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PhD Thesis

Biomaterial based vectors for gene delivery in the peripheral nervous system: a non-viral gene therapy approach towards neuroregeneration

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Abstract

The peripheral nervous system (PNS) may be subject to damage by a variety of insults. As current therapies to treat peripheral neuropathies are mainly palliative than curative, new strategies are awaited. In the recent years, enormous progress has been made in our understanding on the biology of neurotrophic factors and how they may be applied in the treatment of neurologic diseases. These advances have paved the way for new therapeutic approaches that may arrest or reverse the disease process underlying many types of peripheral neuropathies. However, these potent peptides have short systemic half-life and cannot be administrated to patients for long periods of time, due to unwanted systemic effects. To overcome these limitations, the principle of gene therapy, which relies on the use of DNA as a pro-drug that can lead to the production of therapeutic proteins within specific cells, recently got much attention in the field.

The main objective of the present work was the development of safe and effective non-viral DNA delivery systems targeted to the PNS neuron cell population.

As a starting material poly(ethylene imine) (PEI) was used, as it is the gold standard cationic polymer for gene delivery, which has been shown to efficiently transfect cells both *in vitro* and *in vivo*. PEI thiolation was performed in order to prepare nanoparticles with reduced overall charge, with the aim of limiting non-specific interaction of the nanoparticles with the cell surface, and providing the nanoparticles with reactive groups that allow the grafting of targeting moieties to the nanoparticle surface. In the present study, the non-toxic fragment from the tetanus toxin (HC) was chosen as the targeting moiety, due to its ability to specifically interact with peripheral neurons and to undergo retrograde transport. The developed system showed to transfect, in a targeted fashion, a sensorial neuron cell line (ND7/23) and neurons in primary cultures of dissociated dorsal root ganglia. The designed system is based on a modular structure, which would allow the control of the targeting moieties amount on the nanoparticle surface. Indeed, it was demonstrated that the quantity of the targeting moieties at the nanoparticle surface is of critical importance to the transfection outcome. This was further established by molecular recognition force spectroscopy, using atomic force microscopy (AFM), technique that was proposed as

an efficient tool for the optimization of the density of targeting moieties linked to a nanoparticle towards achieving cell-specific interactions.

In view of a regenerative medicine application, the development of a chitosan (CH)-based system was also pursued. CH presents several advantages over PEI, namely, biodegradability and biocompatibility. However, CH has limited transfection efficiency, attributed to its reduced buffering capacity. To overcome this limitation CH was modified with imidazole moieties, which constitute the side group of the amino acid histidine. Imidazole-grafted CH (CHimi) showed superior transfection results, in comparison to the parental polymer, in two different cell line models - human embryonic kidney (293T) and human liver carcinoma (HepG2) cells - *via* the improvement of its buffering capacity, which was hypothesized to promote the endosomal escaping ability of the CHimi-based complexes. Following the lessons learned from the designed PEI systems, the development of a thiolated CHimi-based neuron targeted system able to transfect the ND7/23 cell line, as well as neurons in dorsal root ganglia primary dissociated cultures, at no cost of cell viability, was achieved.

Finally, the efficiency of the proposed strategy to achieve peripheral neuron targeting after a minimally invasive peripheral administration was assessed *in vivo* using the developed PEI-based nanoparticles. The PEI-based vectors promoted the transgene expression in the lumbar dorsal root ganglia, with marginal expression in other tissues, and apparent lack of inflammatory associated reactions and toxic effects.

The obtained results, as well as the versatility and ease of preparation of the developed targeted systems renders these type of nanoparticles promising tools for *in vivo* gene therapy applications in the treatment of peripheral neuropathies.

Resumo

O sistema nervoso periférico (SNP) pode ser danificado em consequência de uma grande variedade de lesões. Dado que as terapias actualmente existentes para o tratamento de neuropatias periféricas são principalmente paliativas em vez de curativas, novas estratégias são necessárias. Ao longo dos últimos anos o nosso conhecimento sobre factores neurotróficos e as suas aplicações no tratamento de doenças neurológicas tem sofrido um enorme avanço. Efectivamente, o conhecimento adquirido abriu caminho para o desenvolvimento de novas estratégias terapêuticas que visam retardar ou reverter o processo patológico subjacente a muitos dos tipos de neuropatias periféricas. No entanto, estes péptidos apresentam um tempo de semi-vida sistémica curto e, adicionalmente, não podem ser administrados por longos períodos de tempo, devido a efeitos sistémicos indesejados. O princípio da terapia génica, baseado no uso de ADN como um pró-fármaco que pode levar à produção de proteínas terapêuticas em células específicas, pode, no entanto, todavia as referidas limitações.

O principal objectivo do presente trabalho é o desenvolvimento de sistemas não-virais para a entrega de genes de uma forma específica e segura na população de neurónios do SNP.

A poli(etileno imina) (PEI) foi usada neste estudo como material de base, dado ser um polímero catiónico que demonstrou uma grande eficiência na transfecção celular, quer *in vitro*, quer *in vivo*. De forma a reduzir a carga das nanopartículas produzidas, e desta forma limitar a interacção não específica destas com a superfície das células, procedeu-se à tiolação do PEI. Tal modificação permitiu também provir as mesmas de grupos reactivos aos quais se podem ligar moléculas de reconhecimento celular específico. No presente estudo, o fragmento não-tóxico da toxina do tétano (HC) foi escolhido como molécula de reconhecimento específico, devido à sua capacidade de interagir especificamente com neurónios periféricos e desempenhar transporte retrógrado.

O sistema desenvolvido demonstrou transfectar, de forma específica, uma linha celular de neurónios sensoriais (ND7/23) e neurónios de uma cultura primária de gânglios da raiz dorsal dissociados. Dado o sistema descrito apresentar uma estrutura modular, permite o controle da quantidade de molécula de reconhecimento

específico que se encontra à superfície da nanopartícula. De facto, demonstrámos que a quantidade de molécula de reconhecimento específico presente na superfície das nanopartículas tem uma importância crucial para o resultado final de transfecção. Este facto foi comprovado e aprofundado pelo uso de espectroscopia de força de reconhecimento molecular, utilizando microscopia de força atómica (AFM), técnica esta proposta como uma ferramenta inovadora para o processo de optimização de nanopartículas com capacidade de reconhecimento celular específico.

Com vista de uma aplicação em medicina regenerativa, um sistema baseado em quitosano (CH) foi igualmente desenvolvido. O CH apresenta várias vantagens em relação ao PEI, incluindo biodegradabilidade e biocompatibilidade. No entanto, o CH enquanto vector de genes possui uma eficiência de transfecção limitada, que fica a dever-se à sua reduzida capacidade tampão. De forma a superar essa limitação procedeu-se à modificação do CH com moléculas de imidazole, que constituem o grupo lateral do aminoácido histidina. A modificação do CH com grupos imidazole (CHimi) demonstrou melhorar a sua eficiência de transfecção em dois modelos de linhas celulares – células embrionárias humanas do epitélio do rim (293T) e células humanas de carcinoma do fígado (HepG2) - através da melhoria capacidade tampão do polímero. Tendo em conta o conhecimento adquirido com o sistema baseado em PEI, procedemos ao desenvolvimento de um sistema de entrega de genes usando CHimi tiolado, que se mostrou capaz de transfectar, de uma forma específica, a linha celular de neurónios sensoriais ND7/23 e neurónios de uma cultura primária de gânglios da raiz dorsal dissociados.

Por fim, o uso dos vectores baseados em PEI foi testada num cenário *in vivo*, de forma a avaliar a sua eficiência para mediar a entrega de genes em neurónios periféricos após uma administração minimamente invasiva. Os vectores baseados em PEI foram capazes de promover a expressão de um gene repórter nos gânglios lombares da raiz dorsal, com uma expressão marginal noutros tecidos. Adicionalmente, não foram detectadas reacções inflamatórias e/ou efeitos tóxicos associados.

Os resultados obtidos, bem como a versatilidade e a facilidade de preparação dos sistemas desenvolvidos, tornam estas nanopartículas promissoras para futuras aplicações de terapia genética *in vivo*, no possível tratamento de neuropatias periféricas.

Resumé

Le système nerveux périphérique (SNP) peut être soumis à différents types de dommages causant son disfonctionnement. Étant donné que les thérapies proposées de nos jours ont un pouvoir palliatif plus que curatif, de nouvelles stratégies pour traiter les neuropathies périphériques apparaissent nécessaires. Récemment, la fonction et le mécanisme d'action des facteurs neutrophiques ont été décrits et leur utilisation pour traiter des maladies neurologiques est envisagée. Ces avancées scientifiques ouvrent ainsi la voie au développement de nouvelles stratégies thérapeutiques pouvant retarder ou inverser le processus pathologique touchant plusieurs types de maladies neurologiques du SNP. Cependant, après leur administration par voie systémique, les peptides neurotrophiques ont une durée de vie limitée et peuvent provoquer des effets secondaires indésirables pour le patient. De ce fait, le principe de thérapie génique basée sur l'utilisation d'ADN comme « pro-médicament » conduisant à la production au sein de cellules spécifiques de protéines thérapeutiques, fait l'objet d'attentions particulières.

L'objectif de ce travail de thèse a été de développer un système non-viral, sûr et efficace de délivrance d'ADN ayant la capacité de cibler spécifiquement les cellules neuronales du SNP.

Considéré comme le polymère de référence dans la délivrance de gènes et étant capable de transfecter efficacement des cellules de manière *in vitro* et *in vivo*, le poly(ethylene imine) (PEI) a tout d'abord été utilisé. Des groupes thiol ont été ajoutés au PEI, ce qui a permis de réduire la charge totale des nanoparticules formées, limitant ainsi les interactions non spécifiques à la surface cellulaire. De plus, cette modification chimique crée des groupes réactifs permettant le greffage de molécules de ciblage à la surface des nanoparticules. Ayant la capacité d'interagir spécifiquement avec les neurones du SNP et d'être internalisé puis transporté jusqu'au noyau, le fragment non toxique (HC) de la toxine tétanique a été utilisé comme molécule de ciblage dans cette étude. Il a été démontré que le système développé était capable de transfecter spécifiquement la lignée cellulaire de neurones sensoriels ND7/23 ainsi que des neurones de culture primaire issus de la dissociation du ganglion spinal. La quantité de fragment HC greffé à la surface des nanoparticules peut être contrôlée de manière précise et il a été montré que l'efficacité de transfection du système dépend de cette densité. D'autre part, cette

relation a été confirmée par la technique de reconnaissance moléculaire par force spectroscopique, en utilisant la microscopie à force atomique (AFM). Ainsi, cette technique a été proposée comme étant un outil efficace permettant d'optimiser la densité de molécules de ciblage présentes à la surface de nanoparticules conduisant à une interaction cellulaire spécifique.

Afin d'appliquer ce système en médecine régénérative, nous avons poursuivi ce travail en développant un système basé sur l'utilisation du chitosan (CH), polymère naturel présentant les avantages d'être biodégradable et biocompatible. Néanmoins, le CH a un pouvoir de transfection limité à cause de sa capacité réduite à tamponner le pH de son environnement. Afin d'outrepasser cette limitation, des molécules d'imidazole, qui correspondent au groupe amine terminal de l'histidine, ont été greffées au CH (CHimi). Grâce à cette modification, la transfection de deux types de lignées cellulaires humaines, les cellules embryonnaires de reins (293T) et les cellules tumorales de foie (HepG2) a été plus efficace, en comparaison avec les nanoparticules formées à partir du CH non modifié. En effet, l'ajout d'imidazole permet au CHimi de tamponner le pH à l'intérieur des endosomes de manière plus efficace et les nanoparticules peuvent être libérées au sein du cytoplasme avant d'être dégradées. De la même manière que pour le PEI, des groupements thiol ont été ajoutés au CHimi et les nanoparticules formées ont montré une transfection spécifique efficace de la lignée cellulaire ND7/23 ainsi que des neurones de culture primaire issus de la dissociation du ganglion spinal, sans entraîner de cytotoxicité.

