Neubauer chamber under light microscope.
Two hours before transfection, the cell culture medium was discarded and 500 μl of fresh medium was added to each well. At transfection time, complexes prepared as described in section 2.1.3, were added to the cell culture. Each treatment was always performed in triplicate. The final plasmid DNA concentration applied was 1.3 $\mu g/cm^2$. Culture medium was refreshed every 24 hours.

2.2. Gene expression studies

Several parameters concerning gene expression mediated by CHimi-based vectors were investigated. Gene expression was evaluated for periods up to 168 hours post-transfection (section 2.2.1 – long term studies). The stability of the transfection activity was also analyzed after freezing and thawing transfected cells (section 2.2.2 – freeze/thaw studies) and the effect of re-transfection on the gene expression was evaluated as described in section 2.2.3. In all gene expression studies, the β-galactosidase gene encoded in pCMV-Sport-βgal plasmid was used as reporter gene. During the present work transfection activity was quantified as the specific activity of β-galactosidase (β-gal) enzyme. Escort™ V (Sigma), a commercially available branched poly(ethylenimine) (PEI), was used as a transfection positive control according to manufacturer instructions (final DNA concentration: 0.35 $\mu g/cm^2$).
Cell viability was also assessed in long term and re-transfection studies for the whole experiments period.

2.2.1. Long term studies

According to previously reported results [28], imidazole-grafted chitosan mediated transfection tends to increase up to 96 hours post-transfection. The doubling time of 293T cells in the conditions of the current study (initial cell density $2.7x10^4$ cells/cm^2) is about 17 hours (data not shown) and, consequently, 96 hours post-transfection cell confluence is approximately 100%. In order to circumvent this limiting factor and to increase the gene expression evaluation period a step of trypsinization was introduced 72 hours post-transfection. In brief, the cell monolayer was rinsed with pre-warmed PBS and 150 μl of trypsin solution was added to each well. After 5 minutes of incubation at 37 °C, cells were carefully resuspended in supplemented DMEM (final volume 1 ml/well). Replicates were mixed in a centrifuge tube, homogenized, diluted (7 times) and re-seeded on PDL-coated 24-well plates.
The described protocol allowed transfection activity evaluation at 48, 72, 96, 120, 144 and 168 hours post-transfection, as illustrated in Figure 13.

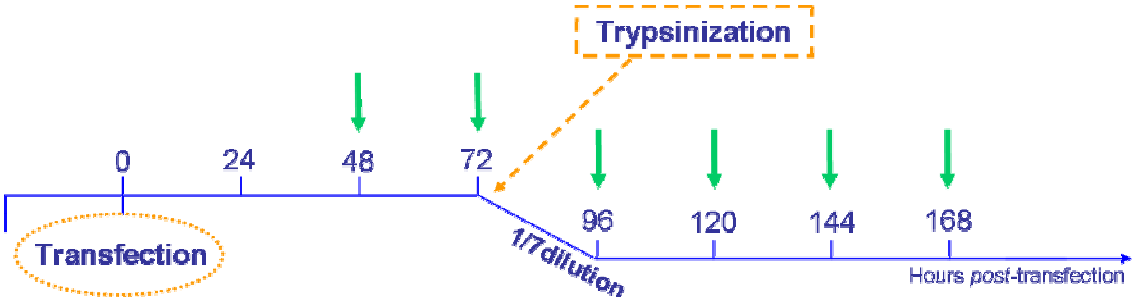

Figure 13: Experimental design for long term gene expression evaluation (green arrows indicate time points for transfection activity evaluation).

To assess the importance of cell density when re-seeding cells 72 hours post-transfection on the transfection activity, an additional experiment was performed. For all the treatments, after trypsinization $2.7x10^4$ viable cells/cm^2 were re-seeded rather than a 1/7 dilution of the cell suspension (Figure 14). In this case, cell viability was also assessed up to 168 hours post-transfection.

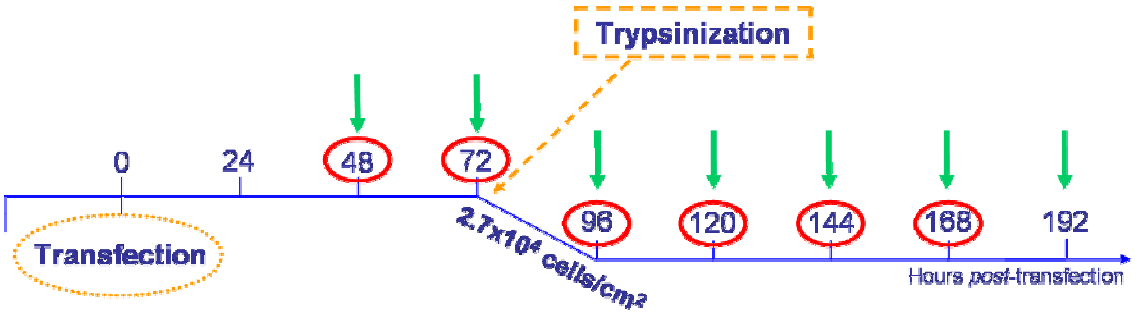

Figure 14: Experimental design to evaluate long term gene expression after re-seeding cells at $2.7x10^4$ viable cells/cm^2 (green arrows represent time points for transfection activity assessment and red circles indicate cell viability evaluation).

2.2.2. Freeze/thaw studies

To evaluate the ability of cultures to withstand a freeze/thaw cycle and to study the specific β-gal activity stability after a freeze/thaw process, transfected cells were frozen and β–gal activity was again evaluated after thawing. In brief, 72 hours post-transfection, cells were trypsinized as described in section 2.2.1. The obtained cell suspension was pelleted by centrifugation (200 g, 4 minutes) and resuspended in 1 ml of supplemented medium. Cells were transferred to cryogenic vials and dimethyl sulfoxide (DMSO) was added drop-by-drop (final concentration of 10% (v/v) in culture medium). After a cryopreservation period larger than 4 weeks, vials were thawed in a water bath (37 °C). Afterwards, cells were pelleted by centrifugation (200 g, 4 minutes), counted and

seeded on PDL-coated 24-well plates at a cell density of $2.7x10^4$ viable cells/cm^2. Samples for transfection activity evaluation were collected 48, 72 and 96 hours after thawing, corresponding to 120, 144 and 168 hours post-transfection (see Figure 15). Transfection activity of cells that were transfected but not frozen was followed for comparison.

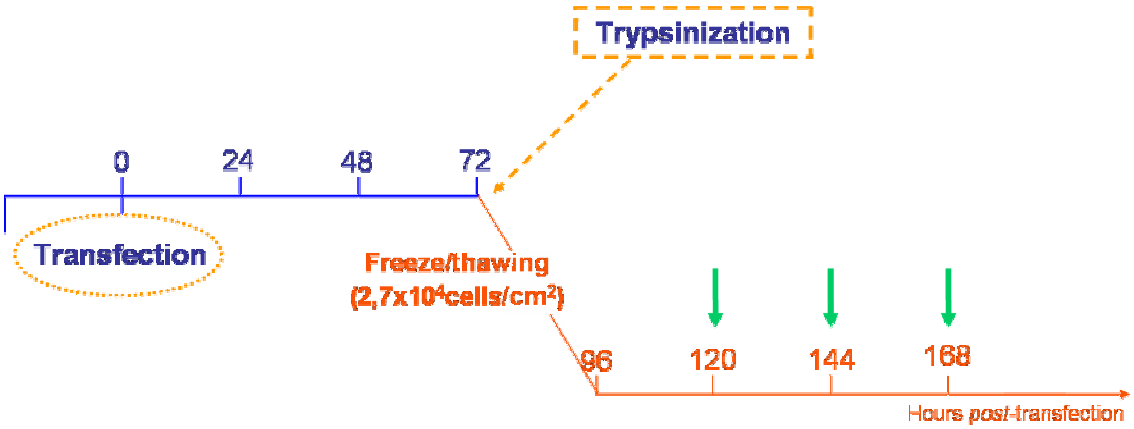

Figure 15: Experimental design for evaluation of transfection activity after freeze/thaw process (green arrows indicate time points for transfection activity evaluation).

2.2.3. Re-transfection studies

To analyze the effect of a second transfection mediated by CHimi-based vectors on gene expression and cell viability the following procedure was performed (see Figure 16).

Cells were trypsinized 72 hours post-transfection, according to the procedure described in section 2.2.1 and, by means of trypan blue exclusion test, cells were counted and seeded at $2.7x10^4$ viable cells/cm^2 on PDL-coated 24-well plates. 24 hours after seeding, cells were re-transfected in similar conditions to that described for transfection in section 2.1.4. Transfection activity was evaluated 48, 72 and 96 hours post re-transfection, corresponding to 144, 168 and 192 hours after the first transfection (Figure 16).

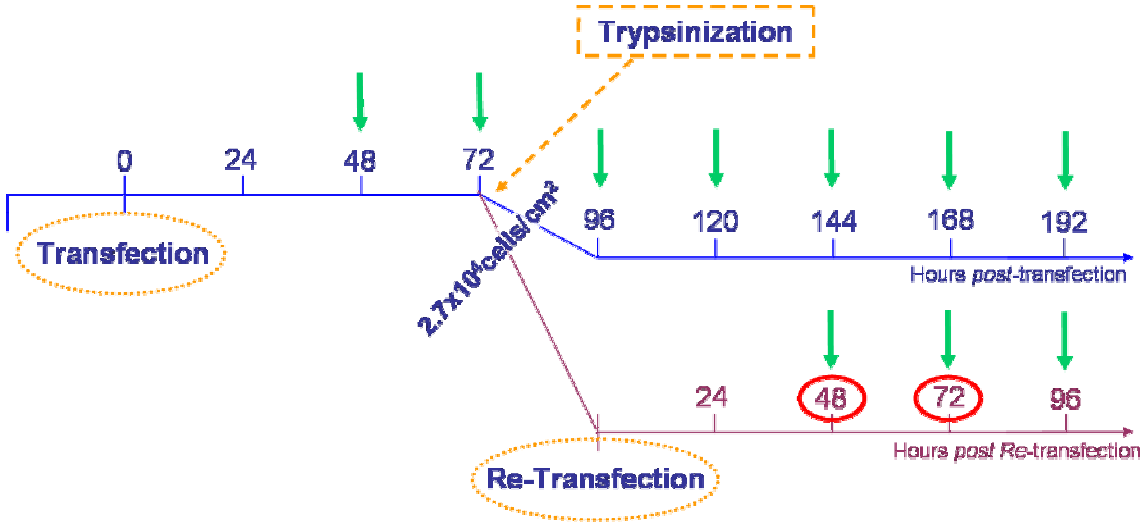

Figure 16: Experimental design for re-transfection studies (green arrows indicate time points for transfection activity evaluation and red circles show time points for cell viability assessment).

As control of the re-transfection process, cells that were not transfected (negative control) in the first transfection step were transfected at the same time. By means of this procedure, transfection activity of transfected and re-transfected cells can be compared.

2.2.4. Cell viability

To assess cell viability of cells transfected with CHimi-based complexes a colorimetric/fluorometric assay based on the reduction of resazurin by viable cells was used [142]. Resazurin is a blue, non-fluorescent molecule that is reduced by several mitochondrial and cytoplasmatic enzymes to a pink fluorescent product, called resofurin. This test is non-destructive, meaning that cell viability and transfection activity of the same cells can be measured.

To perform the resazurin-based assay 10% (v/v) of a resazurin (Sigma) solution (0.1 mg/ml, in PBS) was added to each well. After 4 hours of incubation at 37 °C, 200 μl of the medium was transferred into a black walled 96-well plate (Greiner bio-one) and fluorescence (λex=530 nm, λem=590 nm) was measured (Spectra Max Gemini XS – Molecular Devices). Cells were maintained in culture after refreshing the cell culture medium. Results are represented as percentage of viability relative to control cells (non-transfected cells).

2.2.5. β-gal assay

At the selected evaluation time points, cells were collected and processed for β-gal activity evaluation as follows. Cultures were rinsed with pre-warmed (37 °C) PBS and, subsequently, trypsinized for 5 minutes at 37 °C. Each cell pellet was resuspended in PBS (final volume 1ml) and

transferred to a standard micro test tube. After 10 minutes of centrifugation at 18000 g (room temperature), supernatants were discarded and the cell pellet was resuspended in 100 μl of lysis buffer (0.25 M Tris in water, pH 8). Samples were frozen at -20 °C, till further use. Before starting the β-gal assay, samples were thawed at 37 °C and centrifuged for 15 minutes at 18000 g (4 °C). The resulting lysate (supernatant after centrifugation) was transferred into a new tube and maintained on ice. The β-galactosidase activity was determined in accordance with the β-gal assay protocol of Invitrogen. Lysates of non-transfected cells were used as blank. Briefly, the expression of the β-gal gene produces an enzyme able to hydrolyze β-galactosides, such as ortho-nitrophenyl-β-D-galactopyranoside (ONPG). ONPG hydrolysis produces a bright yellow compound (o-nitrophenyl-β-pyranoside – ONP), measurable by spectrophotometric analysis at 420 nm. To assess the linearity of ONP absorbance a β-gal standard curve obtained by serial dilutions from a stock solution of 4440 U/ml (β-galactosidase from *E. Coli*) was performed for each assay. Linearity was achieved for standards ranging between 3.9 U/ml to $1.53x10^{-2}$ U/ml.
Transfection activity is expressed as specific activity of β-galactosidase: nmoles of ONPG hydrolyzed per minute and normalized per mg of total protein. The total protein was determined by the BCA assay (Pierce). The protein concentration of the samples was interpolated from a bovine serum albumin (BSA, Merck) calibration curve obtained by serial dilutions from a stock solution of 5 mg/ml. A linear curve was obtained with standards of BSA ranging between 2.5 mg/ml and 0.078 mg/ml.

2.3. Intracellular trafficking

In order to further characterize the intracellular mechanisms underlying CHimi-mediated transfection the intracellular trafficking of complexes was studied by confocal laser scanning microscopy (CLSM). The intracellular distribution of complexes formed by the fluorescently labeled CHimi and pCMV-GFP (section 2.3.1 and 2.3.2, respectively) was studied in 293T cells, after cell fixation and cytoskeleton and cell nuclei staining with specific dyes (section 2.3.3).
To evaluate the effect of the fluorescent labeling of CHimi on the complexes properties, the zeta potential and the particle mean size were assessed before and after the chemical modification (see section 2.3.1.1).

2.3.1. Chitosan fluorescent labeling

Imidazole-grafted chitosan was fluorescently-labeled with rhodamine (λex=575 nm and λem=600 nm). By reacting a rhodamine activated derivative [5(6)-Carboxy-X-rhodamine N-succinimidyl ester, ROX (Fluka), see Figure 17] with chitosan, an amide bond can be established, between the

succinimidyl ester terminal and free amino groups of chitosan. CHimi2 (DS=13%) was chosen for the reaction due to the higher number of free amino groups available to react with rhodamine in comparison with CHimi3.

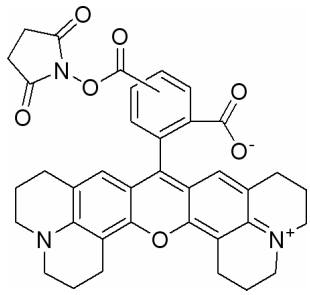

Figure 17: Molecular structure of 5(6)-Carboxy-X-rhodamine N-succinimidyl ester (ROX).

The reaction between the fluorescent tracer and CHimi2 was carried out in conditions in which a 5% replacement of free amino groups of chitosan can be expected [143-145]. In brief, 10 mg of CHimi2 was dissolved overnight in 10 ml of a 1% (v/v) acetic acid solution and added to an equal volume ROX solution (0.13 mg/ml in dehydrated methanol, Molecular Sieves, Merck). The reaction was let to occur for 3 hours, under constant stirring and protected from light. The fluorescently-labeled CHimi2 ($CHimi2_{ROX}$) was precipitated with 5 ml of 0.5 M NaOH solution and pelleted by centrifugation (4000 g, 5 minutes). Afterwards, the polymer was thoroughly washed with deionized water until no fluorescence was detected in the supernatant (λex=575 nm; λem=600 nm). After freeze-drying, $CHimi2_{ROX}$ stock solutions were prepared as previously described in the section 2.1.2 and stored in the dark at 4 °C till further use.

2.3.1.1. Fluorescent chitosan-DNA nanoparticles characterization

Chitosan-DNA complexes prepared with the fluorescently-labeled polymer were characterized in terms of zeta potential and particle size (Zetasizer Nano ZS, Malvern Instruments).

Complexes were prepared as previously described (section 2.1.3). Polymer mass (134 μg) and solution volume (210 μl) of both CHimi2 and $CHimi2_{ROX}$ based complexes were similar. For each complex solution 10 μg of DNA was used. The final complex solution volume was completed to 1000 μl with sodium acetate buffer (5 mM, pH 5.5). All measurements were performed at 25 °C in sodium acetate buffer 5 mM (pH 5.5). The Smoluchowski model was used in zeta potential calculations.

2.3.2. DNA fluorescent labeling

pCMV-GFP plasmid was fluorescently-labeled using the commercial kit Label IT Cy5 (Mirus™) according to the manufacturer's instructions. Covalent modification can take place on reactive heteroatoms on any nucleotide of the nucleic acid (Figure 18). For each 4 µg of plasmid DNA 1 µl of the labeling reagent was added. Following labeling reaction, fluorescent DNA (DNA_{Cy5}) was precipitated in ethanol and quantified spectrophotometrically (λ=260 nm).

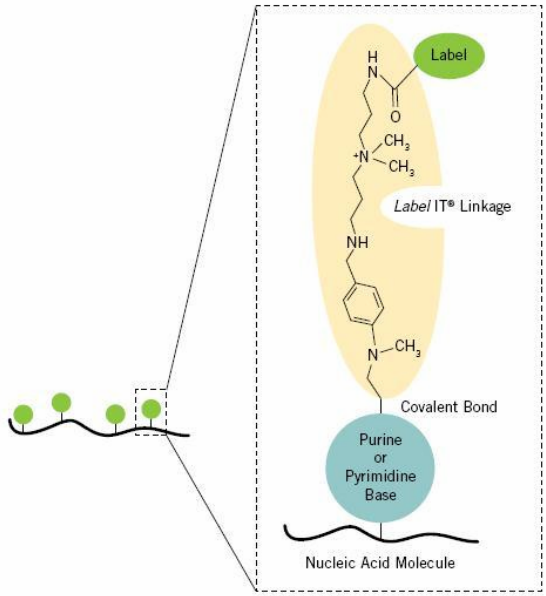

Figure 18: Schematic drawing representing the covalent bonding between DNA and Label IT® reagent (available at [146]).

2.3.3. Fluorescence microscopy

293T cells were cultured as previously described (section 2.1.4) on PDL-coated glass coverslips. Glass coverslips (Menzel Glaser) were treated according to the following procedure. Coverslips were washed in 1 M HCl solution for about 16 hours at 55 °C. A pre-wash in acid solution improves PDL adhesion to coverslips. After cooling, the coverslips were washed with deionized water and ultimately with ethanol. Subsequently, coverslips were air dried on filter paper (Whatman). Afterwards, the coverslips were immersed in a 0.1 mg/ml PDL solution and agitated at 200 rpm (orbital shaker, KM-2 Edmund Buhler) for 90 minutes. Coverslips were thoroughly washed with deionized water to remove free PDL and, ultimately rinsed in 100% ethanol solution and air dried individually prior to further use.

For the fluorescence microscopy studies, cells were transfected with complexes formed with both fluorescently labeled $CHimi2_{ROX}$ and DNA_{Cy5}, at an N/P molar ratio of 18. Five time points were analyzed: 2, 4, 6, 24 and 48 hours post-transfection. For each sample, cultures were rinsed twice

with pre-warmed PBS and fixed 15 minutes at 37 °C with paraformaldehyde (4% (w/v), in PBS), supplemented with 2% sucrose. Cells were stored in PBS at 4 °C till further use.
For staining cell cytoskeleton and nuclei, fixed cells were permeabilized according to a previously described procedure [147] and incubated with 1% (w/v) BSA for 1 hour. Cell nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI, 0.1 μg/ml in PBS, Sigma) and cell cytoskeleton filamentous actin (F-actin) was counterstained with Alexafluor 488-conjugated phalloidin (5 U/ml, in 1% (w/v) BSA, Molecular Probes). Finally, samples were mounted in Vectashield (Vector) and observed under the confocal microscope (Leica Microsystems). Cytoskeleton staining was not performed in samples collected 48 hours post-transfection, in order to allow the detection of GFP positive cells.

2.4. Cell-free gene expression

Concerning intracellular pathways in transfection, one question that needs to be addressed is whether the disassembling of complexes is critical for the gene transcription and protein production. Therefore, in order to assess the importance of CHimi-based complexes disassembling for the protein synthesis, gene expression in the presence of CHimi-based vectors was evaluated in cell-free extracts.

2.4.1. *In vitro* transcription/translation assay

The *in vitro* transcription/translation assay is constituted by cell-free extracts that allow protein synthesis from a DNA template. This kind of system is commonly used tool in molecular biology for the identification of gene products, screening constructs for either naturally occurring or engineered mutations and also in protein folding studies. Although *in vitro* transcription/translation systems are not a typical tool in gene delivery studies, it was applied herein as mean to understand if gene expression is impaired when plasmid DNA is assembled with CHimi-based vectors.
A TNT® Quick Coupled Transcription/Translation System (Promega) was used in this experiment. It combines a reticulocyte lysate, a T7 RNA polymerase, nucleotides, salts and a Recombinant RNasin® Ribonuclease. By adding ^{35}S[methionine] to the reaction mixture, the synthesized protein can be easily detected by phosphorimaging. The TNT system is configured for transcription and translation of genes cloned downstream from a T7 RNA polymerase promoter. The plasmids used in the transfection experiments as reporter genes have CMV promoters (Figure 12) and, consequently, are unsuitable to be used in the Promega system. Therefore, a pET-3a based plasmid, with a T7 promoter, encoding for a TTR protein [148] was applied in this experiment. The plasmid encoding for TTR will be identified as pTTR during this thesis.

In accordance with the manufacturer instructions, 1 μg of pTTR was used in each reaction. Consistent with the intracellular trafficking studies previously described, CHimi2 was selected for this experiment and CHimi2-based complexes were prepared at an N/P molar ratio of 18.

The final volume for a standard TNT reaction is 50 μl. In order to adjust the final volume of the reaction with the one proposed in the manufacturer procedure, a modification on the complexes preparation procedure was required. Therefore, the DNA solution volume was reduced to the minimum (i.e. 2 μl, in 25 mM Na_2SO_4 solution) and, after heating (55 °C), it was mixed with a CHimi2 solution, while vortexing. Complexes were let to stabilize for 10 minutes at room temperature according to the procedure described in the section 2.1.3.
Four reactions were performed as represented in Table 2.

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Table 2: Conditions tested in the *in vitro* transcription/translation assay. (a): Manufacturer suggested conditions.

	Control (a)	**Control** Dilution effect	**Control** pH effect	**CHimi2-based complexes**
	1	2	3	4
plasmid DNA (1 μg/μl)	1μg	1μg	1μg	1μg
Na_2SO_4			1μl	1μl
CHimi2 (0.1%, in acetate buffer)				13.4 μl
Acetate buffer			13.4 μl	
H_2O	7.5 μl	14.4 μl		
TNT mix	40 μl	40 μl	40 μl	40 μl
^{35}S[methionine](15mCi/ml)	1.5 μl	1.5 μl	1.5 μl	1.5 μl
Final volume	**50 μl**	**56.9 μl**	**56.9 μl**	**56.9 μl**

The reaction was let to occur for 90 minutes at 30 °C. The products of each reaction were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel. After drying, the gel was exposed to a phosphor screen overnight. Phosphorimaging analysis of the dried gel was performed using a Typhoon 8600 variable mode imager (Molecular Dynamics).
In order to assess the protein position within the gel and its molecular weight, the recombinant human TTR (produced by *E. coli*) along with a molecular weight marker were loaded on the same SDS-PAGE gel and stained with Coomassie blue.