Enfin, le pouvoir de transfection des nanoparticules formées à partir de PEI ainsi que leur spécificité envers les neurones périphériques ont été étudiés *in vivo* après injection chez le rat. L'injection des nanoparticules au niveau de la patte n'a pas entraîné de réaction inflammatoire ni de toxicité apparentes. De plus, l'expression du transgène a été détectée de manière évidente au sein des neurones du ganglion spinal au niveau lombaire, et faiblement dans les autres tissus.

Au regard des résultats obtenus et grâce à sa préparation relativement simple permettant de moduler à volonté ce système de délivrance génique, l'utilisation de ces nanoparticules *in vivo*, dans un contexte de thérapie génique pour le traitement des pathologies du SNP paraît prometteur.

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Chapter I

Aim and structure of this thesis

A variety of diseases affecting the peripheral nervous system (PNS) still remain untreatable. These may include sensory neuropathies, motor neuron diseases and peripheral nerve injury [1]. Depending on their extent these can have unrecoverable consequences to the patient with total loss of functionality in the more severe cases. One of the challenges currently facing neuroscientists is the development of effective therapies based on the advances achieved in basic research.

The use of genes as pro-drugs can be faced as a strategy in order to reduce this gap. The principle of gene therapy, which relies on the use of DNA as a pro-drug that can lead to the expression of therapeutic proteins within specific cells, recently got much attention. Indeed, the development of a vector that could, locally and in a time controlled fashion, lead to the production of a neurotrophic factor that would augment the cell survival or regeneration is an approach that has been proposed to solve this problem.

In gene therapy, viruses have proved to be the most efficient system to mediate gene delivery and expression. However, their use in a clinic scenario raises obvious safety concerns [2-5]. Over the past decades there have been many attempts to design non-viral vectors that could overcome the safety concerns of viral systems, be biocompatible, and present no limitations in terms of large scale production in a cost effective way. The majority of these systems is cationic in nature and interacts with the negatively charged DNA trough electrostatic interactions forming a nanoparticle.

The main aim of this work was the development of a safe and efficient non-viral gene delivery vector that could achieve the delivery of therapeutic genes targeted to the PNS neuron cell population.

In the design of a targeted nanoparticle-based system several points have to be considered. Namely, the size and charge of the nanoparticle, its stability in physiological milieu, the nature, density and exposure of the targeting moieties, the delivery efficiency of the system, the duration and kinetics of the transgene expression and the toxicity profile.

In **Chapter II**, an overview of the main viral and non-viral gene delivery vectors and strategies applied are exposed. The drawbacks and promises of the application of each vector type to the nervous system are described with a final focus on non-viral

vectors. The main barriers that have to be overcome by a non-viral system towards achieving a targeted and efficient gene delivery are also discussed.

Among the polycationic non-viral vectors, poly(ethylene imine) (PEI)-based delivery systems have been shown to efficiently transfect cells, both *in vitro* and *in vivo* [6] and even to be able to mediate transfection activity in levels comparable to the ones obtained by viral vectors after brain injection [7]. However, PEI based complexes interact with cells in a non-specific manner, mainly due to electrostatic interactions resulting from the high positive charge of PEI-DNA complexes prepared with an excess of PEI content [8], as this is *sine qua non* in order to attain gene protection and efficient transfection.

Aiming at circumventing the depicted problems, in **Chapter III,** we describe a simple and efficient system that could specifically transfect peripheral sensorial neurons, based on PEI and on the 50 kDa non-toxic fragment from the tetanus toxin (HC). Initially, by means of polymer thiolation one could reduce the PEI-DNA complex overall charge, reducing non-specific interactions with the surface of cells, expectedely increase complex stabilization in the extracellular milieu, due to the formation of disulfide bonds, and, finally, to allow the grafting of the HC targeting moieties to the nanoparticle surface. The HC fragment was chosen as targeting moiety as it has been shown to interact specifically with peripheral neurons and to undergo retrograde transport [9]. Additionally, previous studies showed that PEI-poly(ethylene glycol) (PEG) copolymer-based complexes had increased blood circulation and reduced toxicity, when injected intravenously [10]. Therefore the design of the proposed system considered the grafting of the HC fragment to the nanoparticle surface *via* a bifunctional thiol reactive 5 kDa PEG, acting the PEG as non-fouling coverage and as a spacer, towards achieving an optimal exposure of the protein to interact with cell surface. Producing the targeted nanoparticles in two steps allowed the independent tuning of the nanoparticle system core and of the amount of the targeting moieties at the nanoparticle surface. The developed system was shown to be able to transfect in a targeted fashion a sensorial neuron cell line (ND7/23) and neurons in primary cultures of dissociated dorsal root ganglia.

In fact, the amount and exposure of the targeting moieties in the nanoparticle surface are critical parameters regarding the targeting potential of a nanosized delivery system [11, 12]. Following that premise, in **Chapter IV** we establish, in an *in vitro* scenario and by means of atomic force microscopy (AFM) studies a new screening

method for the tailoring of targeted nanoparticle systems. Here, we demonstrate that molecular recognition force spectroscopy could be used as a tool to optimize the targeting moiety density of a nanoparticle system towards attaining optimal cell-specific interaction, as showed for the chosen neuronal cell model - ND7/23 cells.

When aiming at an application in a regenerative medicine scenario, the biodegradability and cytotoxicity of the selected delivery system are a major cause of concern. Aware of this hurdle, we pursued the development of such systems using chitosan (CH) as a starting material, a biodegradable polymer with a well-established biocompatibility [13]. CH has been previously proposed as a good candidate for gene delivery but it presents low transfection efficiency on its own. The high efficiency of PEI as a transfection agent has been attributed to its buffering capacity in the endosomal pH range (7.4-5) [14]. Indeed, CH has a limited buffering capacity [15] and taking into account the CH limitations, in **Chapter V** the imidazole grafting of CH (CHimi) is described. By increasing the buffering capacity of CH, an improvement of transfection efficiency was observed in both 293T (human embryonic kidney 293 cells) and HEPG2 (human hepatocellular carcinoma) cell lines, without compromising its biocompatibility [16].

Following the lessons learned in **Chapter III**, in which the design of the targeted PEI-based vectors is described, and inspired with the promising outcome of the grafting of CH with imidazole functionalities, in **Chapter VI** we pursued the development of a neuron targeted systems using CHimi as a starting material. In this chapter it is described the development of a thiolated CHimi-based neuron targeted system able to transfect a neuronal cell line (ND7/23), as well as neurons in dorsal root ganglia primary dissociated cultures, at no cost of cell viability [17].

The ultimate test to a gene delivery system is its applicability in an *in vivo* scenario. Ensuing the promising results obtained for the PEI-based system *in vitro,* we tested, in a minimally invasive peripheral administration, the applicability and biodistribution of the targeted nanoparticles in a rat model, as described in **Chapter VII**. Subcutaneous peripheral administration of the developed vectors led to the transgene gene delivery and respective protein expression in the lumbar dorsal root ganglia, with neglectable expression in other tissues. Moreover, the apparent lack of local inflammatory reactions and toxic effects could render these vectors as very promising one for use in a clinical scenario. However, aditional studies are necessary

to further assess their potential. Indeed, we are currently performing new studies in order to confirm the targeting potential of this system.

Finally, **Chapter VIII** provides the concluding remarks, correlating the overall obtained results and suggesting new avenues for future research.

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Chapter II

A non-viral gene delivery approach targeted towards the Peripheral Nervous System

1. Peripheral Neuropathies

The peripheral nervous system (PNS) is constituted by sensory and motor neurons outside the brain and the spinal cord. Sensory nerves, originated from clusters of neurons called ganglia, conduct sensory signals from organs and skin to the central nervous system (CNS). Motor nerves, originated from motor neurons situated in the spinal cord, transmit stimulatory signals from the CNS towards muscles and glands.

PNS problems are common and encompass a large spectrum of traumatic injuries, diseases, tumors or iatrogenic lesions [1]. Injuries to the peripheral nerves may result in a partial or total loss of motor, sensory and autonomic functions in the involved segments of the body. One of the most common peripheral neuropathy is due to diabetes mellitus. It has been estimated that about 50% of the individuals who have had diabetes for more than 25 years suffer symptomatic peripheral neuropathy [2]. Indeed neuropathies are one of the major contributors to morbidity in patients with diabetes mellitus [3]. In the course of HIV-1 (human immunodeficiency virus type 1) infection, up to 50% of the patients suffer from neurological complications affecting either the central CNS or the PNS [4]. Genetic factors may also contribute to the onset of PNS pathologies. Familial amyloidotic polyneuropathy is one example. A mutation in a single base pair of the transthyretin gene leads to amyloid depositions in the extracellular matrix throughout the connective tissue, with exception of the brain and liver parenchyma and affecting particularly the PNS, leading to organ dysfunction and ultimately death [5, 6]. Sensory impairment is amongst the first symptoms, with pain and temperature sensations being the most severely affected. Motor involvement occurs later in the course of the disease causing wasting and weakness. There is a progressive loss of reflexes with upper limb involvement occurring months to years after lower limb manifestations [7].

Conventional treatments for peripheral neuropathies have primarily been palliative rather than curative. Perhaps most importantly, they have often been ineffective [8]. In the recent years, enormous progress has been made in our understanding of the biology of neurotrophic factors and how they may be applied in the context of neurological diseases. These advances have suggested new therapeutic approaches that may arrest or reverse the disease process underlying many types of peripheral neuropathies [8]. Studies with recombinant peptides have demonstrated that a number of neurotrophic factors, including nerve growth factor (NGF), neurotrophin-3 (NT-3), insulin-like growth factor (IGF) and vascular epithelial growth factor (VEGF)

can prevent the degeneration of peripheral sensory axons [8]. But these potent short-lived peptides cannot be administrated to patients for long time, due to unwanted systemic effects [9].

The lack of effective response presented by modern therapeutics and the crescent need for new treatments has brought new interest in the gene therapy approach. There are several reasons for the use of therapeutic gene transfer or gene therapy in the treatment of neuropathologies. More unique RNA sequences are expressed in the nervous system and a large proportion of the identified genetic diseases display a neurologic component to the phenotype. In many cases, the regional specialization of nervous system function dictates that a therapeutic intervention may be best achieved by the local and specific expression of a transgene product, such as a neurotrophic or anti-apoptotic factor. In addition, the local expression of neurotrophic factors achieved by gene transfer may be used to achieve desired outcomes avoiding unwanted adverse effects that could result from systemically administrated drugs [10].

Extensive work has been developed in the recent years in order to optimize and accomplish successful gene transfer to mammalian cells. Non-viral and viral strategies have been attempted, but an effective and safe system has yet to be achieved, as the drawbacks presented by each of the strategies were never solved. Nevertheless, the number of clinical gene therapy trials continues to grow, with over 1579 completed, ongoing or pending worldwide [11].

2. Gene Therapy

Gene therapy can be defined as a strategy that provides to somatic cells the genetic information required for producing specific therapeutic proteins in order to correct or modulate determined pathologies. Somatic gene therapy aims at overcoming some of the drawbacks presented by direct protein administration, including low bioavailability, immune response, *in vivo* instability, high hepatic and renal clearance, and high cost of production [12]. Therefore, providing a therapeutic gene as a “pro-drug” to a patient may circumvent some of the limitations associated with the direct use of recombinant proteins.

An effective gene delivery system should achieve the protection of the complex from premature degradation in the extracellular environment, cell specificity of the gene targeting, delivery of the gene to sufficient cells, and expression of the gene at high

enough concentrations and with sufficient persistence to produce a therapeutic effect [13, 14].