2.5. Statistical data analysis

Data are presented as average ± standard deviation (SD). Results were statistically treated using the nonparametric Mann-Whitney *U*-test. For multiple comparisons, homogeneity of variances was assessed by Barlett's test and after assuming variances as non-significantly different, two-way ANOVA, follow by post hoc Bonferroni test were performed. Results were considered statistically significant when $p \leq 0.05$. Calculations were performed using SPSS® software for Windows (version 15.0).

CHAPTER IV – RESULTS

1. GENE EXPRESSION STUDIES

In a previous study we had proved that grafting imidazole moieties to a chitosan backbone is an advantageous strategy to increase the transfection activity of chitosan-based vectors [28]. The aim of the present work was to further characterize and understand the transfection mechanism mediated by CHimi-based vectors, envisaging an application in a regenerative medicine scenario. In a first approach, a detailed study on gene expression mediated by CHimi-based vectors was performed.

1.1. Long term studies

Previous results suggested that CHimi-mediated transfection activity tends to increase up to 96 hours post-transfection [28]. Accordingly, it had been published that transfection activity mediated by chitosan vectors increases over time [112]. In consequence, it was found of particular interest to study gene expression mediated by CHimi-based vectors over an extended time period.

To increase the time period for β-gal evaluation in the transfected cultures, the addition of a dilution step to the initial experimental conditions was required. Therefore, 72 hours post-transfection cells were trypsinized, diluted (7 times) and re-seeded. By means of this procedure, transfection activity was evaluated up to 168 hours post-transfection. Results are presented in Figure 19 for cultures transfected with both CHimi2 and CHimi3.

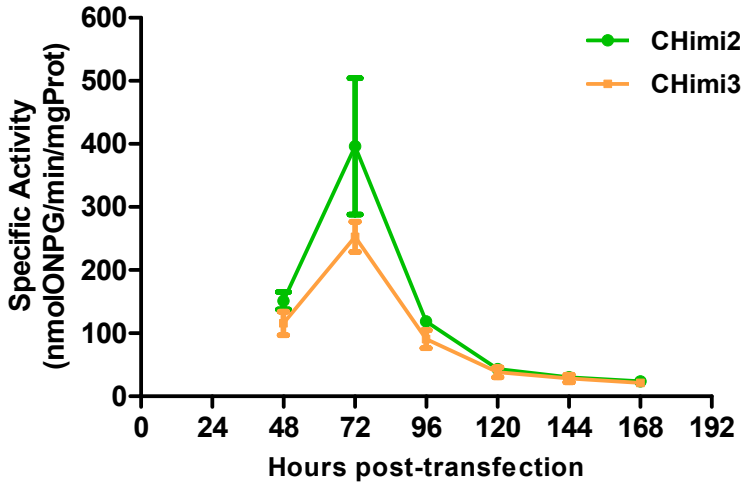

Figure 19: Transfection activity as function of time of cultures treated with CHimi2- and CHimi3-based vectors. Representative experiment out of the four performed (average ± SD; n=3).

As it can be observed in Figure 19, transfection activity is maximum 72 hours post-transfection for both CHimi2 and CHimi3. After trypsinization and dilution (72 hours post-transfection), β-gal specific activity decreased up to 120 hours post-transfection. From this time period up to the last evaluation point transfection activity reduction slowed down reaching a minimum value at 168

hours post-transfection. The results were consistent for both polymers studied and the reduction observed after trypsinization seems to be consequence of the dilution step performed, as discussed in the following paragraph.

In the present study transfection activity is defined as the activity of β-gal enzyme normalized by the total protein content of the sample. If one dissects the transfection activity results by representing separately activity of β-gal and the total protein content as a function of time (see Figure 20) it is clear that, after trypsinization, β-gal activity is maintained at stable values. The tendency for reduction of β-gal specific activity (Figure 19) is a consequence of the cell growth and the resulting increase in the total protein content (Figure 20). Furthermore, at 96 hours post-transfection the decrease on β-gal activity after trypsinization is comparable to the dilution factor introduced (7 times).

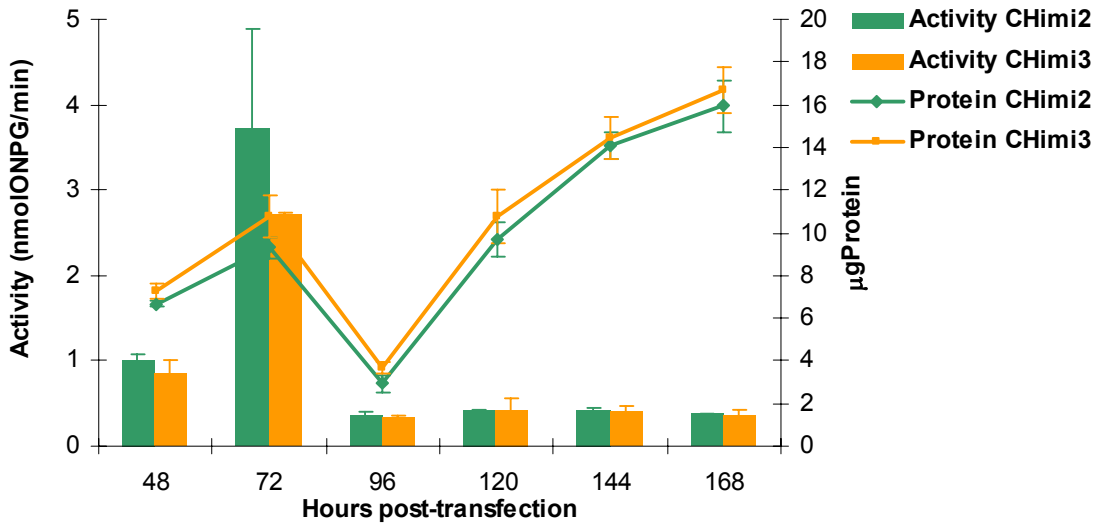

Figure 20: β-gal activity and the total protein content of CHimi-based vectors transfected cells as a function of time.

A commercially available PEI (Escort V) was used as transfection positive control. PEI is the gold standard of cationic polymers used for transfection due to its high efficiency. Therefore, PEI-mediated gene expression kinetics was also analyzed (Figure 21).

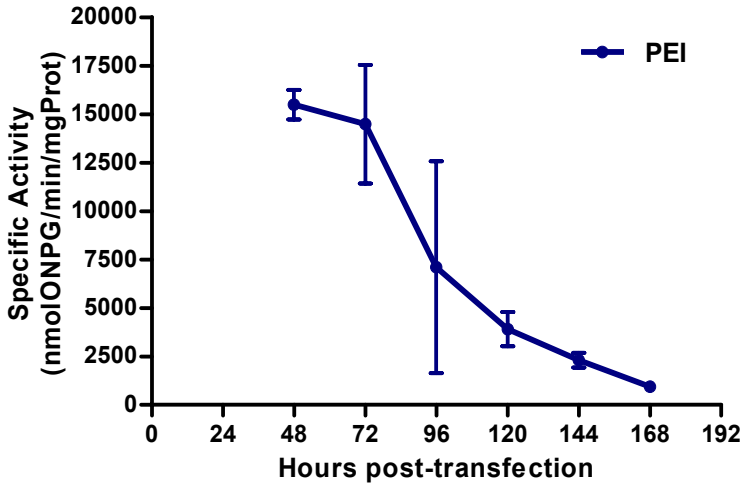

Figure 21: Transfection activity as a function of time of cells transfected with PEI (Escort V). Representative experiment out of the four performed (average ± SD; n=3).

PEI-mediated gene expression achieves the maximum 48 hours after transfection. After cell trypsinization, β-gal specific activity declines over time (Figure 21).

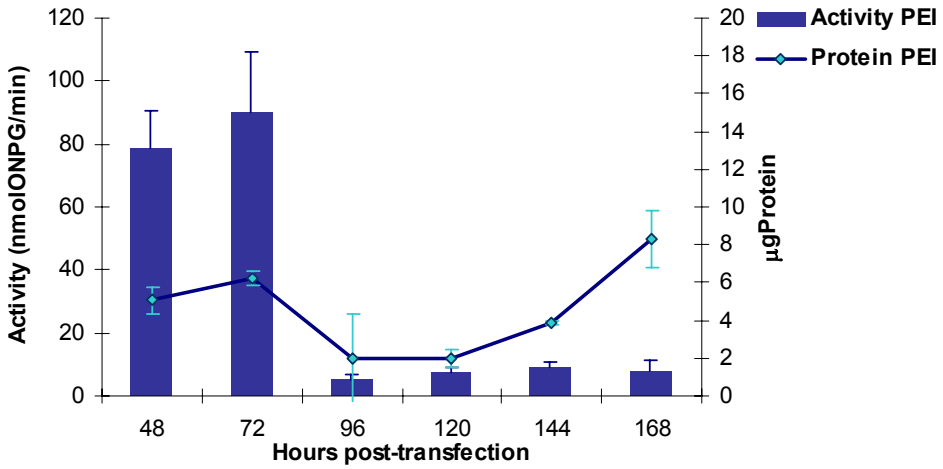

Figure 22: β-gal activity and the total protein content of PEI (Escort V) transfected cells. At 96 hours post-transfection it was difficult to determine the total protein and the β-gal activity due to the reduced number of cells. In consequence a marked error bar was obtained.

If we analyze total protein and β-gal activity as a function of time (Figure 22) the former does not significantly decrease over time, after cell trypsinization. Again, the decrease on β-gal specific activity is consequence of the increase in the total protein content. However, it should be noted that the total protein content of the PEI-treated cultures is significantly lower than that obtained in CHimi mediated transfection.

In the experimental conditions defined for the study previously described, after trypsinization, cells were diluted (7 times) and re-seeded at an unknown concentration. This procedure intends to

simulate a continuous assay. The dilution was only the way to extend the evaluation time and did not intend to change the conditions, for each treatment, at which cells were at the time of trypsinization. However, after considering the effect of the total protein content and the cell growth on the β-gal specific activity, it was thought important to reset differences in terms of number of cells before re-seeding. The dilution of different cell populations could induce a marked effect on the total protein content, due to differences on cell growth rates, resultant of cell-cell contact inhibition. Seeding different cell densities leads to different cell growth rates. Therefore, after trypsinization, cells were re-seeded at the initial cell density ($2.7x10^4$ viable cells/cm^2) and β-gal specific activity was evaluated up to 192 hours post-transfection (Figure 23).

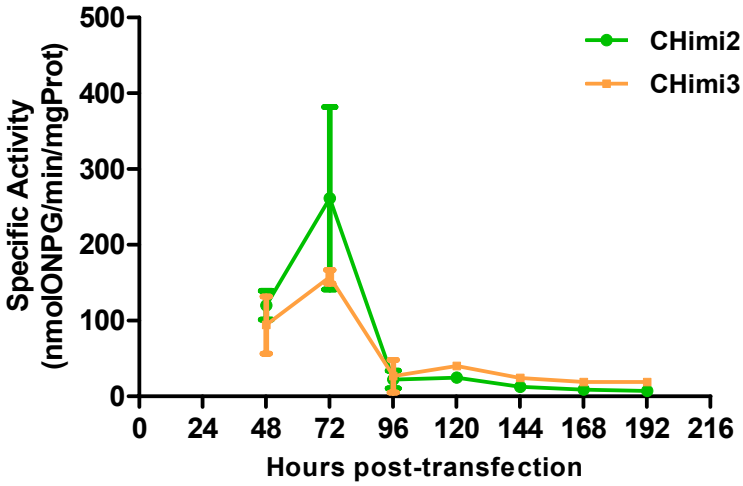

Figure 23: Evaluation of β-gal specific activity over time. After trypsinization (72 hours post-transfection), cells were seeded at $2.7x10^4$ viable cells/cm^2. Representative experiment out of the three performed (average ± SD; n=3).

The overall specific activity profile obtained is similar to that reported in Figure 19. Different re-seeding cell densities did not alter gene expression kinetics (Figure 19 and Figure 23). For both CHimi-based vectors studied the kinetics are maintained independently on the cell density used to re-seed cells after trypsinization.

This experiment was also performed for PEI-mediated transfection and no differences on β-gal specific activity kinetics were detected, comparing to that shown in Figure 21.

We have previously shown that CHimi is not cytotoxic over the concentration range used for transfection and cells transfected with CHimi-based complexes (N/P=3) show over 100% of relative viability, 24 hours post-transfection [28]. After the extension of the experiment period for β-gal assessment and the increase in the polymer amount used to complex DNA (N/P=18), an evaluation of the relative viability of cells transfected with CHimi-based complexes (N/P=18) for the all period studied was required. Cell viability was assessed at the same time points of β-gal evaluation using the resazurin-based test (Figure 24).

For this study, after trypsinization, the cultures were re-seeded at fixed cell density ($2.7x10^4$ viable cells/cm^2), so that one can observe the effect on cell viability of the contact with complexes without influence of the cell density used in the second seeding step.

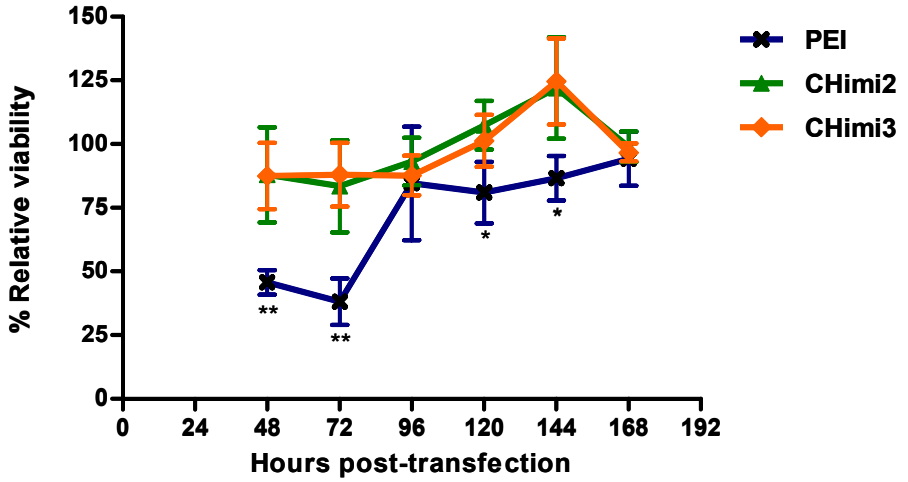

Figure 24: Cell viability as a function of post-transfection time. Relative viability was calculated relative to non-transfected cells. Representative experiment out of the three performed (average ± SD; n=6). *p≤0.05; **p<0.001. Homogeneity of variance tested by Barlett's test. Two-way ANOVA and Bonferroni post hoc test. Statistical differences were calculated comparing the three groups.

Cell viability was calculated relative to control cultures (non-transfected cells). Cells transfected with CHimi-based complexes maintain 100% of relative viability for all the time period evaluated – up to 168 hours post-transfection. Conversely, only approximately 50% of PEI-transfected cells were viable in the first two time points evaluated – 48 and 72 hours post-transfection (Figure 24). At 120 and 144 hours post-transfection statistically significant differences were still detected between relative viability of cultures treated with PEI and CHimi-based vectors. Even though, in the conditions defined for this experiment, PEI-transfected cells viability tends to increase, reaching 100% of relative viability at 168 hours post-transfection.

1.2. Freeze/thaw

In order to evaluate the effect of freezing and thawing on the β-gal gene expression, CHimi transfected cells were frozen 72 hours post-transfection and β-gal specific activity was evaluated again after thawing.

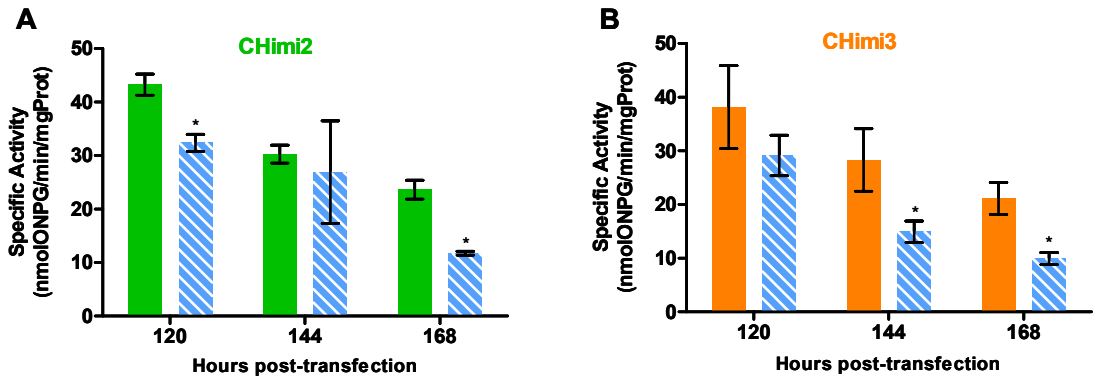

Figure 25: Effect of a freeze/thawing cycle on β-gal specific activity as a function of time in culture after transfection.
A: Cells were transfected with CHimi2 and maintained in culture (green) or sustained a freeze/thawing cycle (dashed bars in blue).
B: Cells transfected with CHimi3 maintained in culture (green) or sustained a freeze/thawing cycle (dashed bars in blue).
Representative experiment out of the three performed ((average ± SD; n=3). * p≤0.05 relative to non-frozen cells calculated by Mann-Whitney test for non-parametric samples.

Cultures transfected with CHimi-based complexes were able to withstand one freeze/thawing cycle. Although there is a statistically significant decrease in terms of β-gal specific activity between the cells maintained in culture and those freeze/thawed at some time points (see Figure 25), the cells maintain the ability to produce new β-gal and the gene was not lost during the process. Moreover, comparing the overall decay in time it is very similar for freeze/thawed to non-freeze/thawed cells. Specific activity is maintained in the same order of magnitude in both cases. These observations are consistent for both CHimi-based polymers studied.

This study suggests stability on transfection to a freeze/thawing cycle and further confirms the non-toxicity of CHimi-based vectors. A freeze/thaw cycle is a relatively harsh process to cells; only healthy cells can survive. Additionally, when seeding cells after thawing, the cell viability was assessed by means of trypan blue exclusion assay. Comparing the total number of viable cells, no differences were detected between CHimi-transfected cells and non-transfected cells. This observation suggests that did not occur any additional loss of viable cells due to transfection, during the freeze/thawing process. The trypan blue viability (live cells relative to the total number of cells) was higher than 90% for both treatments. Conversely, when cultures transfected with the PEI-based vector were frozen the trypan blue cell viability after thawing is reduced up to 70%. In terms of transfection activity behavior, the tendency for reduction after freeze/thawing is similar to that presented for CHimi-based vectors.

1.3. Re-transfection studies

To evaluate if repeated transfection steps could be a strategy to uphold β-gal gene expression in time if found necessary in a clinical application, CHimi transfected cells were re-transfected 96

hours after the first transfection. β-gal specific activity was evaluated 48, 72 and 96 hours post re-transfection, corresponding to 144, 168 and 192 hours after the first transfection.

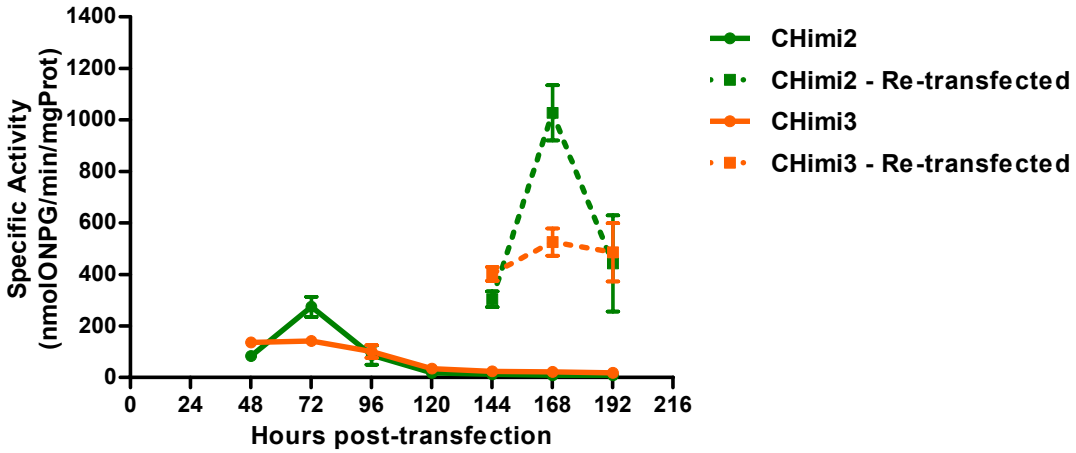

Figure 26: Evaluation of β-gal specific activity as a function of time after re-transfection with CHimi2 and CHimi3, comparing to non-re-transfected cells. Representative experiment out of the five performed (average ± SD; n=3).

The results shown in Figure 26 suggest that transfection activity is improved after a second addition of CHimi-based complexes, for both the degrees of substitution of chitosan tested. Specific activity achieves higher values, greater than that obtained at the specific activity peak in long term studies – 72 hours post-transfection.

To further explore the significant improvement on β-gal specific activity after re-transfection, an additional study was performed, as a control of the re-transfection process. The experimental procedure is represented in Figure 27.

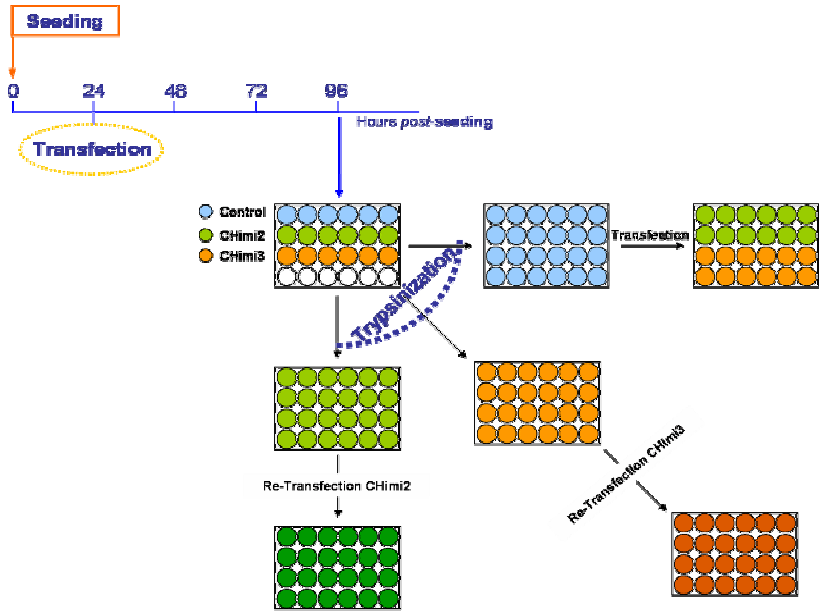

Figure 27: Experimental procedure performed as re-transfection control. Note that in the time line hours post-seeding are represented rather than hours post-transfection.

96 hours after seeding, corresponding to 72 hours post-transfection, both transfected cells (CHimi2, CHimi3) and controls (non-transfected cells) were trypsinized and re-seeded. 24 hours later, CHimi2 and CHimi3 transfected cells were re-transfected – second addition of CHimi-based complexes. Simultaneously, control cells were transfected for the first time (see Figure 27). This procedure intends to compare β-gal specific activity in cells transfected only once with re-transfected cells. With this settings, both transfected and re-transfected cells were in the same experimental conditions (time in culture, changes of culture medium), allowing a more accurate comparison between results.