Currently, the major obstacle to the actual realization of gene therapy is the development of a non-toxic and effective delivery system [15]. Gene expression results when genetic material is transported inside the target cell and it is currently possible to obtain local transient transgene expression when naked plasmid deoxyribonucleic acid (DNA) is injected in muscle tissue [16]. However, to obtain a systemic effect with the injection of naked DNA has shown to be difficult, resulting in low levels of gene expression in all major organs [17]. There are several reasons for that: (1) the use of naked DNA by itself will not lead to a targeted expression *in vivo*, (2) free oligonucleotides and DNA can be rapidly degraded by serum nucleases when injected intravenously [18], and (3) the arrival of a DNA plasmid in to the aimed cellular compartment (i.e. nucleus) by a passive process is a rare event due to the presence of barriers within the cell [19]. Therefore, the use of a carrier could be considered *sine qua non* for the delivery of genetic material in an efficient and safe manner [20]. Vehicles for gene delivery, which have successfully demonstrated the delivery of exogenous genes *in vivo*, can be divided into two major groups: viral and non-viral vectors. In the following paragraphs, an overview of the most relevant gene delivery systems from both groups will be presented.

2.1. Viral vectors in gene therapy

Viruses are the most popular vectors in laboratory studies and clinical trials, mainly due to their efficiency, with the spectrum of virus used in research still surpassing the few used in clinical trials. The most commonly used viruses include: retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV) [21].

In Table 1 an overview of the main differences presented by the aforementioned viral vectors can be observed. A more detailed description of each of the vectors will be displayed in the following paragraphs, focusing on their application in the nervous system.

Table 1- Comparison of properties of various viral vector systems (Adapted from [22-26]).

Features	***Retroviral***	***Lentiviral***	***Adenoviral***	***AAV***	***HSV***
Genome	RNA	RNA	Linear double stranded DNA	Single stranded DNA	Double stranded DNA
Maximum insert size	7 – 7.5 kb	7 – 7.5 kb	8 - 10 kb	4.5 kb	30 kb
Concentrations (viral particles per mL)	$> 10^8$	$> 10^8$	$> 10^{12}$	$> 10^8$	$> 10^{10}$
Route of gene delivery	*Ex vivo*	*Ex/In vivo*	*Ex/In vivo*	*Ex/In vivo*	*Ex vivo*
Integration	Yes	Yes	No	Yes/No	No
Duration of expression *in vivo*	Short	Long	Short	Long	Short
Immunological problems	Few	Few	Extensive	Few	Extensive
Pre-existing host immunity	Unlikely	Unlikely, except maybe AIDS patients	Yes	Yes	-
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Inflammatory response, toxicity	Inflammatory response, toxicity	Inflammatory response, toxicity

2.1.1. Retroviral vectors

Retroviral vectors have several characteristics that make them extremely appealing for gene therapy. They integrate their genome into the host cell chromosomes as double stranded DNA, generated by reverse transcription, allowing the therapeutic gene to be stable in the progeny for long-term expression. This is especially important in the case of highly replicating cells, where non-integrative vectors are rapidly lost due to dilution. Nevertheless, there are several disadvantages for the use of this class of recombinant viral vectors. These include a variable rate of gene expression, large sensitivity to silencer effects induced by the surrounding chromosomal sequences in the site of integration, the inability of some types of virus to transfect non-dividing cells, sensitivity to human complement system and potential problems concerning insertional mutagenesis [27-30]. However, as most neural cells that are transduced with these vectors are terminally differentiated, the concerns about the activation of proto-oncogenes following integration are in principle diminished [31]. Examples of retrovirus used as recombinant vectors include oncoretrovirus, lentivirus and spumavirus [32]. In opposition to oncoretrovirus, the lentivirus and spumavirus have been shown to infect both dividing and non-dividing cells [27]. In particular, it has been demonstrated that lentivirus could infect neurons [33, 34], enabling this type of virus with a more efficient and wide infection capability as viral vectors. The best-known member of the lentivirinae subfamily is the human immunodeficiency virus type I (HIV-1) that has been used to develop vectors for *in vivo* gene delivery to non-dividing cells [35-37]. Although preclinical studies seem very promising, the use of HIV-based vectors in clinical trials continues to be a very controversial issue [38, 39]. There were many concerns in the matter of safety, in part justified by the lack of clinical experience to predict possible adverse effects induced by gene transfer conjugated to the fact that many aspects of the pathogenesis of AIDS have not yet been completely understood [40].

2.1.2. Adenoviral vectors

The second most commonly used form of gene delivery vectors is based on recombinant adenovirus. This type of virus has relatively large linear double stranded DNA genomes (36 kb) that contain many structural and regulatory genes that are expressed at different moments during the infection, and has the capability to infect both dividing and non-dividing cells [32]. Adenoviruses do not usually integrate their genetic material into the host DNA. Instead, they are replicated as episomal (extrachromosomal) elements in the nucleus of host cells [22]. There are several

advantages in the use of this type of viral vector, including a highly efficient rate of gene transfer associated with a large number of vector copies per cell, the ability to transfer relatively large therapeutic genes and it presents a low risk of mutagenesis as it does not integrate into the host genome [41]. After intracranial injection, this vector has been shown to infect neurons, type 2 astrocytes, oligodendroglia and microglia [42, 43]. Moreover, adenoviral vectors have been shown to undergo retrograde transport from nerve terminals [44-47], which makes them potential candidates for the use in the nervous system. The major drawback of this type of vector concerns the nature of transient gene transfer and expression, which often lasts only a couple of months in non-dividing cells and a much shorter time in dividing cells. Moreover, this type of vectors is immunogenic, due to the large amount of protein present in the capsids and to the fact that most of the viral proteins are expressed by transduced cells [27, 32].

2.1.3. Adeno-associated viral vectors

AAV is a simple, non-pathogenic, single stranded DNA virus (5 kb) that encodes only two gene products. As the name implies, AAV are dependent on the presence of adenovirus for replication. They can replicate both as an episome and as integrated provirus, depending on several factors including the cell type [48]. They have a wide tropism, can readily infect non-dividing cells, and have no known pathologic effect in humans [22, 32]. Vectors based on recombinant AAV are similar to adenovirus vectors in the capability of highly efficient gene transfer. They are also similar to retrovirus in the capability of stable transgene expression, at least is some settings, as they also have the potential to integrate into the target cell genome [49]. The serotype 2 is so far the most studied and therefore the most used, in *in vivo* studies and clinical trials. A differential transduction of distinct AAV serotypes has been tested for adult and neonatal CNS of rat and mice. It has been shown that after intrastriatal injection, the type 2 serotype transduces predominantly neurons, while serotypes 1, 4 and 5 show a broader tropism, also transducing astrocytes and ependymal cells [50-52]. Furthermore, the ability of these vectors to be transported retrogradely both in the CNS [53] and PNS [54, 55] make them attractive for the use in the nervous system.

The main drawback of this class of vectors is that they can only accommodate small transgenes. Apparently, transfer and expression are not always stable due to tissue-specific variations in the rate of vector integration [49]. Despite their low immunogenicity, some immune response has been observed, which has been implied with the viral capsid and the transgene expression [49].

2.1.4. Herpes simplex virus based vectors

HSV family naturally infects the human mucosa (ocular, oral and vaginal) causing lytic curable effects. During its life cycle, it infects the sensory nerve endings and migrates to the neuronal cell bodies, resulting in a latent infection [56]. The targeting efficiency to the PNS is due, in part, to the expression of the high affinity receptor for HSV (nectin-1) on peripheral sensory axons [57]. The large linear double strand DNA genome of HSV (about 150 kb), which is 15 and 4 times larger than lentivirus and adenovirus, respectively, can be replaced by 30 kb of foreign genes, exhibiting one of the biggest viral vector cargo capability [58]. Vectors derived from HSV-1 have been shown to infect neurons *in vitro* and *in vivo* and undergo retrograde transport [59-62]. On the other hand, the original pathologic and latent infectious nature of these viruses and the inability to obtain sustained expression can limit their therapeutic applications [63].

2.1.5. Viral infection process

The expression of cell surface molecules varies in different tissues due to distinct function of the cells. Specific virus-cell recognition events are essential in viral tropism and pathogenesis of the infection. In some cases, the clinical outcome of the infection process can be largely explained by the receptor specificity. In the case of Epstein-Barr virus infection, B-cells expressing the complement receptors, recognized by the virus, are the main target [64]. In rabies, the virus interacts with the acethylcholine receptor during migration from the exposed peripheral area to the CNS [65]. The viral infection process may either use several receptor molecules during the early events (attachment, entry and un-coating) or, in some cases, only one molecule may be sufficient to fulfill all of these steps. After recognition of the cell surface receptor, viruses usually enter the cells utilizing processes, which are used for internalization of extracellular material necessary for the physiological cell functions [66]. After the interaction between the virus and the receptor, the virus particle moves into clathrin-coated pits and the vesicles are internalized into the cytoplasm. These can then fuse with an early endosome, and the virus can be further delivered to the late endosomes or to other intracellular organelles. The viral genome needs, therefore, to be released from the membranous vesicle. This procedure varies between viruses and may take advantage of viral fusion peptides or virus-induced rupture of the vesicle. Some viruses (*e.g.* HIV and measles virus) are able to enter the cells by direct fusion of the viral envelope with the plasma membrane, which leads to the release of the viral core directly into the cell cytoplasm [66].

Because many viruses replicate in the nucleus of their host cells, they have developed several efficient methods for transporting their genome into this compartment [67]. They utilize the complex machinery that cells have evolved for protein and nucleic acid trafficking.

2.1.6. Risks presented by the use of viral vectors in gene therapy

The in vivo preclinical application has demonstrated toxicity, immunogenicity and inflammatory potential of viral vectors. And while phenomena of insertional mutagenesis and oncogenesis are related with integrating viruses, immune and inflammatory response, and the ability to recombine are transversal to all [22, 68-70]. Indeed, the potential of viruses to adapt to new or changing cellular environments or ecological niches via genetic variation appears to be an important point to have in mind. Mutations occur in the genomes of DNA as well as RNA viruses. Although mutations occur slower in DNA virus than in the RNA virus, due to the proofreading function of many DNA polymerases. Furthermore, mutations in RNA virus can occur up to a million-fold more frequently than in DNA virus [71, 72]. And in spite of the fact that the major mechanism that drives adaptation is based on accumulation of point mutations, evolution of viruses also occurs through recombination, which occurs in both DNA and RNA viruses, leading to the exchange of parts of genomes. Consequently, this process may result in the emergence of new virus variants. For recombination to take place, at least co-infection of a cell by two different virus variants is required. In some cases, recombination between viruses and cellular nucleic acid can lead to the capture of cellular coding sequences [73]. Despite new generations of safer viral vectors are currently being developed; the described facts underpin the risk regarding the use of virus for clinical applications.

A variety of experimental conditions is applied in the laboratory for propagation and isolation of virus and genetically modified derivatives. As a result, these viruses are subject to selective forces that are likely to differ from those experienced in nature. These forces could have unpredictable influences on the virus. As highlighted by the emergence of new viral diseases in the last two decades, the process of adaptation often involves the acquisition of an altered cell tropism of host range [74]. Against this background the intensified use of viruses and their genetically modified variants as viral gene transfer vectors for biomedical research is again a cause for concern.

2.1.7. Prospects to the future use of viral vectors

In order to develop an efficient and safe gene delivery system applicable in a clinical scenario, new approaches are awaited. Fundamental drawbacks presented by the

generality of viral-vectors, including pathogenicity, insertional mutagenesis, toxicity, limitations in scaling up, cost and ethical concerns [75], have drawn interest in non-viral vectors. Nevertheless, one cannot neglect the efficiency and specificity attained by some virus, product of millions of years of evolution. The merge of some qualities of viral vectors associated with non-viral systems might show the solution to a safe and efficient gene delivery system.

2.2. Non-viral vectors in gene therapy

Over the past decades there have been many attempts to design a non-viral vector that could mediate the level of gene expression and specificity attained by viral vectors, allow a greater flexibility in terms of the size of the DNA that can be transported, bypass the immune system, and improve safety concerns. Several lipid-, peptide-, nanoparticle- and polymer-based systems are currently being developed for gene delivery [21, 76-78]. The majority of these systems are cationic in nature. They interact with the negatively charged DNA trough electrostatic interactions. Classically, the complex total charge (zeta potential) should present a positive net value. This will allow the carrier to efficiently interact with the negative charged cell membrane and undergo internalization by the cell, which occurs mainly trough endocytosis [79].

Although in a broader sense non-viral gene therapy includes other examples of nucleic acid delivery applications, i.e. anti–sense or interfering RNAs, those are not included in the scope of this thesis (see reference [80] for review). In the following paragraphs different material and strategies applied in the design of non-viral gene delivery systems will be discussed.

2.2.1. Cationic lipids

Since the introduction of (N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylamonium chloride) (DOTMA) as gene carrier in 1987 [81], liposomes have become one of the most studied non-viral vector. Since then, several other cationic lipids have been synthesized and studied for gene delivery [82-91].

In addition to amine groups, all cationic lipids possess a hydrophobic group, which may be comprised of one or two fatty acid or an alkyl moiety of 12-18 carbons in length or a cholesterol moiety. The hydrophobic tails assure that the cationic lipids assemble into bilayer vesicles and disperse in aqueous media, by effectively exposing the amine head groups towards the aqueous medium while shielding the hydrophobic portion of the molecule. Furthermore, the amine group represents an absolute necessity for the transfection competence of these vectors, as this group interacts electrostatically with the negatively charged groups of DNA, condensing the

large anionic molecule and forming small transportable units – lipoplexes [20]. Lipoplexes can range from 50 nm to just over a micrometer in size [92-94] and the influence of lipoplex size on transfection efficacy is contrary to what would be expected, with larger lipoplexes being reported to improve transfection *in vitro* [94-97].

Based on freeze-fracture electron micrographs and X-ray diffraction studies, it was suggested that DNA is sandwiched between many liposomal particles [98, 99]. The transfection mechanism of cationic lipoplexes was initially associated with direct plasma membrane fusion [100, 101], but it is nowadays accepted that gene transfer occurs primarily *via* endocytosis [102, 103]. Many physical factors may influence the stability, formation and transfection efficiency of lipoplexes such as particle size, zeta potential, DNA/liposome ratio and ionic strength of the medium [96, 104, 105]. Additionally, many cationic lipid molecules cannot form lipoplexes with DNA by themself, and are usually associated with neutral lipids (or lipid like molecules) such as dioleyl phosphatidylethanolamine (DOPE) or cholesterol [95, 106-108]. DNA/cationic lipid complexes seem promising candidates for gene delivery since they deliver nucleic acids efficiently both *in vitro* and *in vivo* [100, 109-111]. Indeed, 6.7% of the current gene therapy clinical trials concern the evaluation of lipoplex formulations to deliver genes, active molecules or drugs [11]. However and despite the great success for *in vitro* transfection, cationic lipoplexes often exhibit significant drawbacks when used for *in vivo* delivery. The interaction with plasma proteins leads to a rapid clearance from the circulation via the reticulo-endothelial system [110]. Moreover, the *in vivo* assessment of lipid-base systems has revealed that, in the case of systemic administration, gene transfer was mainly observed in highly vascularized tissues (i.e. heart, lung, liver, spleen) [109, 110, 112]. To overcome these drawbacks, efforts have been made to allow the use of these systems in a *in vivo* scenario (see [113] for a review) however further improvements regarding vector toxicity, efficiency and stability are still awaited.

2.2.2. Cationic polymers

Polymers bearing groups that are protonated, or at least partially protonated at physiological pH, have been also employed as gene carriers. The electrostatic interaction between the cationic amino groups on the polymer and the negatively charged phosphate groups in DNA results in a particulate complex, the polyplex, which is the transfecting unit [20]. The interaction between cationic polymer/DNA complexes and negatively charged cell membranes can enhance DNA uptake by the cells and thus improve transfection efficiency [114]. Moreover, polyplexes seem to be

more stable than lipoplexes, less toxic, and have shown to improve the protection of DNA from nuclease degradation [115]. They show structural versatility including the possibility of covalent binding of targeting moieties for gene expression mediated trough specific receptors [63, 116]. However, compared to viral systems, the efficiency of gene delivery from cationic polymers is still relatively low [117]. Further improvements are therefore awaited in order to prone these vectors for a clinical use. The most widely studied cationic polymers for gene therapy will be further discussed in the following paragraphs.

Poly(L-lysine)

Poly(L-lysine) (PLL) is a linear polypeptide having the amino acid lysine (positively charged at physiological pH) as the repeating unit (Figure 1). The first polycation to be employed for gene delivery was PLL conjugated with asialoorosomucoid for hepatocellular gene targeted delivery [118]. The gene transfer activity of PLL polyplexes is poor, unless endosomolytic (e.g. imidazole) or lysosomotropic agents (e.g. chloroquine) are added [119, 120]. In an elegant study Akinc and Langer determined that after internalization, most of the polyplexes were retained in the lysosomal pathway instead of been released to the cytoplasm [121]. This is an important difference in the biological activity of the amphiphilic cationic lipids and the soluble polymer PLL [20]. In fact, PLL has poor transfection ability when applied alone or without modifications. However, it has been shown that DNA condensation and transfection efficiency increased with the increase of PLL molecular weight, however associated also with a toxicity increase [122]. The creation of amphiphilic PLL, by linking both poly(ethylene glycol) (PEG) and palmitoyl groups to the polymer, reduced toxicity without compromising the gene delivery efficiency [122]. The conjugation of histidine residues to PLL resulted in a more efficient transfection than the one mediated by the PLL-cloroquine mixture [123, 124]. Poly(L-histidine) (PLH) has been shown to destabilize lipid bilayers when in a slightly acidic medium and induce fusion upon protonation of the imidazole groups by the increase of interaction between this polymeric cation and the membrane phospholipids [125]; explaining in part the effect of histidine on the increased transfection efficiency of PLL copolymers with PLH.

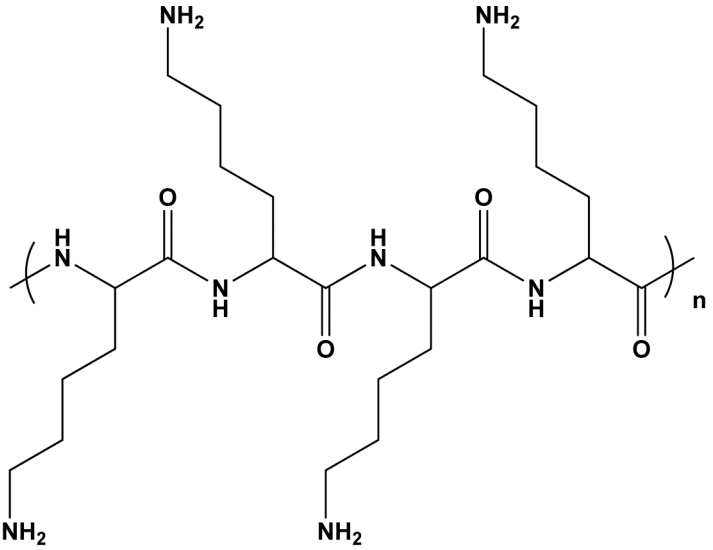

Figure 1 – Chemical structure of poly(L-lysine) (PLL).

The chain length heterogeneity of the commercially available PLL is one of the major causes of variability in the preparation of reproducible and stable formulations. The extreme heterogeneity greatly complicates both the kinetics of DNA-PLL interaction and the thermodynamic stability of the final DNA complexes [122]. Although the peptide linkage is naturally found in proteins, the repeating unit of PLL, the L-lysine amino acid, is not found in nature. As a result, PLL has been found to be cytotoxic [126], what is possibly arising from its slow degradation *in vivo*. Moreover PLL polyplexes are rapidly bound to plasma proteins and cleared from circulation [127, 128].

Due to the above mentioned drawbacks the wide spread application of this polymer for gene delivery purposes has been limited.

Dendrimers

Dendrimers were introduced in the late 70s and early 80s and due to their unique properties have found use for drug and gene delivery, as well as in other nanoengineering applications [129].

Polyamidoamine (PAMAM) dendrimers consist of a class of highly branched spherical polymers (Figure 2) that due to the ease of synthesis and commercial availability have been the most used for gene transfer applications. The surface charge and diameter of any given PAMAM dendrimers is determined by the number of synthetic steps taken to produce it – generations, therefore, five polymerization cycles produce a five-generation dendrimer [130].

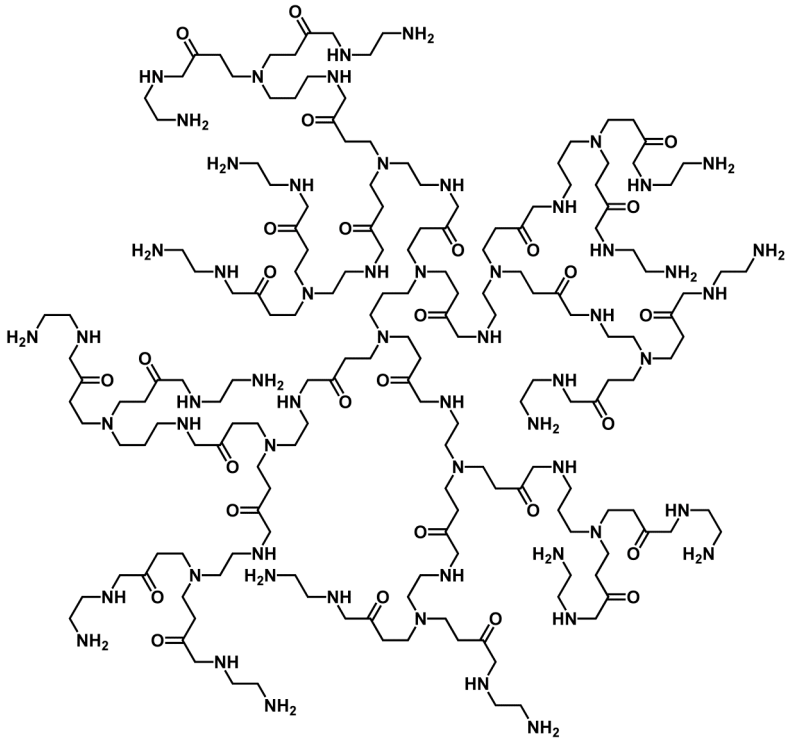

Figure 2 – Chemical structure of a second-generation poly(amidoamine) (PAMAM) dendrimer.

One of the major major structural differences in PAMAM dendrimers are related to the core molecules, either ammonia (NH_3) or ethylenediamine (EDA), with which the stepwise polymerization process begins and which dictates the final overall shape, density and surface charge of the molecule. With each new generation, the molecular weight of the dendrimers more than doubles, and the number of surface amine groups exactly doubles [131]. Additional dendrimer chemistries have been successfully developed, including poly(propylene imine) (PPI) [132] and poly(L-Lysine) dendrimers [133], among others (see [129] for a review).

Dendrimers condense DNA via electrostatic interactions of their terminal primary amines with the DNA phosphate groups. The particle size, surface charge and gene transfer efficiency of dendrimer/plasmid complexes has been shown to be influenced by the dendrimer concentration in the complexes [134]. Moreover the increase in the amount of terminal amino groups appears to enhance gene delivery. The dendrimer/DNA complexes are presumably internalized by endocytosis and there are several advantages associated with the star shape of the polymer, as DNA appears to interact with the surface primary amines only, leaving the internal tertiary amines available for the neutralization of the acidic pH within the endosomal/lysosomal compartment [135]. After internalization, the release of the complexes by the endosome as been associated with the protonation of the internal tertiary amine

groups, leading to the swelling of the endosome and subsequent release of the DNA to the cytoplasm [131]. Dendrimers with free amine groups in the macromolecule periphery are reported to have a concentration- and generation number–dependent toxicity [136, 137] what, without doubt, has limited their application in clinical scenario. Even so, several alterations to the basic PAMAM structure have been performed aiming at reducing these polymers cytotoxicity [138, 139], as well as to improve cell binding [130, 140, 141] and cell targeting [142].