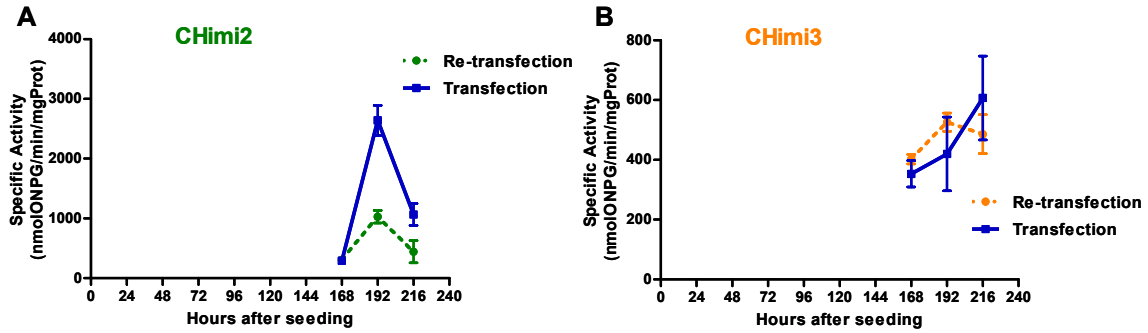

Figure 28: Transfection activity of cultures transfected only once (solid line) or two times (dashed line) with (A) CHimi2 and (B) CHimi3. Representative experiment out of the three performed (average ± SD; n=3).

Figure 28 shows that when transfecting or re-transfecting cells at the same time point, no differences in terms of β-gal specific activity were detected. The increase over the maximum values after re-transfection described in Figure 26 seems to be consequence of the experimental procedure. Results are consistent for both CHimi-based polymers studied.

Re-transfection and re-transfection control studies were also performed for PEI-based transfection (Figure 29).

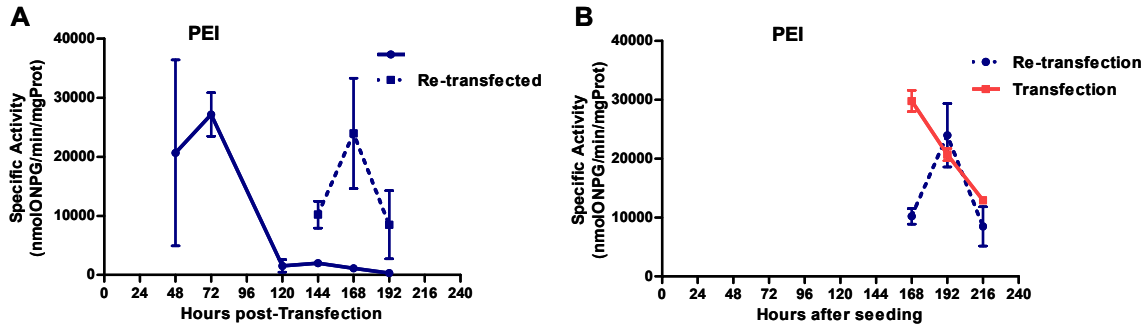

Figure 29: Evaluation of β-gal specific activity after re-transfecting cells with PEI-based vectors. (A) Compares long term transfection activity with the re-transfected cells, while (B) shows the re-transfection control. Representative experiment out of the three performed (average ± SD; n=3).

Re-transfecting cells with PEI-based vectors results in an increase on transfection activity to maximum values (Figure 29A). In this case, the initial peak is not surpassed, in opposition to that described for CHimi-mediated re-transfection. Even though, when cells were transfected and re-transfected in parallel, PEI-mediated transfection activity is comparable – in agreement to what happens in CHimi transfection (Figure 29B).

We showed that transfection activity could be maintained at the highest levels by a repeated administration of the vector. However, to be a useful strategy to increase levels of gene expression, re-transfection process should maintain the cells viable. Therefore, the upshot of this second addition of the vector on the cell viability was evaluated in the following experiment.

No changes on cell viability were detected in cells re-transfected with CHimi-based vectors. A second dose of CHimi-based complexes does not impair the cell metabolic activity (Figure 30). Conversely, PEI-mediated transfection induces some toxicity, similar to that reported in the first time points of the long term studies (see Figure 24). Similar results are obtained if the viability after re-transfection is calculated relative to cells transfected only once.

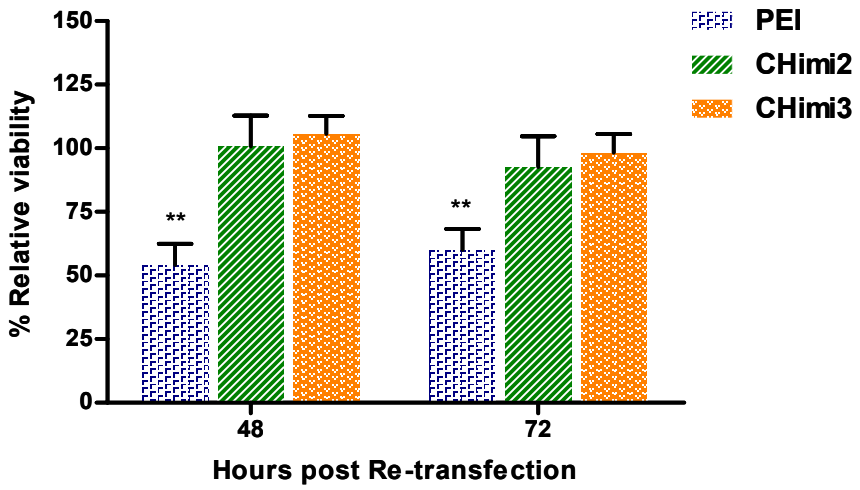

Figure 30: Relative cell viability after re-transfection with CHimi and PEI based vectors. Relative viability was calculated relative to control (non-transfected cells). Representative experiment out of the three performed (average ± SD; n=3). **p<0.001. Homogeneity of variance tested by Barlett's test. Two-way ANOVA and Bonferroni post hoc test. Statistical differences were calculated between the three groups.

2. INTRACELLULAR TRAFFICKING

2.1. Fluorescence microscopy

Intracellular trafficking studies were performed in order to characterize, at the cellular level, the mechanism of transfection mediated by CHimi-based vectors. By means of confocal laser scanning microscopy (CLSM) experiments it is possible to detect particles and to follow their route inside cells. To an accurate intracellular localization of CHimi-DNA complexes, the nucleus (genomic DNA) was fluorescently labeled as well as the filamentous actin (F-actin) of the cytoskeleton. Additionally, both the modified polymer and DNA were fluorescently labeled by covalent bonding with fluorescent tracers (rhodamine and Cy5, respectively).

2.1.1. Fluorescent chitosan-DNA nanoparticles characterization

Among the two polymers CHimi2 was selected for this study as it has the higher number of free amines to react with the fluorescent tracer. CHimi2 was fluorescently labeled with rhodamine. Due to the relevance of complex's physical properties to the cell entrance process and intracellular trafficking [87], after labeling, $CHimi2_{ROX}$ based complexes were characterized in terms of size and zeta potential. Complex characterization intends to evaluate the effect of grafting rhodamine to the CHimi based polymer on the overall complex's properties. The covalent bond with the fluorescent tracer blocks some chitosan free amino groups what could alter the net charge of the polymer and its ability to form complexes (by electrostatic interaction) with DNA. In consequence, the physical properties of the CHimi-DNA complexes could also be affected. Therefore, this characterization was mandatory.

Table 3: Zeta potential, average size (Z-Average) and polydispersity index (PdI) of CHimi2-DNA based complexes. Measurements were performed in acetate buffer 5 mM (pH 5.5) at 25 °C. Zeta potential was calculated according to the Smoluchowski model. $CHimi2_{ROX}$ indicates the fluorescent labeled polymer (average ± SD; n=3).

	Zeta Potential (mV)	Z-Average (nm)	PdI
CHimi2	18.55 ± 1.85	222.50 ± 26.26	0.358
$CHimi2_{ROX}$	20.45 ± 4.66	216.11 ± 45.17	0.333

No significant differences were detected in terms of particle mean size and zeta potential between complexes based on the fluorescently labeled CHimi2 and the non-labeled counterpart (Table 2). These results suggest that the chemical grafting of the fluorescent tracer did not affect CHimi2 ability to complex DNA or CHimi2-based complexes overall physical properties.

Complexes with both fluorescently labeled DNA and CHimi2 were not characterized in terms of particle size and zeta potential since it is not expected that the DNA labeling would significantly change complex properties. The fluorescent tracer covalently binds to DNA nucleotides. Therefore, it is not expected to alter the net negative charge of DNA neither the complex properties, because it does not interfere with DNA phosphate groups, the chemical groups responsible for the electrostatic interaction with CHimi positive groups. Additionally, complexes were prepared with an excess of polymer (N/P molar ratio of 18) and, consequently, the polymer will be predominant on complex properties.

The influence of DNA covalent bonding with fluorescent markers on the DNA trafficking had been reported elsewhere [149]. The authors showed that the plasmid DNA nuclear retention after mitosis differs, depending on the method used to the DNA fluorescent labeling. The present work does not attempt at a detailed study on the fate of plasmid DNA inside cell nuclei and, of our knowledge, there are no reports describing differences on the trafficking of fluorescently labeled complexes.

2.1.2. Confocal Laser Scanning Microscopy

The intracellular trafficking of CHimi-based complexes was studied by CLSM in 293T cells after cell fixation at five time points: 2, 4, 6, 24 and 48 hours post-transfection. For each time point one slide was analyzed per experiment, from a total of three independent experiments. A minimum of 20 images from different areas of the sample were analyzed per evaluation time. Representative images of each evaluation period are presented.

The first hours after transfection are known to be critical on the intracellular trafficking of polymer-DNA complexes [114]. Therefore, the first analyzed time point was **2 hours post-transfection**.
$CHimi2_{ROX}$-DNA_{Cy5} complexes (colored in pink) can enter the cell early after transfection and 2 hours post-transfection they were found in the cell cytoplasm (Figure 31B). However, the majority of complexes were bound to the cell membrane (Figure 31A). Though rarely, during this study, it was possible to find cells during mitosis (Figure 31C).