Poly(ethylene imine)

Poly(ethylene imine) (PEI) was introduced as a gene delivery vector by Behr in 1995 [143], and has become one of the gold standard among the non-viral based vectors.. PEI can be synthesized in two forms, branched and linear. Branched PEI is a cationic polymer composed of primary, secondary and tertiary amines (Figure 3). It has been shown to effectively condense plasmids into colloidal particles that achieve transfection into a variety of cells, both in vitro and *in vivo* [143]. These condensed particles are of spherical shape and have a narrow particle size distribution, which presumably allows high cellular uptake of the plasmids leading to high transfection efficiency [144].

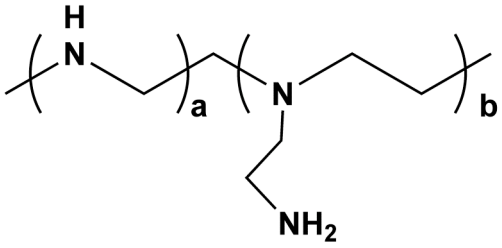

Figure 3 – Chemical structure of poly(ethylene imine) (PEI).

PEI has a high charge density, due to the fact of having in every three atoms of the backbone a nitrogen atom, and in linear PEI, all of those nitrogen atoms are protonable, whereas in branched PEI, only two thirds of them can be charged [117]. With the overall protonation level of PEI increases from 20 to 40% at pH 7 and 5, respectively [114]. It is thought that the presence of PEI in the endosome leads to an accumulation of protons brought in by the endosomal ATPase, coupled to an influx of chloride anion. PEI ability to accept the protons pumped to the endolysosome will oppose the pH decrease, inhibiting nucleases and unbalancing the endosome osmolarity by the increase of Cl^- concentration, with resulting osmotic swelling of the endosome. Therefore, PEI may enhance intracellular trafficking by buffering the endosomal compartments, thus protecting the DNA from lysosomal degradation by

endosomal release via lysosomal disruption [143]. Many factors affect the efficiency/cytotoxicity profile of PEI polyplexes such as the polymer molecular weight and degree of branching, ionic strength of the solution, zeta potential, and particle size [145, 146]. The transfection efficiency of PEI has been tested for a wide range of polymer molecular weights and found to increase with the increase of the polymer molecular weight (in the range of 0.6 to 70 kDa) [147]. However, the toxicity associated to the transfection process mediated by PEI polymers was also shown to augment with the PEI molecular weight [148-150]. The PEI toxicity mechanism has been associated with its aggregation and adherence to the cell surface, eliciting cell necrosis [151]. Finally, it is currently accepted that the optimal exists between 11.9 and 70 kDa [20]. Besides molecular weight the degree of PEI branching has also shown to play a role, with linear PEI shown to be less efficient on complex condensation in relation to similar molecular weight branched PEI [152]. Moreover, complex stability was shown to be higher when PEI with more primary amines was used, making branched PEI a more suitable polymer for gene delivery [153].

Focus has been drawn on the improvement of PEI transfection efficiency and toxicity profile. One of the most common modifications is polymer PEGylation, which allows the formation of complexes with a more hydrophilic surface, which reduces the interaction of complexes with proteins. *In vivo* studies have shown that PEI-PEG copolymer-based complexes had increased blood circulation and reduced toxicity, when injected intravenously. But no gene expression was detected even for the higher doses tested (25 μg of pDNA per mouse) [154]. This outcome was associated to the lower stability of the copolymer based complexes, in relation with PEI complexes, leading to rapid clearance [154]. Nonetheless, the reduced charge of the copolymer-based complexes can also be a factor, by impairing their interaction with cell membrane, the first step of cell trafficking. Additionally, the authors also proposed that the reduced transfection efficiency could be attributed to hindered gene transfer steps that occur posterior to cell internalization. To test this hypothesis a PEI-PEG copolymer that could dissociate in a pH triggered fashion was developed, these complexes exhibited a gene expression 2 orders of magnitude and 1 order of magnitude higher in vitro and in vivo (using a mouse model), respectively, in relation to the unmodified PEI-PEG copolymers [155].

Other molecules have been grafted to PEI in order to improve transfection efficiency. The grafting of low molecular weight branched PEI (1.8 kDa) with cholesterol moieties was shown to increase its transfection efficiency while reducing its toxicity, regardless of the amine group modified [156]. Cyclodextrin-modified PEIs (CD-PEIs, branched and linear) were shown to have reduced toxicity, in a manner inversely

proportional to the density of grafted cyclodextrin, although at the cost of transfection efficiency. Nevertheless, the transfection efficiency of both branched and linear CD-PEI were higher than the unmodified analogues in the presence of chloroquine, suggesting that cyclodextrin grafting was hindering the buffering capacity of PEI [157]. Dexamethasone conjugated low molecular weight PEI (2 kDa) was produced with the aim of enhancing the translocation of the polymer/DNA complex into the nucleus. This vector demonstrated transfection efficiency comparable to the one mediated by 25 kDa branched PEI based vectors, with reduced signs of toxicity [158].

The synthesis of PEI compounds that could break down to less toxic lower molecular weight compounds was also attempted. Reducible PEI derivatives were produced by the treatment of low molecular weight branched PEI (800 Da) with dithiobis(succinimidylpropionate) or dimethyl·3,3‘-dithiobispropionimidate. The obtained polymer-based complexes elicit lower transfection levels than the 25 kDa branched PEI, nonetheless with significant lower toxicity [159]. A more recent study concerning reducible linear PEI derivatives has also shown low citoxicity levels for these materials and transfection efficiencies similar to branched PEI [160].

Efforts have been made to combine or even exchange the non-specific electrostatic polyplex-cell surface interaction with a specific receptor mediated cellular uptake, by the incorporation of cell binding ligands into transfection complexes. One of the targeting ligands that have been used in PEI is transferrin. The coupling of transferrin to PEI (800 kDa) resulted in a 30 to 1000 fold increase in transfection efficiency in the neuroblastoma cell line Neuro2A, the T cell leukemia cell line Jurkat and the erythroleukemic cell line K562 [161]. The coupling of anti-CD3 antibodies with PEI also mediated specific gene delivery targeted to CD3-expressing cells [161]. A variety of other ligands has also been successfully coupled to PEI, including galactose for hepatocyte targeting [162], mannose for enhanced uptake by dendritic cells [163], epidermal growth factor (EGF) for enhanced uptake by epithelial cells [164], and integrin-binding peptides [165]. Despite some of the drawbacks already described, PEI is still one of the most applied cationic polymer in gene delivery, mainly due to its high efficiency even in difficult to transfect cells, like neurons [166].

Chitosan

Chitosan (CH) is a linear polysaccharide composed of glucosamine and N-acetyl-D-glucosamine (Figure 4), where the molar ratio of glucosamine to N-acetyl-D-glucosamine is greater than 1 [167].

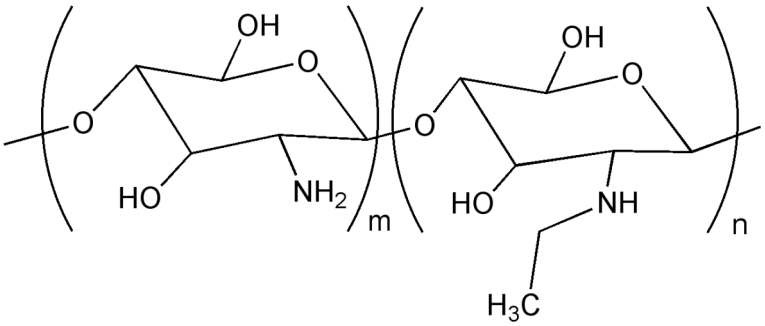

Figure 4 - Structure of Chitosan, considering the deacetylated monomer (m) and the acetylated monomer (n).

CH is the N-deacetylated derivative of chitin, although this N-deacetylation is almost never complete. Both chitin and CH have shown excellent biological properties, such as biodegradation in the human body [168, 169], antibacterial [170], and wound-healing activity [171]. CH is a biocompatible polymer that can be broke down slowly to harmless products (amino sugars), which are completely absorbed by the human body. This polymer is a weak base with a pKa value of about 6.2 - 7.0 and is therefore insoluble at neutral and alkaline pH values. In acidic mediums, the amine groups of CH will be positively charged, conferring to the polysaccharide a high charge density. CH cationic characteristics provide strong electrostatic interactions with mucus, negatively charged mucosal surfaces and other macromolecules such as DNA [172]. Since the early application of CH for gene delivery, pioneered by Mumper et al. [173], CH has become one of the most prominent naturally derived non-viral vectors. CH-based DNA transfer depends greatly on the ionic and non-ionic interactions established between the carbohydrate backbone of CH and the surface proteins of cells [174]. In similarity with other polymeric based gene delivery vectors, CH mediated gene delivery can encompass a number of cellular barriers, enzymatic degradation, cellular uptake, escape from the endolysosome pathway, complex dissociation and nuclear import [175, 176].

The preparation of self-assembling and oligomeric CH/DNA complexes was first described in 1995, by mixing a solution of the respective CH with plasmidic DNA [173]. CH particles can be prepared by different methods, including coacervation [177], gelation [178] and desolvation [179]. The use of the different methods together with other parameters like nucleic acid concentration, salt concentration, pH, charge ratios and temperature can influence the size of the formed complexes [180-183]. Additionally, complex size was also shown to increase with the increase of the polymer molecular weight [184]. The smaller size complexes have the advantage of entering the cells through endocytosis and/or pinocytosis, therefore increasing the

transfection rate. Nevertheless, it has also been shown that high molecular CH forms more stable complexes due to a chain entanglement effect [185].

DNA binding with CH is dependent on both the molecular weight and the degree of deacetylation, where lower molecular weight and lower deacetylation degree both require higher ratios of polymer to completely complex DNA. Based on these observations one can be lead to think that CH with higher degrees of deacetylation will increase the transfection efficacy, however when the degree of deacetylation of a CH with a starting molecular weight of 390 kDa was decreased from 90 to 70%, its transfection efficacy increased in two fold [167]. This was associated with a more rapid release of DNA from the complexes, since the DNA is more readily available to the surrounding cells. Additionally, *in vivo* CH degradation has been shown to significantly increase between 73 and 69% degree of deacetylation [176]. It has also been shown that the complexation of highly purified CH fractions (molecular weights of <5 kDa, 5-10 kDa, and >10 kDa) at a charge ratio of 1:1 resulted in almost complete inhibition of degradation by DNase II [186], indicating that CHs are able to efficiently protect DNA against degradation, associated with a change in the tertiary DNA structure causing steric hindrance [181].

One of the practical problems for gene therapy *in vivo* is that gene delivery can be inhibited by the presence of serum [187]. Indeed the presence of serum (20%) promoted gene expression 2 to 3 times higher than that without serum for CH based complexes [188], making CH an interesting vector for *in vivo* applications. However, CH-based delivery systems, as other non-viral gene delivery vectors, have shown transfection efficiencies that vary greatly with cell type, with higher gene transfer being observed in HEK293 cells, as compared with HT-1080, Caco-2, MG63 or mesenchymal stem cell lines. This difference has been associated with differences in cell uptake levels as well as differences in CH degradation inside the endosomal compartment [176, 189].

Copolymerization is a common method to tune properties of macromolecules. Copolimerization of CH with PEG (Mw=5 kDa, with 9.6% of CH grafting) showed to increase stability of polyplex even in the presence of serum or bile [190]. After bile duct infusion pegylated CH it was shown to mediate higher transfection at day 1 and at day 3 after portal vein infusion compared of that of unmodified CH [190]. This effect was hypothesized to be due to the decrease of CH-based polyplex opsonization, therefore prolonging the complex circulation time. More recently, this assumption was reinforced by studies that show that CH PEGylation could not only avoid rapid clearance but also lead to the increase of the levels of the transfecting agent reaching tumor tissues and liver [191].

PEI/CH copolimerization has also shown to lead to improvements in terms of transfection levels, with transfection levels reaching higher values than for 25 kDa PEI, with a reduction of cell toxicity being observed in relation to the PEI homopolymers [192].

CH functionalization remains still the most popular approach regarding CH-based vector optimization. Lactosylated CH based vectors when tested in HeLa cells, and when in the presence of 10% fetal calf serum, demonstrate to have transfection levels comparable to that of PEI [193]. Trimethylated CH oligomers were explored in order to improve CH-based vectors, allowing increased solubility and stability in physiological conditions. This process is based on a reductive methylation procedure using methyl iodide in an alkaline environment [194]. These polycations were characterized and tested for their efficiency on transfecting COS-1 cells (kidney) and Caco-2 cells (epithelial). All synthesized derivatives showed excellent solubility in water at different pH values and at both 2:100 and 2:10 DNA/oligomer ratios, the quaternized CH oligomers formed smaller complexes compared to unmodified CH oligomers [194].

In order to increase the CH-based systems transfection efficiency, the formation of CH/DNA/ligand complexes has also been explored. Transferrin receptor was one of the first to be exploited for receptor-mediated gene delivery. Transfection efficiency depended on the cell type and the level of surface transferrin receptor expression, and was enhanced when an endosomolytic agent, such as chloroquine, was used [188]. In a similar manner, conjugation of the C-terminal globular domain of the fiber protein (KNOB) to CH–DNA nanoparticles resulted in a 130-fold increase in the transfection efficiency in HeLa cells and several fold in HEK293.

The qualities presented by CH have boosted the interest on this polymer regarding gene delivery applications. However, the modest transfection potential of CH has limited its success. Indeed new approaches are needed in order to be able to apply this polymer to neuronal cell transfection applications.

2.2.3. Other non-viral vectors

Rather than aiming at presenting an exhaustive overview, in this chapter we focused the discussion on the most studied non-viral gene delivery systems. However the list of proposed non-viral vectors is much longer, including, but no confined to: (1) nanoparticle based systems based on gold [195], silica [196] or carbon nanotubes [197], (2) biodegradable polymers, namely, poly(4-hydroxy-L-proline ester) [198], poly[α-(4-aminobutyl)-L-glycolic acid] [199], poly(amino-ester) [200], phosphorus containing degradable polymers, and (3) cationic synthetic peptides [77]. Although for

certain applications some of these vectors have found limited success they were not in our scope. For a further detailed review see [78].

3. How can we develop a non-viral gene delivery vector PNS specific?

New therapeutic approaches that may arrest or reverse the disease process underlying many types of peripheral neuropathies are awaited. In the search of such a solution, focus has been drawn to the gene therapy. Due to unsolved hurdles presented by viral vectors crescent interest is currently being drawn to non-viral vectors. However, when trying to design a gene delivery system to the PNS, one question that immediately surfaces is - How can one enable these simple vectors to target the aimed cells? In the following paragraphs an overview of the strategies proposed so far to target such systems to neuronal cells will be presented.

The association of the vector with the neuron cell membrane is the initial step of the vector internalization process. The majority of the non-viral gene delivery systems developed so far associates to cells by non-specific interactions. Conversely, targeted systems have primarily to interact with specific cell surface molecules. The differential expression of surface molecules in neuron cells has been explored in order to design targeted non-viral gene delivery systems. The receptors that have been explored to achieve vector targeting can be divided in three major groups: neuropeptides, neurothrophins and neuro-toxin receptors.

In a previous study, neurotensin (NT), a 13 amino acid neuropeptide was conjugated with PLL in order to attain a vector that specifically transfects cells expressing the NT high affinity receptor (NTRH) [201]. The vector specificity was further proved by the absence of internalization observed in cell lines lacking the NTRH (Cos-7 and L-929) [201]. Further work included the use of the hemagglutinin HA2 fusogenic peptide and the Vp1 nuclear localization signal of SV40 to the NT-polyplex. Both internalization in and transfection of dopamine neurons [202] improved, enabling these vectors as promising vectors for the treatment of Parkinson's disease. More recently this group has applied the same vector with a gene encoding for the human glial cell line-derived neurotrophic factor to hemiparkinsonism-induced rats, and showed biochemical, anatomical and functional recovery in this animal model of the disease [203].

Neurotrophin receptors have also been explored in order to design targeted gene delivery vectors to neurons, as their expression is specific for determined neuron cell

populations. A chimeric peptide composed of a targeting sequence derived from the NGF loop 4 and a DNA-binding moiety (10 lysine residues) was used to form complexes with plasmid DNA and shown to increase reporter gene expression in PC1 cells in a DNA dose dependent manner [204]. Moreover, the transfection levels were shown to be inhibited by an NGF excess in the media but not by the use of a low-affinity NGF receptor p75 antibody, suggesting that the internalization was due to interaction with the high-affinity NGF receptor TrkA [204]. The electrostatic association of this peptide to low Mw PEI (600 Da) was able to increase the targeted transfection levels in cell lines, which express TrkA, as well as to the dorsal root ganglia after intrathecal injection in rats [205].

The targeting of upregulated receptors in injured neurons has also been demonstrated. It has been shown that upon injury the $p75^{NTR}$ neurotrophin receptor is up regulated in peripheral nerves [206]. By using a PLL based system coupled to a monoclonal antibody (MC192) to the $p75^{NTR}$ a previous study demonstrated transgene expression (glial derived neurotrophic factor) in targeted neurons leading to an almost complete neuronal rescue after axotomy [207].

Neurotoxins have also been exploited due to their natural ability to bypass the cellular barriers in order to access neurons. A 31 amino acids bi-functional peptide (PLL-molossin) has been developed for gene delivery and was tested in primary culture of rat cerebral cortex cells. With the best combination, more than 30% of cells strongly expressed the reporter gene, with no observed toxicity [208]. The peptide consisted of the 15 amino acid integrin target domain from the venom of the American pit viper *Crotalus molossus molossus* [209] RGDNP [210], which was found to bind strongly to the CNS of rats [209], and a 16-lysine chain for electrostatic binding of DNA [208]. The non-toxic pentameric CTb chain of the *vibrio cholerae* bacterium [211] specifically binds to the pentasacharide moieties of the ganglioside GM1 [212]. The CTb fragment was grafted to a 100-lysine chain and the obtained complexes were shown to increase both transfection activity and efficiency of PC12 cells in relation to unmodified complexes [213]. A novel non-viral gene delivery system targeted to the CNS has been developed employing the non-toxic HC fragment of the tetanus toxin (TeNT). The TeNT is a large protein (150 kDa), which targets with specificity neuronal cells [214]. When applied intramuscularly the TeNT binds to pre-synaptic motor neuron terminals, and after internalization is trafficked to the cell body by retrograde axonal transport [214]. The toxin then undergoes trans-synaptic spread to other cells in the CNS, where it inhibits pre-synaptic

neurotransmitter release by the cleavage of synaptobrevin [215]. TeNT contains three structural and functional domains. The L-chain is responsible for intracellular toxicity [215], while the HC and HN chains contain cell binding and putative endosome disruption activities, respectively [216]. HC has been chemically or genetically conjugated to several enzymes such as superoxide dismutase [217], horseradish peroxidase [218], and β-galactosidase [219]. These conjugates have been applied in a variety of experiments and showed to be targeted to neurons, both *in vivo* and *in vitro*. The intramuscular administration of these conjugates resulted in their delivery to the brain stem, motor neurons of the spinal cord and to a lesser extent the dorsal root ganglia [217, 220]. A previous study using a vector based on the construct of PLL:HC was found to target neuronal cells, N18 RE 105 (neuroblastoma x glioma mouse/rat hybrid) and F98 (glioma) cell lines, and mediated transfection efficiency values ten times higher than without HC [220]. In contrast the transfection of epithelial cell lines (CaCo-2 cells, HeLa cells) showed only a two-fold increase [220]. More recently, a 12 amino acid peptide (Tet1), which mimics the HC fragment receptor binding properties, has been identified by phage display [221]. Tet1 was grafted to 25 kDa branched PEI and showed to improve transfection in PC12 cells, in relation to PEI alone, and also to improve association with dissociated dorsal root ganglia primary culture [222]. In posterior work, the Tet1 peptide was grafted to 25 kDa branched PEI via a 5 kDa PEG and the obtained complexes were shown to improve gene expression in rat brain. Moreover, the Tet1-PEG-PEI based complexes were shown to exclusively transfect neural progenitor cells, whereas the PEG-PEI based complexes were found to transfect a heterogeneous cell population [223].

In many cases, the specialization of nervous system function dictates that a therapeutic intervention may be best achieved by the local and specific expression of a transgene product, such as a neurotrophic or antiapoptotic factor. In addition, the local expression of neurotrophic factors achieved by gene transfer may be used to attain desired outcomes avoiding unwanted adverse effects that could result from systemically administrated drugs [10]. In this sense the ultimate objective on the development of an efficient non-viral gene delivery vector is cell specificity. Nonetheless the vector will have to bear other features enabling it to overcome a wide range of biological barriers.

4. What are the barriers for efficient gene transfer?

Neurons are considered one of the most difficult cell types to transfect [224, 225]. This can be due, at least in part, to the multiple cellular barriers encountered by the vectors. Most of these barriers can be transversal to most cell types, however neurons have additional specifications. Morphologically neurons can be over 1 meter long, which can impair the easy access of a vector to the cell nucleus or perinuclear space, in the case of siRNA. As terminally differentiated neurons do not divide, one can consider the nucleus membrane as one of the main barriers regarding neuron cells. The understanding of the cellular barriers conditioning the route of a non-viral gene delivery vector is of paramount importance in order to efficiently design and improve such a system. The main barriers encountered by a gene carrier are summarized in Figure 5, and will be discussed in further detail in the following paragraphs.

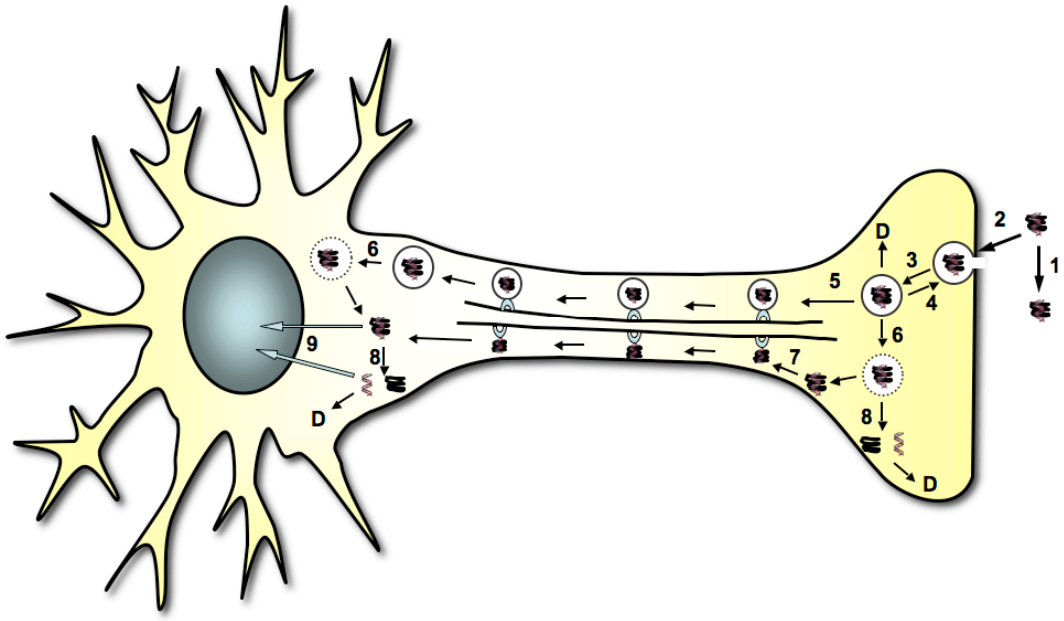

Figure 5 – Possible barriers encountered by a non-viral gene carrier. When in the extracellular milieu a complex can be neutralized by polyanionic compounds (1) or bind to cell surface and be endocytosed (2). Normally, the endocytosed material is trafficked in the early endosome compartment (3), which can be recycled to the cell surface and excreted (4), or can then undergo association with cell active transport (i.e. retrograde transport in the case of a neuronal cell) (5), susquencial the endosome maturate and lead lead to endosomal escape (6) or to the fusion with lysosomes, leading to degradation (D). When free in the cytoplasm the complex can: associate with active transport mechanism and go trough retrograde transport towards the nucleus (7) or can disassemble (8), then enter the nucleus, free or associated with the carrier (9) or free DNA can be quickly degraded (D).