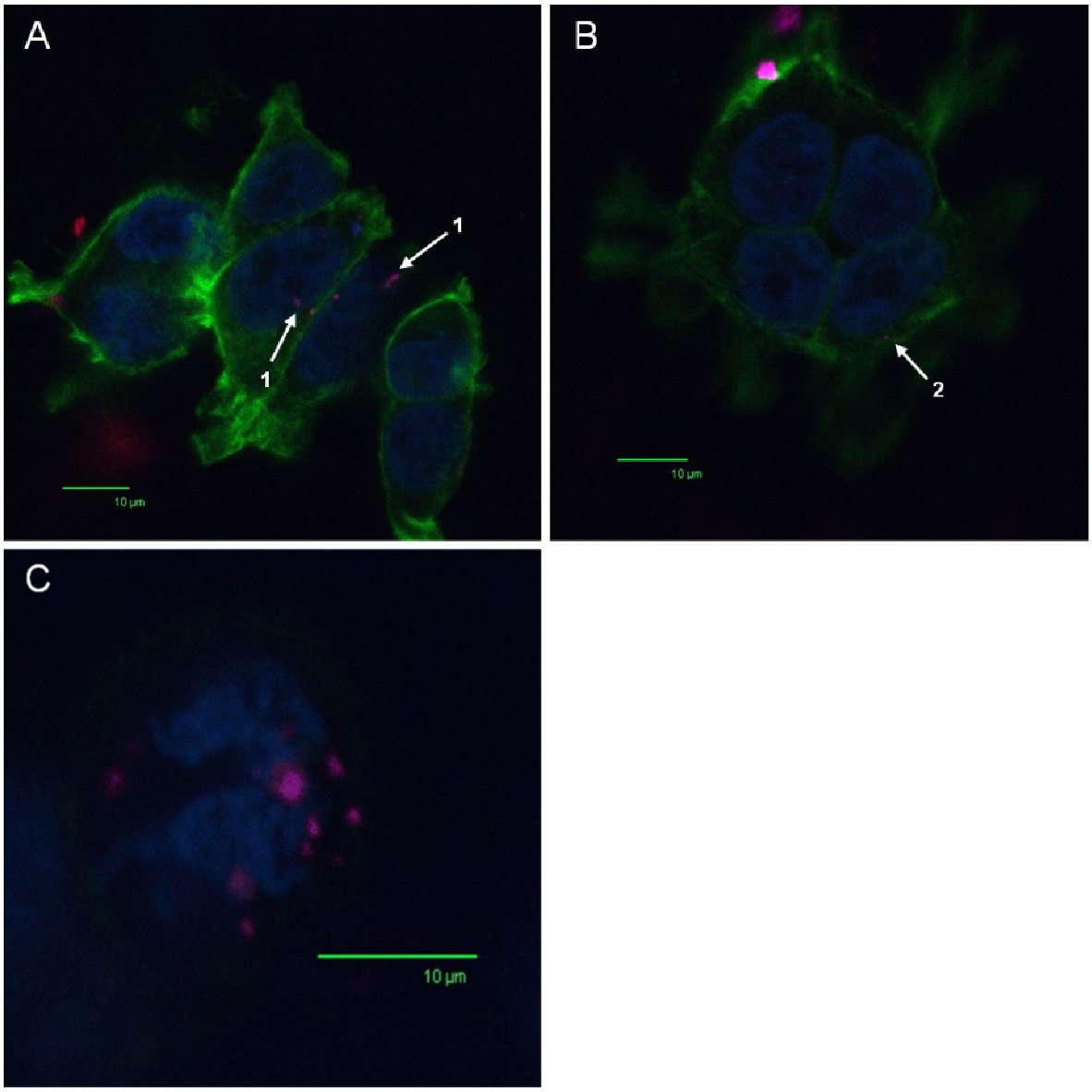

Figure 31: CLSM images obtained 2 hours after transfecting 293T cells with CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes (pink). White arrows indicate complexes: 1- bound to cell membrane; 2- inside the cytoplasm. Cells were stained with phalloidin (F-actin, green) and counterstained with DAPI (genomic DNA, blue).

4 hours after transfection complexes can be found either bound to the cell membrane or within the cell cytoplasm (Figure 32B). Moreover, CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes were found to co-localize with genomic DNA, meaning that the complexes were able to get into the cell nucleus (Figure 32A). From all the complexes detected inside the cell, less than 10% were detected inside the nucleus.

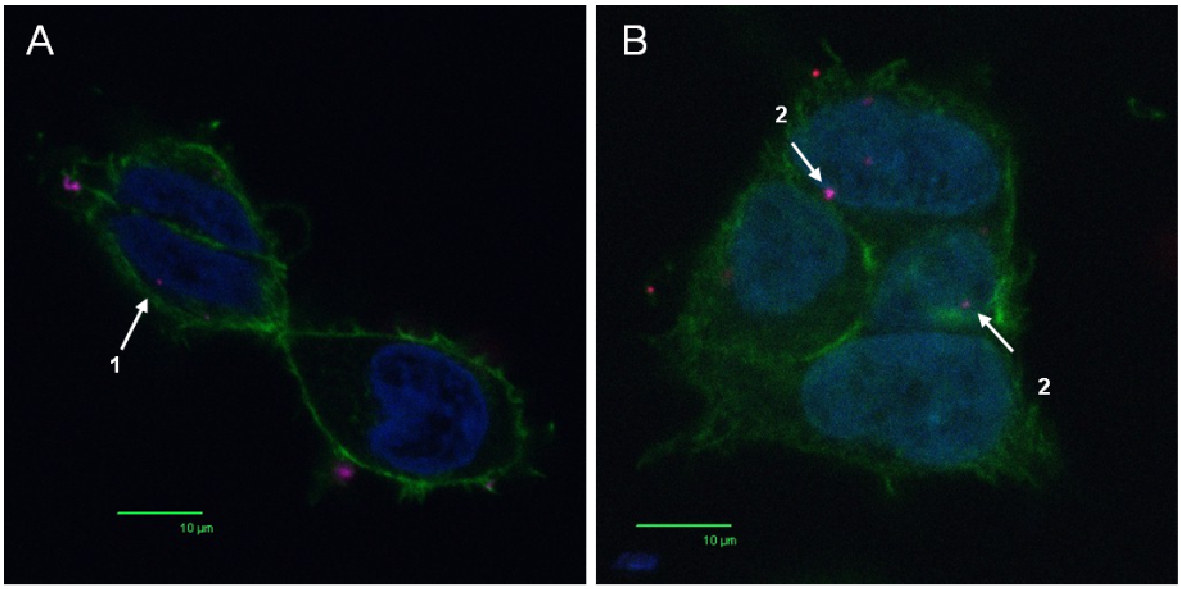

Figure 32: CLSM images of 293T cells 4 hours after transfection with CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes (pink). Cells were stained with phalloidin (F-actin, green) and counterstained with DAPI (genomic DNA, blue). White arrows point: 1- CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes inside the nucleus; 2- complexes bound to the cell membrane.

6 hours post-transfection the scenario is very similar to that described for 4 hours post-transfection. CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes can be found attached to the cell membrane (Figure 33B, 3), complexes can also be localized in the cell cytoplasm or nucleus (see Figure 33A, 1 and Figure 33B, 2, respectively). The detection of complexes inside cell nucleus corresponds to less than 10% of the complexes detected within the cytoplasm.

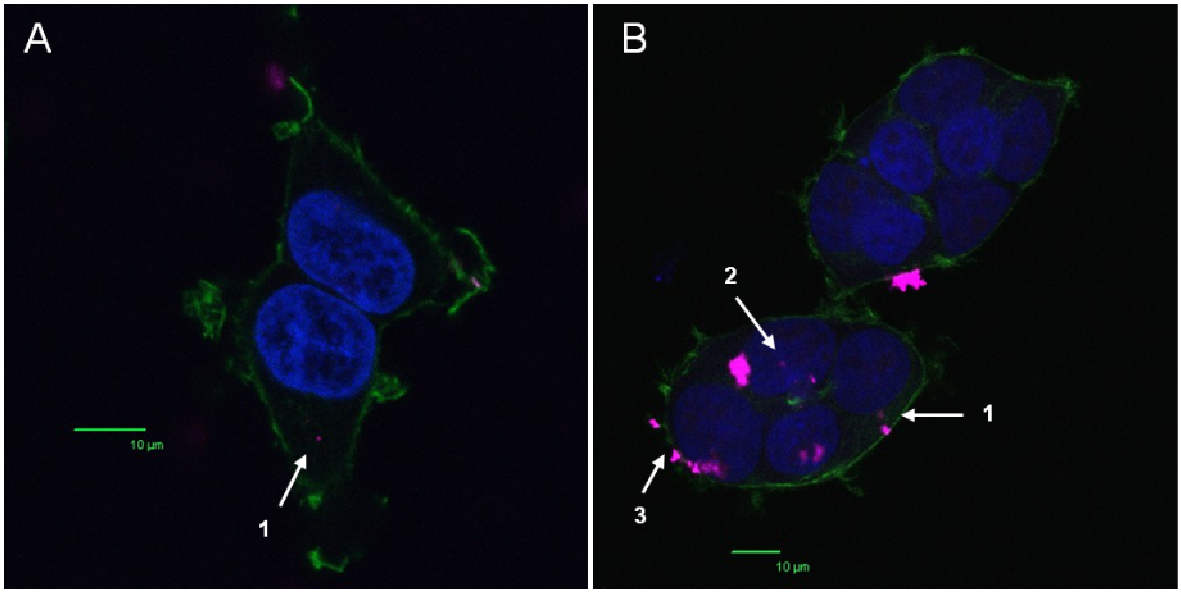

Figure 33: CLSM images obtained 6 hours after transfecting 293T cells with CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes (pink). Cells were stained with phalloidin (F-actin, green) and counterstained with DAPI (genomic DNA, blue). White arrows indicate CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes: 1- in the cell cytoplasm; 2- inside cell nucleus; 3- bound to cell membrane.

Since we were interested in the long term transfection mechanism, cells were also fixed 24 and 48 hours post-transfection and analyzed by CLSM.

At **24 hours post-transfection** the total number of cells in the sample greatly increased. The overall intracellular distribution of CHimi-based particles was maintained. CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes were found bound to the cell membrane and also inside the cell. Intracellularly, complexes were observed in the cytoplasm and in the nucleus (Figure 34B). For the first time it was identified free CHimi2$_{ROX}$ inside the cell (Figure 34A), but no free DNA$_{Cy5}$ was found. Even though, in all the samples analyzed, free CHimi2$_{ROX}$ was only detected at this time point in a rare event.

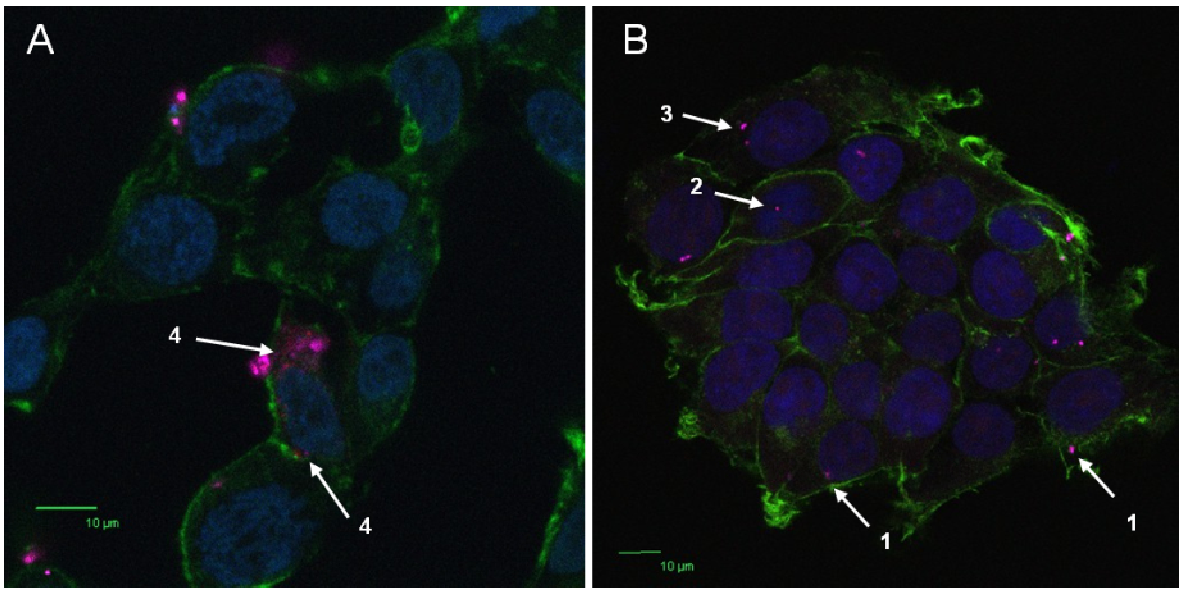

Figure 34: CLSM images of 293T cells 24 hours after being transfected with CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes (pink). Cells were stained with phalloidin (F-actin, green) and counterstained with DAPI (genomic DNA, blue). White arrows indicate: 1- CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes bound to cell membrane; 2- complexes inside the nucleus; 3- complexes inside the cell; 4- free CHimi$_{ROX}$.

The samples collected **48 hours post-transfection** were not fluorescently labeled for F-actin, because, due to GFP expression, transfected cells are naturally labeled green. At this time point, only GFP positive cells were analyzed.

The detection of GFP positive cells suggests that the labeling of DNA with the fluorescent tracer Cy5 did not impair the transcription and/or translation of the plasmid DNA (Figure 35).

CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes were identified inside transfected cells (Figure 35A), within the cell cytoplasm and bound to the cell membrane (Figure 35B).

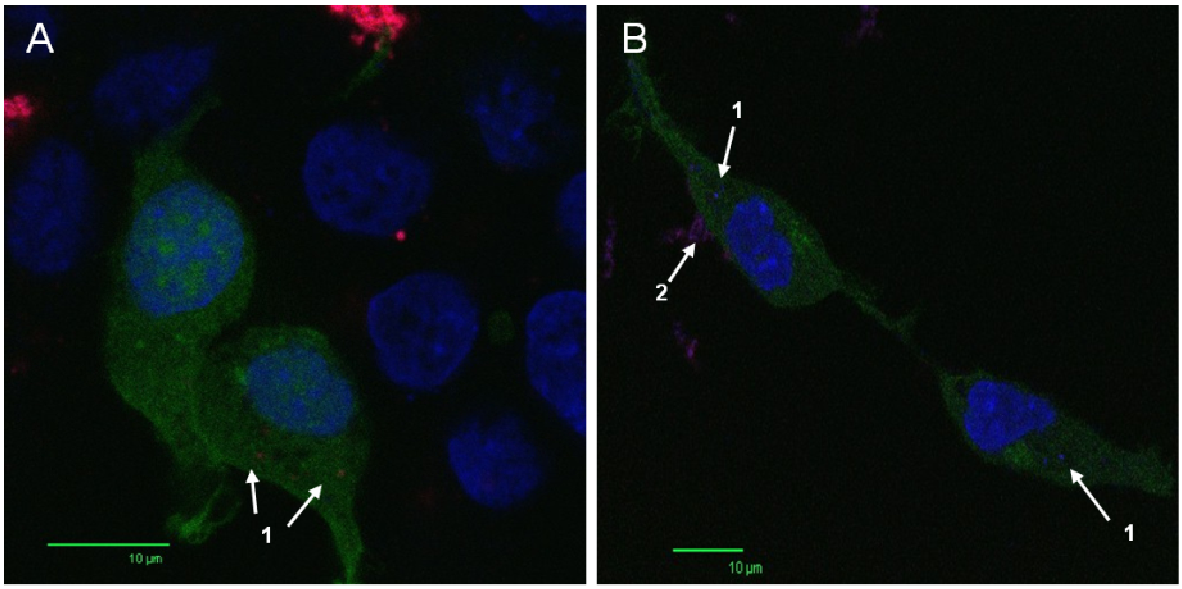

Figure 35: CLSM images of GFP positive 293T cells 48 hours after transfection with CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes (pink). Cell nuclei were stained with DAPI (genomic DNA, blue). White arrows indicate: 1- CHimi2$_{ROX}$-DNA$_{Cy5}$ complexes inside transfected cell; 2- complexes bound to the cell membrane.

2.2. Cell-free gene expression

In order to assess the importance of CHimi-DNA complexes disassembling to the overall gene expression process an *in vitro* transcription/translation assay was performed. The assay consists in a solution based on a reticulocyte lysate that is able to produce a specific protein from a DNA template. The commercial kit used is configured to genes cloned downstream a T7 promoter, therefore, a pET-3a plasmid encoding for the TTR protein [148] was applied. TTR is a homotetrameric protein of 55 kDa, and each monomer has 14 kDa.

Consistent with the intracellular trafficking studies, CHimi2 was selected for this experiment. The stability of CHimi2-based complexes under the experimental conditions of the *in vitro* transcription/translation assay was evaluated before performing the experiment. In brief, CHimi2-pTTR complexes were incubated for 90 minutes at 30 °C – experimental conditions of the cell-free assay – in phosphate buffer solution (0.1 M, pH 7.8). According to information provided by the manufacturer the final pH of the reticulocyte reaction mixture is in the range of 7.7-7.8. Temperature, pH and time are critical parameters for polymer-based complexes, namely in polymer charge density and its ability to complex and protect DNA. Therefore, after incubation, complexes were loaded in a 1% agarose gel and exposed to an electric field, as mean to check if any free DNA exists. As CHimi2 is in a sodium acetate buffer solution (5 mM, pH 5.5), a solution of pTTR in sodium acetate buffer submitted to the same conditions was used as positive control (Figure 36).

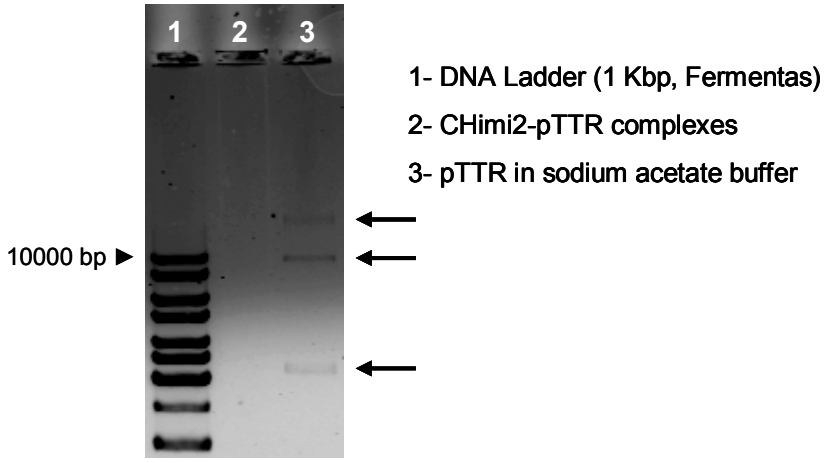

Figure 36: Gel electrophoresis of CHimi2-based complexes after incubation in phosphate buffer (0.1 M, pH 7.8), 90 minutes at 30 °C (lane 2). pTTR in sodium acetate buffer (5 mM, pH 5.5) was used as positive control (lane 3).

No free DNA was detected after the incubation of CHimi-based complexes in a phosphate buffer solution (Figure 36, lane 2). The result suggests that the plasmid remains complexed with CHimi-based polymer at the pH and temperature conditions of the assay. Moreover, the complete retention of DNA shows that the modification in the complex's formation procedure does not interfere with the complexation process.

Due to the modifications introduced in the transcription/translation assay experimental procedure, namely in the final reaction volume, a number of controls were performed (section 2.4.1, chapter III). Reaction 2 aims at controlling the dilution effect caused by the increase in the reaction final volume, whereas the effect of the presence of sodium acetate buffer (pH 5.5) and the possible change in the reaction final pH was equated in reaction 3. Reaction 1 is recommended by the manufacturer as positive control.

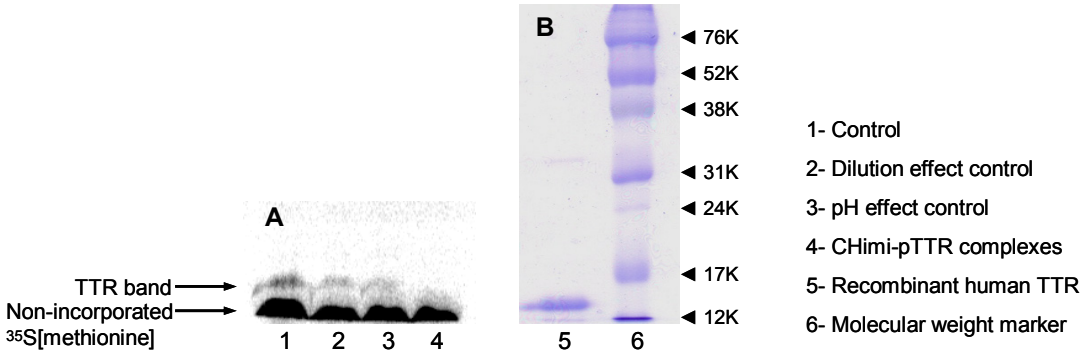

Figure 37: Effect of CHimi-pTTR complexes on the *in vitro* transcription/translation assay of pTTR. All samples run in the same gel, however, due to different subsequent staining procedure, the gel was cut after electrophoresis in two sections: (A) - Phosphor imaging of TNT reaction products; (B)- Coommasie staining of recombinant human TTR and a molecular weight marker (Rainbow molecular weight marker, GE Life Sciences).

In the gel section A (Figure 37A) the radioactive products of the *in vitro* transcription and translation assay can be visualized. A radioactive signal corresponding to the TTR protein is detected in all the controls (lane 1-3). In lane 4 no radioactive signal can be visualized, suggesting that the assembling of plasmid DNA with CHimi-based polymer is impairing the transcription and/or translation in the conditions tested. Figure 37B shows the Coommasie staining of the molecular weight marker and the recombinant human TTR protein (produced in *E. Coli*), confirming the molecular weight of the protein and its position within the gel.

CHAPTER V – DISCUSSION

In a regenerative medicine approach, gene delivery can be used as mean to induce the production of therapeutic proteins *in loci* to promote or modulate regeneration. The delivery of genes encoding for growth factors is particularly appealing due to the important role of growth factors in cell proliferation and differentiation.
To be applied in regenerative medicine a gene vehicle should promote a sustained and time limited protein production. The delivery of growth factors is required only during the healing process and an uncontrolled production of these proteins could be unsafe. Additionally, gene delivery vectors should also guarantee non-toxicity and biodegradability.

In a previous work we showed that grafting imidazole moieties to chitosan results in improved transfection efficiency of the vectors. Chitosan is a cationic polymer of natural origin that, due to its biocompatibility and biodegradability is very attractive for many clinical applications, as for regenerative therapies. In this context, the main objective of the current work is to study the mechanism of transfection mediated by CHimi-based vectors, in a regenerative context. Specifically, we aim to understand if this vector could be applied in a regenerative medicine application and how it can be improved. To achieve this goal, the first step was to perform a detailed study on CHimi mediated gene expression.
Gene expression mediated by CHimi based vectors was assessed for periods up to 8 days. It was shown that the maximum of β-gal specific activity is achieved 72 hours post-transfection and it decreases upon trypsinization and dilution of the cultures. This result does not corroborate our first study on CHimi-mediated transfection, which suggests that transfection efficiency tend to increase up to 96 hours post-transfection [28]. However, these differences seem to be caused by different experimental settings used in both studies. A reduction on transfection activity after cell trypsinization has already been reported in chitosan-mediated transfection [81]. Nevertheless, the analysis of β-gal activity results (without normalizing per mg of protein) showed that the decrease after trypsinization is comparable to the dilution performed. Additionally, after that dilution, β-gal activity was maintained in quite stable values up to the end of the experiment. Since β-gal half-life is about 30 hours in mammalian cells (estimated calculation performed at [150]), the result suggests that new protein is being produced long after transfection.

Non-viral gene delivery vectors are characterized to promote a transient gene expression, due to their inability to induce the transgene integration in the host cell genome. Even though, herein it was demonstrated that CHimi-based vectors promote a sustained gene expression for at least 8 days after the transfection moment. Furthermore, we showed that cells transfected with CHimi-based vectors maintain 100% of cell viability for the all period of the experiment. It remains to be proven if the attained protein levels are sufficient for a therapeutic effect.

In cases where levels of expression obtained with these systems are proven to be insufficient to attain the required therapeutic effect, a repetitive administration of the vector could be the strategy

not only to further increase the protein production, but also to uphold the levels, prolonging the therapeutic effect. It is important to note that non-viral vectors promote a transient gene expression; therefore, for applications where a prolonged therapeutic is required, repeated administration may be necessary. As transfection of 293T cells with CHimi-based vectors showed no signs of cytotoxicity, these vectors are potential candidates for repeated administration of the therapeutic gene. Consequently; the possibility of re-transfect 293T cells has been explored *in vitro*.
When re-transfecting cells with CHimi2 or CHimi3 based vectors the overall β-gal specific activity increased, reaching values higher than the peak obtained at 72 hours after the first transfection. Therefore, a second addition of CHimi-based complexes proved to be a successful strategy to increase β-gal expression. Additionally, re-transfecting cells with these vectors did not induce any toxic effect and cells maintain 100% of relative viability. However, when comparing transfection activity of cells maintained in culture for the same period of time under the same experimental conditions, no differences are detected between cultures transfected one or two times. The above mentioned transfection activity overshoot seems to be dependent on the experimental conditions, rather than the number of CHimi-based complex additions.
To our knowledge there are no published reports where a second transfection was performed *in vitro*. In a similar approach, Cohen and co-workers tried a second addition of plasmid DNA, 48 hours after transfection with liposome-based vector. However, in that case, no increase on transfection activity was reported [151].