Vector stability

The first step to consider in the journey of an efficient non-viral gene delivery vectors is the carrier stability in the biological milieu. The existence of nucleases in the extra cellular environment imposes the necessity of genetic material protection at this step. The delivery systems have to display colloidal stability in physiological ionic strength and when in contact with existent endogenous anionic molecules (i.e. glycosaminoglycans, serum albumin and other extra-cellular proteins) [226-229].

Cell entrance and intracellular traficking

The route followed by non-viral gene delivery vectors may differ in the cases in which the vector does or does not have targeting moieties. In the case of non-targeted vectors data suggests that the complexes first associate with cell membrane via electrostatic interactions with the anionic cell surface proteoglycans. It was shown that for PLL-DNA complexes, the inhibition of proteoglycan sulfation by the use of sodium chlorate, the removal of cell surface glycoseaminoglycans (GAGS), or the addition of GAGS to the transfection media, dramatically decreases transfection levels [230]. A similar trend was observed for cationic liposome complexes, where liopplexes where unable to transfect a cell line lacking proteoglycans [231]. *In vivo* studies also showed that cationic liposome mediated transfection was inhibited when mice where pre-treated intravenously with heparinases [232].

Evidence exists indicating that cationic substances lead to adsorptive endocytosis and subsequent complex internalization [233, 234]. It has been reported the involvement of clathrin-coated pits in the internalization of the complexes of DNA with cationic lipids and polymers [235, 236]. Phagocytosis of cationic lipid or polymer/DNA complexes have also been described, even in cell lines that are not professional phagocytes [92, 237, 238]. The possibility that caveolae may be involved in the internalization process is supported by the increased internalization of folate (which resides in caveolae) targeted cationic lipid or polymer based complexes in tumor cells [239-241]. Another possible route of entry is macropinocytosys, indeed it has been proposed to mediate the uptake of non-viral gene delivery vectors due to its ability to internalize large structures [242]. However, macropinocytosys is a rare event in non-professional phagocytic cells. One can postulate that several of these pathways can participate to different extents in individual cell types. However, further systematic studies are necessary to clarify which are the most important entry routes for particular vectors and/or for different cell types.

The size of the complexes has also shown to affect cellular uptake in several cell lines. Poly(lactic acid-co-glycolic acid) (50:50)–DNA complex uptake by Caco-2 cells was shown to be particle size dependent, with the highest levels of internalization being observed for the 100 nm particles, in relation with 1 and 10 micra particles [243]. Moreover, in another study, the same particles showed higher transfection levels for 70 nm average diameter particles in COS-7 and HEK-293 cells as compared with 200 nm particles, with comparable internalization levels for both particle dimensions [244]. Additionally, PEI/DNA nanogels were prepared by photo-Fenton reaction with different sizes - 38, 75, 87, 121, 132 and 167 nm of average diameter – and were tested in several cancer cell lines. The highest transfection efficiency was obtained for the 75 and 87 nm particles [245]. Regarding these results one can suggest that the optimal size of a non-targeted complex internalization and transfection-wise is in the range of 70 to 100 nm.

The incorporation of targeting moieties is expected to modify the particle uptake, with the ligand/receptor interaction governing the internalization pathway and the subsequent intracellular route. A wide range of ligands has been used to target cationic nanoparticles, including, but not limited to, asialo orosomucoid [246-248], mannose [249-251], lactose [252-254], transferrin [255-257], and metabolites such as folate [258-261]. One of the major influences of ligand incorporation in a complex resides in the modulation of the cell uptake rate. Transferrin-PEI polyplexes were shown to enter K562 cells in a 1 hr period, while PEI800 polyplexes required up to 4 hrs [262]. With the targeted polyplexes promoting higher transfection efficiency (25 fold). In another study the grafting of CH with lactose (8%) has shown similar internalization levels in HepG2 cells as CH alone, however, the intracellular trafficking towards the nucleus was faster and promoted higher transfection efficiency [253].

Despite the fact that receptor mediated gene delivery can augment selectivity and the rate of particle internalization, the intracellular fate seems to be more important to an efficient gene delivery. ATP-mediated proton accumulation provides the endosome and lysosome of cells with an acidic environment (pH 5.0-6.2) in contrast with the cytosol conditions (pH 7.4) [263]. Non-viral gene delivery systems that can use the acidic environment to escape the endosomal and lysosomal degradation often mediate efficient transfection. Chloroquine is a well-defined lysosomotropic agent that raises the pH of the lysosomal environment, preventing enzymatic degradation [264]. So, one of the strategies used considers the incorporation of chloroquine in non-viral gene delivery system. In the same way, the incorporation of membrane destabilizing peptides, such as the synthetic N-terminal peptides of rhinovirus VP-1

or influenza virus HA-2, into cationic complexes can mediate endosomal release. Under acidic conditions, these peptides rearrange to form an amphipathic alpha-helical structure that can interact with the endosomal membrane and promote the endosomal escape [265]. Alternatively, several polymers that bear amine groups with low pKa have been shown to exhibit “proton sponge” potential. These compounds are able to buffer the acidification process inside the endosome vesicle, leading to swelling and finally lysis, allowing the release for the cytosol [266].

Following endosomal escape, the complex/DNA must traverse the cytosol to access the nucleus. Diffusion of free DNA in the cytoplasm has been shown to be significantly lower than in a dilute solution [267]. Indeed a plasmid DNA with over 2000 bp in the cytosol has less than 1% of the diffusion coefficient then when in water. This difference may reside in cytoskeletal elements in the cytoplasm that function as molecular sieves, preventing the free diffusion of large molecules [268]. In that sense carriers that could compact genetic material in smaller particles could be beneficial. However, in the case of cationic lipid carriers the release of DNA seems to occur during endosomal escape. The injection of cationic lipid-based complexes in the cytoplasm has shown to be much less efficient in terms of transfection than injection of DNA alone [269]. Conversely, in cationic polymer based systems a partial association of DNA and the carrier seems to occur after endosomal release. A beneficial effect in transgene expression was observed when PEI and PLL based complexes were injected in the cytoplasm [270], indicating that an association of the complexes with cell transport processes could be occurring. However, this mechanism is still not fully understood. Enhanced diffusion due to the condensed nature of the complex and the enhanced protection of DNA could be partially involved. In the case of neurons, microtubule-based transport is fundamental for normal cellular function since it allows the rapid transport of molecules between the cell body and the axon. The main motor for retrograde transport, towards the cell nucleus, is associated with dynein [271], and it has been shown to be critical in the infectious process of neurotropic viruses like HSV and adenovirus [272]. As non-viral vectors are in a similar range of size of those of viruses it is expected that the association with active retrograde transport could be beneficial for the transfection process. However, until now, non-viral delivery systems that could specifically recruit the motor retrograde machinery have not yet been demonstrated.

Nuclear intake

In order to access the transcriptional machinery of the nucleus, plasmid DNA must cross the nuclear membrane. The nucleus is the cellular compartment that encloses

chromatin and the machinery necessary for gene transcription. Its composition has to remain intact in order to maintain the integrity of the nuclear structure. Although access to the nucleus is a highly restricted process, a multitude of macromolecules has to enter and exit the nucleus, for the control of the basis cellular metabolism and to respond to changing environmental conditions [273]. Except during mitosis, when the nuclear envelope disappears, the only way macromolecules can enter the nucleus is through the nuclear pore complex. Indeed, dividing cells often exhibit higher transfection levels than non-dividing cells, indicating that the plasmid DNA access to the nucleus is improved during the nuclear envelope disruption that occurs during mitosis [274]. Given the post-mitotic nature of neurons, the nuclear import of plasmid DNA is sine qua non for the efficient gene expression.

Three possible nuclear entry routes have been proposed for the genetic material: trough nuclear pores, it can become physical associated with chromatin during mitosis or can simply transverse the nuclear envelope. Although the last seems less obvious, as no experimental evidence has been found so far.

The nuclear pore complex is built of a diverse set of nuclearporins and associated nuclear and cytoplasmic filaments surrounding a central channel structure [275]. This structure allows passive diffusion of small molecules (up to 9 nm in diameter, or proteins up to 50 kDa) or active transport of larger molecules (up to 25 nm in diameter, or ≈ 1000 kDa) [275]. The nuclear import mechanism of macromolecules (proteins, RNAs and ribonucleoproteins) is energy dependent and carrier-mediated [276].

Studies regarding cationic vectors have shown a beneficial effect in comparison with DNA alone, suggesting that the cationic nature of the carrier could exert a nuclear localizing effect, not an unexpected result as most of the nuclear-homing sequences bear a cationic nature [277].

Targeting and transport of viral genomes to the nucleus have shown to depend on nuclear localizing sequence (NLS) exposed in the surface of the capsid particle [278]. Karyophilic proteins bear one or more nuclear targeting signal peptides called NLS, from which some examples are presented in Table 2.

Table 2 – Examples of aminoacid sequences required for nuclear transport of proteins (Adapted from [279]).

SV40 large T-antigen	PKKKRKV
HIV-1 Rev	RQARRNRRNRRRRWR

Depending on the imported protein, one or two cytoplasmic transport factor, called kariopherin, associates with the NLS sequence to form a pore-targeting complex. The complex docks on the distal end of the fibrils protruding from the cytoplasmic ring of the NPC. It is then translocated thought the pore by an energy-dependent mechanism, which has not yet been fully elucidated, but, probably, consists of a series of dissociation/re-association steps of the complex with nuclearporins [273]. Covalent attachment of NLS to plasmid DNA has shown to increase nuclear translocation and improve gene transfer [280-284]. Nonetheless, conflicting studies have shown no improvement upon incorporation of NLS moieties in plasmid DNA [285, 286], indicating that additional variables can influence the nuclear import process. It has also been shown that a optimal degree of NLS exists, as too many NLS signals can lead to translocation inhibition due to the interaction with multiple nuclear pores [280]. Additionally, the grafting to the plasmid DNA can lead to the inhibition of the transgene by blocking the enzymatic access to the expression cassette. In order to circumvent this problem peptide nucleic acid sequences have been developed, which function as sequence-specific DNA binding domains, allowing the control of the number and the binding site of the NLS sequences [287].

The main challenge ahead consists on the development of an effective non-viral gene delivery vector aiming at a therapeutic clinical application. In face of the large number of barriers that such a system has to face *in vitro* and *in vivo,* new designs are awaited. As the nervous system presents a number of specific characteristics that hinder the action of a gene delivery vector, such system will have to include a number of features that may enable the efficient delivery of the transgene. Indeed a simple, safe, cheap and efficient system that can specifically transfect peripheral sensorial neurons can bring new answers to address peripheral neuropathies.

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Chapter III

Targeted gene delivery into peripheral sensorial neurons mediated by self assembled vectors composed of poly(ethylene imine) and tetanus toxin fragment c*

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Abstract

A simple, safe and efficient system that can specifically transfect peripheral sensorial neurons can bring new answers to address peripheral neuropathies. A multi-component non-viral gene delivery vector targeted to peripheral nervous system cells was developed, using poly(ethylene imine) (PEI) as starting material. A binary DNA/polymer complex based on thiolated PEI (PEISH) was optimized, considering complex size and zeta potential and the ability to transfect a sensorial neuron cell line (ND7/23). The 50 kDa non-toxic fragment from tetanus toxin (HC), which has been previously shown to interact specifically with peripheral neurons and to undergo retrograde transport, was grafted to the complex core via a bi-functional PEG (HC-PEG) reactive for the thiol moieties present in the complex surface. Several formulations of HC-PEG ternary complexes were tested for targeting, by assessing the extent of cellular internalization and levels of transfection, in both the ND7/23 and NIH 3T3 (fibroblast) cell lines. Targeted gene transfer to the neuronal cell line was observed for the complex formulations containing 5 and 7.5µg of HC-PEG. Finally, our results demonstrate that the developed ternary vectors are able to transfect primary cultures of dorsal root ganglion dissociated neurons in a targeted manner and elicit the expression of a relevant neurotrophic factor.