To own a stock of cryopreseved cells expressing a therapeutic protein can be of interest prospecting *ex vivo* therapy or a tissue engineering application. To assess the transfection activity stability after a freeze/thawing cycle, cells transfected with CHimi-based vectors were frozen and after thawing β-gal specific activity was again evaluated. Cells transfected with CHimi-based vectors were successfully frozen/thawed without significant decrease of the total cell number relatively to frozen/thawed non-transfected cells. However, in terms of transfection activity of treated cultures it slightly decreased after the freeze/thawing process. Even though, the tendency to a sustained gene expression over time was maintained.

In this study, a commercially available PEI was used as positive control in transfection experiments, since PEI is the cationic polymer most studied for gene delivery. In terms of long term gene expression, a similar profile to the one mediated by CHimi-based vectors was observed. However, the peak on transfection efficiency was achieved 48 hours post-transfection; 24 hours before of that observed in CHimi-mediated transfection. This result is in accordance with Koping-Hoggard and colleagues results [112]. These authors showed that the onset of gene expression on cultures treated with chitosan-based polyplexes is later than that observed when cultures are treated with PEI. The differences on gene expression kinetics were attributed to different onset on the endosomal rupture, since no proton sponge capacity was ascribed to chitosan. However, in our case, some buffering capacity was already proved for CHimi-based complexes (unpublished data).

The improvement on CHimi transfection activity induced by grafting imidazole moieties is impaired in the presence of bafilomycin A1 – a specific blocker of vacuolar proton ATPases. Nevertheless, the "delay" on gene expression promoted by CHimi, comparing to PEI, is maintained, suggesting that processes other than the endosomal rupture can be delaying CHimi-mediated gene expression.

When re-transfecting cells with PEI-based vectors, transfection efficiency increased to the maximum values. In this case the peak attained at 48 hours post-transfection was not surpassed. Due to the relatively higher transfection efficiency of PEI-based vectors comparing to CHimi a possible explanation for this observation may be the saturation of gene expression [36]; i.e. is achieved at each transfection the maximum on β-gal expression in the transfected cells.

An important difference between PEI and CHimi-based vectors is the toxicity. At 48 and 72 hours post-transfection, the relative cell viability of cells transfected with PEI is only 50%. Although statistically significant differences relative to CHimi-based vectors were detected 120 and 144 hours post-transfection, in the defined experimental conditions, cells transfected with PEI gradually reach 100% of relative viability. However, after re-transfecting cells, the same toxic effect was detected. The changes on relative cell viability following re-transfection with PEI are comparable to that detected 48 and 72 hours after the first transfection. This result could be a consequence of a membrane destabilization induced by the high positive charge density of PEI [74, 152]. The toxicity of PEI-based vectors could be hampering the increase on transfection efficiency over the maximum after re-transfection.

Grafting imidazole moieties to chitosan has proved to be an advantageous strategy to improve chitosan-based vectors efficiency [28]. Herein, we show that this polymer has interesting properties to be applied in a regenerative medicine approach. CHimi promotes a sustained gene expression at least for 192 hours after the transfection moment. Additionally, a second addition of the vector could be a strategy to further improve transfection activity or to uphold the levels of the therapeutic protein production. CHimi-based vectors are also suitable to set up a stock of cryopreserved transfected cells expressing a therapeutic gene.

To study the intracellular trafficking of gene vehicles has been pointed up as a mean to design functional and efficient gene delivery systems [26]. To further characterize CHimi-based vectors transfection mechanism, the subsequent step was to follow the intracellular routing of CHimi-DNA complexes.

Cells transfected with complexes based on both fluorescently-labeled $CHimi2_{ROX}$ and DNA_{Cy5} were fixed at 2, 4, 6, 24 and 48 hours post-transfection and observed under CLSM after cell filamentous actin (F-actin) and genomic DNA fluorescent labeling.

$CHimi2_{ROX}$-DNA_{Cy5} complexes were detected within the cell cytoplasm at all the time points analyzed. Some authors showed that chitosan-based vectors could be detected inside the cell since 0.5 hours post-transfection [87]. In the present study, complex distribution was not evaluated

at that time point, but the results confirmed that CHimi-based complexes were able to get into the cell early after transfection (2 hours). Additionally, it has been shown that complexes can also be observed in the cytoplasm of GFP positive cells (transfected cells), two days after the transfection moment. Taking this observation together with the prolonged expression over time mediated by CHimi-based vectors and the experimental evidence that plasmid DNA can not promote transgene expression [28] one can suggest that CHimi is still protecting DNA from nucleases degradation, at such late time points post-transfection.
$CHimi2_{ROX}$-DNA_{Cy5} complexes were also found bound to the cell membrane in all the samples examined, up to 48 hours post-transfection, suggesting that complexes uptake may not be restricted to the first hours after transfection, but it is a process prolonged in time.

In previous studies, polymer-DNA complexes have been detected inside cell nuclei [87, 96, 114]. Accordingly, $CHimi2_{ROX}$-DNA_{Cy5} complexes were found inside cell nucleus 4 hours post-transfection. It was possible to co-localize particles with nuclei staining up to 24 hours post-transfection. In the published literature, it is not completely elucidated how polymer-DNA complexes can enter into the cell nuclei. However, it is consensual that it can happen, at least, when the nuclear membrane is disrupted during mitosis [133]. We showed that 2 hours post-transfection with CHimi-based vectors, cells can be found during mitosis. Those cells are potentially more prone to the uptake of complexes into the cell nucleus.
Intracellular tracking studies are normally focused on the first hours post-transfection, since these are considered to be critical on the complexes routing and fate [114]. Moreover, the literature concerning chitosan intracellular trafficking is scarce [87, 96]. For that reason the comparison of our results to other studies is limited.

In studies concerning transfection mechanisms one important question that needs to be addressed is whether and where polymer-DNA complexes disassemble.
It was proposed that the chitosan-DNA descomplexation occurs in the cell nucleus, based on the observation that no significant disassembling was detected in the cytoplasm [87]. However, up to now, there is no evidence confirming this theory. In our CLSM experiments $CHimi2_{ROX}$-DNA_{Cy5} complexes were detected inside cell nuclei and, from all the samples analyzed, free $CHimi2_{ROX}$ was only detected intracellularly in the cytoplasm, 24 hours post-transfection, in an atypical event. DNA_{Cy5} was never detected in the free form, neither in the nucleus nor in the cytoplasm. Based on these results, the disassembling of $CHimi2_{ROX}$-DNA_{Cy5} complexes can not be proved. Some authors have proposed that transcription and translation are not impaired if DNA is tightly complexed with the PEI-based polymer; that is, gene expression can occur without disassembling of the complexes [116]. To test this hypothesis we had performed an *in vitro* transcription/translation assay with CHimi-based complexes. The results showed that when DNA is complexed with CHimi-based polymer, no protein production is detected, pointing to the importance of complex disassembling prior to gene transcription. This event seems to be critical to a

successful expression of the gene, in accordance with the previously proposed by Huang and co-workers [87].
Assuming that disassembling of complexes is critical for gene expression one could expect to find free DNA_{Cy5} by CLSM. Since it did not occur, we hypothesize that the experimental conditions or the technique used were not sensitive enough to detect free plasmid DNA, due to the number of molecules or the small size of such construct. Moreover, when DNA is free it is also more prone to be degraded, particularly if the disassembling takes place in the cytoplasm. A rapid DNA degradation could make arduous its detection.

Concerning the question "where complexes disassemble", some theories are published. It has been proposed that the DNA release from polymer-based complexes occurs in the nucleus by competition with RNA [129], nuclear proteins or DNA [126]. Since we were not able to identify free DNA_{Cy5} in our CLSM experiments we can not suggest about the local of $CHimi_{ROX}$-DNA_{Cy5} complex disassembling. However, one can analyze the proposed theories taken into account the results obtained in the *in vitro* transcription/translation assay. The mixture where the *in vitro* transcription/translation assay occurs consists in a reticulocyte lysate, which exact constitution is not described, but it is known that it contains nuclear proteins as well as RNA. Within the competitors proposed, only genomic DNA is absent of the mixture. However, a recent work of Bertschinger and co-workers consider that it is unlikely that genomic DNA is involved in the intracellular disassembly of PEI-DNA particles [129].
CHimi-based complexes disassembling could not fit in these competition theories. Since we are working with a degradable polymer, we can hypothesize that the disassembling of complexes is dependent on the degradation of the polymer, rather than electrostatic competition within the cell nucleus. Chitosan β-(1–4) linkages between D-glucosamine and N-acetyl glucosamine residues are susceptible to be hydrolyzed by several enzymes, namely in the lysosome [77]. The sustained gene expression mediated by CHimi-based vectors could be a consequence not only on the limited buffering capacity of the polymer – and therefore a prolonged stay in the endosomal route – but also on the degradation of the polymer and the resultant release of DNA. Polyplex disassembling before entering into the cell nucleus have recently been proposed for lPEI-DNA complexes, but the mechanism that triggers the DNA release is not clarified [62].
The present results showed that CHimi-based complexes can be detected inside the cell cytoplasm as well as inside cell nucleus. However, the particles that can enter the cell nucleus seem not to be the ones responsible for the expression of the gene. Tuning the degradation rate of CHimi-based polymers could be a strategy to control the disassembling of complexes and ultimately the production of the therapeutic protein.

CHAPTER VI – CONCLUDING REMARKS AND FUTURE WORK

The obtained results show that the CHimi-based vectors under study promote a sustained gene expression, up to 8 days after transfection. The transfection activity can be increased by a second addition of CHimi-based complexes without compromising cell viability. Additionally, cells transfected with CHimi-based vectors are able to withstand a freeze/thawing cycle, maintaining a sustained transfection activity.
In terms of intracellular trafficking, CHimi-based complexes were found within the cell cytoplasm at all evaluation times, being the longest 48 hours post-transfection. Additionally, our observations show that complexes are able to enter the cell nucleus, co-localizing with genomic DNA. However, the overall number of complexes found in the nucleus was low comparing to the number detected in the cytoplasm. Though rarely, free labeled-CHimi was observed inside the cell cytoplasm. Moreover, fluorescently labeled DNA was not observed in a free form. However, when CHimi-based complexes are tested in a cell free *in vitro* transcription/translation assay, no protein production is detected. These results lead us to pinpoint the importance of CHimi-based vectors disassembling to achieve a successful gene expression. The disassembling seems to be critical for DNA transcription and/or translation.

Based on the results obtained in this work, we hypothesize that CHimi-based complexes disassembling could be dependent on the degradation rate of the polymer before the nuclear uptake and that those complexes that are detected inside nuclei are not the ones responsible for the transcription of the gene. A complementary study, consisting in the injection CHimi-based complexes into the cell nucleus, could attest the relevance of this process to allow the expression of the gene.

The work presented was mainly focused on the study of the intracellular mechanisms of the transfection process and the complex uptake itself was not examined. To a complete understanding on CHimi transfection mechanisms the cellular uptake should be addressed. Additionally, and considering the number of complexes detected in the CLSM studies, to target the uptake of CHimi-based complexes could be an important strategy to improve transfection efficiency.

In the present work, 293T cells were used as model cells. In the framework of the project in which this work is involved, and prospecting an application on neuroregeneration, studies on neuronal cell lines are under investigation.

CHimi-based vectors show important properties to be applied in a regenerative medicine approach. A sustained gene expression can guarantee a prolonged therapeutic effect. If the levels of protein expression are not sufficient or if an extended therapeutic effect is required, a repetitive administration of CHimi-based vectors could be performed as a gainful strategy. Additionally, based on our hypothesis, gene expression profile may also be adjusted to fulfill a therapeutic end

by tuning the degradation rate of the polymer. Currently, the use of CHimi of lower molecular weight is under research. In a regenerative medicine scenario, to have cryopreserved cells expressing a specific growth factor could reduce the time lag to a clinical application. Even though, to evaluate if the attained protein levels are sufficient for one achieve a therapeutic effect is a question that needs to be addressed, ultimately, *in vivo*.

CHAPTER VII – REFERENCES

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