Introduction

Peripheral nervous system (PNS) problems are common and encompass a large spectrum of traumatic injuries, diseases, tumours or iatrogenic lesions [1]. Conventional treatments for peripheral neuropathies have primarily been palliative rather than curative, but most importantly, they have often been ineffective. In the recent years, enormous progress has been made in our understanding of the biology of neurotrophic factors and how they may be applied in the treatment of neurologic diseases. These advances have paved the way for new therapeutic approaches that may arrest or reverse the disease process underlying many types of peripheral neuropathies [2]. Studies with recombinant peptides have demonstrated that a number of neurotrophic factors, including nerve growth factor, neurotrophin-3, brain derived neurotrophic factor (BDNF), insulin-like growth factors and vascular epithelial growth factor can prevent the degeneration of peripheral sensory axons [2, 3]. However, these potent peptides cannot be administrated to patients for long periods of time, due to unwanted systemic effects or short systemic half-life [4]. The lack of effective response presented by modern therapeutics and the crescent need for new treatments have brought new interest in gene therapy approaches. The local expression of neurotrophic factors achieved by gene transfer may be used to attain the desired outcome avoiding unwanted adverse effects that could result from the systemic administration of drugs [5].

The present work aims at the development of a non-viral gene delivery system able to specifically transfect a neuronal cell population with a therapeutic final purpose. In recent years extensive work has been developed in order to accomplish successful gene transfer to mammalian cells. Both viral and non-viral approaches have been attempted, but an effective and safe system has not yet been found as the drawbacks presented by each of the strategies remain to be solved. Poly(ethylene imine) (PEI) was introduced by Behr in 1995 [6], and has become one of the gold standards of non-viral gene delivery. PEI has been shown to effectively condense plasmids into colloidal particles able to transfect cells, both *in vitro* and *in vivo* [7]. These condensed particles are of spherical shape and can be prepared with a narrow particle size distribution, which presumably allows high cellular uptake of the plasmids, resulting in a high transfection efficiency of the system [7]. However, PEI based complexes interact with cells in a non-specific manner mainly due to electrostatic interactions resulting from the complex high positive charge [8]. In order to promote cell-specificity, efforts have been made to combine or even exchange the non-specific electrostatic polyplex-cell surface interaction with a specific receptor-

mediated cellular uptake, by the incorporation of cell binding ligands into the transfection complexes. A variety of ligands has been successfully coupled to PEI, including galactose for hepatocyte targeting [9], mannose for enhanced uptake by dendritic cells [10], epidermal growth factor for enhanced uptake by epithelial cells [11], integrin-binding peptides [12] and anti-CD3 antibodies for gene delivery to CD3-expressing cells [13]. Efforts have also been developed in order to attain neuron-specific complexes. The grafting of PEI with neurotensin and tet-1 peptides was pursued, and although an increased complex uptake was observed in PC-12 and dorsal root ganglia (DRG) primary cultures [14, 15], no transfection was attained in fully differentiated PC12 cells or DRG cultures.

Neurotoxins have been for long explored to target the nervous system. Such an example is the use of the tetanus toxin (TeNT). When applied intramuscularly TeNT binds to the pre-synaptic neuron terminals, and after internalization is trafficked to the cell body by retrograde axonal transport [16], thus allowing the possibility of delivery by a minimally invasive method. The non-toxic carboxylic terminal fragment from TeNT (HC) has been chemically or genetically conjugated to several enzymes such as superoxide dismutase [17], horseradish peroxidase [18], and β-galactosidase [19]. The *in vivo* systemic administration of these conjugates resulted in the efficient delivery to the brain stem, motor neurons of the spinal cord and to the DRG. Fairweather *et al*. showed that HC-grafted poly(lysine) mediated transfection ten times more efficiently than polyplexes without HC, in neuronal cell lines [20].

Focusing on a therapeutic application, we developed a PEI-based system to deliver genes to the PNS. Based on the design of a modular structure, and therefore a more versatile one, we aimed at a targeted non-viral gene delivery system able to undergo retrograde transport. In a first step the core of the complex and its correspondent transfection efficiency was optimized using PEI as starting material. Polymer thiolation was performed in order to obtain a thiol-functionalized complex core. Upon optimization of the basal formulation, the grafting of the targeting moieties - HC fragment - was performed via a bifunctional PEG spacer. Using this strategy the amount of protein on the complex surface was easily tuned in order to obtain neuron specificity and transfection.

Materials and Methods

Polymer modification

Branched PEI (25 kDa, Sigma) was purified by dialysis, using a membrane with a molecular weight cut-off of 2.5 kDa (Spectrum labs, USA), for 3 days against a 5 mM HCl solution at 4ºC (renewed daily). After freeze-drying, the purified PEI was

dissolved in a 5% (w/v) glucose solution (pH 7.4) at 1 mg.ml^{-1}. Alternatively, purified PEI was modified with 2-iminothiolane (Sigma) as described elsewhere [21]. Briefly, a 2-iminothiolane solution in dimethylformamide was added to a PEI solution in phosphate saline buffer (0.05 M NaH_2PO_4/Na_2PO_4, 0.1 M NaCl, 0.01 M EDTA, pH 7.4), at a ratio of one mole of 2-iminothiolane per mole of PEI primary amine, and let to react for 3 hrs while stirring at room temperature (RT). The thiolated PEI (PEISH; Fig.1) was purified as previously described and freeze-dried. PEISH was dissolved in a 5% (w/v) glucose solution (pH 7.4) at 1 mg.ml^{-1} concentration and stored at -80 ºC until further use.

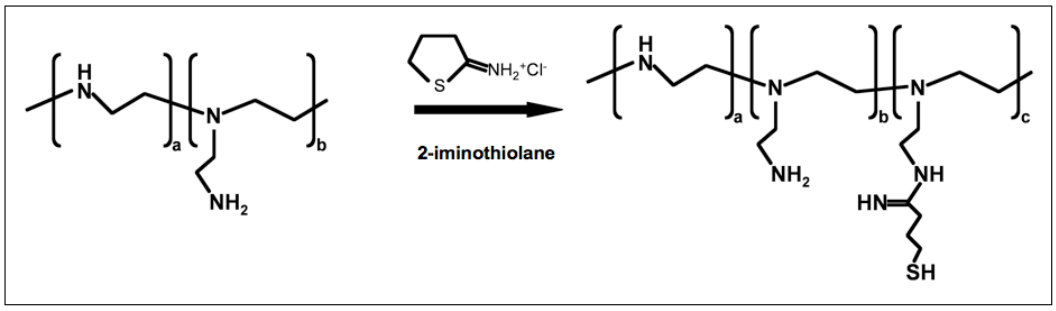


Figure 1- Branched poly(ethylene imine) thiolation reaction with 2-iminothiolane.

Plasmid DNA

The plasmid DNAs used encoded for the β-galactosidase (β-Gal; pCMV-Sport-βGal, 7.8 kb), green fluorescent protein (GFP; pCMV-GFP, 7.4 kb), luciferase (Luc; pCMV-LUC, 6.4 kb) or BDNF (pCMV-BDNF, 7.7 kb). Plasmids were produced in a DH5α competent *E. coli* strain transformed with the respective plasmid. Subsequently, DNA purification was performed using an endotoxin-free Maxiprep kit following the manufacture's instruction (GenElute, Sigma). Plasmid concentration and purity were assessed by UV spectroscopy. Plasmid solutions with Abs (260 nm/280 nm) ratio comprised between 1.7 and 2.0 were used in all studies.

Complex core formation (binary complex)

DNA-polymer complexes were prepared as described elsewhere [22] by mixing, while vortexing, equal volumes of plasmid DNA (in 5% (w/v) glucose aqueous solution, pH 7.4) and PEI or PEISH solutions. PEI-based complexes were allowed to form for 15 min at RT before further use. PEISH based complexes were let to oxidize for 24 hrs at RT. PEI based complexes with different molar ratios of primary amine groups (N) to moles of DNA phosphate groups (P) – N/P molar ratio – were prepared. In the case of PEISH based complexes the weight ratio of polymer/DNA was the same as used for the PEI based complexes.

Ternary complex formation

HC production, purification and modification. The non-toxic fragment of the tetanus toxin (HC) was produced recombinantly using the BL21 *E. coli* strain. The plasmid encoding for the HC fragment, together with a coding sequence for six histidines in the N terminal, was a kind offer from Prof. Neil Fairweather (King's College, UK). The HC production in the BL21 *E. coli* strain and purification was performed as described by Sinha *et al*. [23]. Subsequently, the obtained HC fragment was covalently linked to a PEG spacer bearing a maleimide (MAL) end group. Briefly, a bifunctional 5 kDa PEG (JenkemUSA, China) bearing an N-hydroxysuccinimide (NHS) and an MAL end group was used as indicated by the manufacturer, at a 2.5 PEG/HC protein molar ratio. The amount of reactive MAL groups in the HC protein was determined using a modified Ellman's assay [24], and found to be 1.5±0.4 mol PEG/HC. Hereafter, the PEG-modified HC will be designated as HC-PEG.

Ternary complex formulation. The core complexes were formed using PEISH at an N/P molar ratio of 3 and let to stabilize for 15 min. Subsequently, at a final concentration ranging from 1.25 to 7.5 µg per 2 µg of plasmid DNA, HC-PEG was added to the complex mixture and let to react for 24 hrs at RT. The efficiency of HC-PEG binding to the complex was assessed by means of radiolabelling as follows. HC-PEG was labelled with ^{125}I using the iodo-gen method [25]. Purification of the ^{125}I-HC-PEG was performed using Sephadex G-25 M columns (PD-10, Amersham Pharmacia Biotech). The yield of the iodination reaction was 97%, as estimated by trichloroacetic acid precipitation (TCA, 10% v/v, Merck) of the protein. The HC-PEG working solution was prepared by diluting ^{125}I-HC-PEG with non-labelled HC-PEG (1 mg.ml^{-1}) to a final concentration of (15x10^6 cpm.mg^{-1}) and left at 4ºC. Ternary complexes corresponding to 20 µg of DNA were prepared using the HC-PEG working solution, as previously described. In parallel, the same amount of HC-PEG working solution was diluted to the same final volume with 5% (w/v) glucose solution (pH 7.4) and used as controls. After 24 hrs, the complexes and free -^{125}I-HC-PEG dispersions were separated using a 100 kDa cut-off filter (Vivaspin 500, Sartorius) by centrifugation at 1300 rpm for 30 min, and washed 2 times with 0.2 ml of a 5% (w/v) glucose solution (pH 7.4). The eluted solution was transferred to radioactive immunoassay tubes and the corresponding radioactivities were counted in a gama-counter. The HC-PEG percentage of binding to the complex core was determined by comparing the counts of free ^{125}I-HC-PEG from the ternary complexes and the corresponding control.

Complex size and zeta potential determination

Complexes were prepared as previously described. Ten μg of plasmid DNA (pCMV-GFP) was used for each formulation. The zeta potential and size of the complexes were assessed using a Zetasizer Nano Zs (Malvern, UK) following the manufacturer's instructions. The Smoluchowski model was applied for zeta potential determination and cumulant analysis was used for mean particle size determination. All measurements were performed in triplicate, at 25°C.

Cell culture

Cell lines. ND7/23 (mouse neuroblastoma (N18 tg 2) x rat dorsal root ganglion neurone hybrid) or NIH 3T3 (mouse embryonic fibroblast) cell lines (both obtained from ECACC) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, supplemented with 10% (w/v) foetal bovine serum (FBS) (heat inactivated at 57ºC for 30 min.) and 1% PS (10,000 units.ml^{-1} penicillin and 10,000 μg.ml^{-1} streptomycin), all supplied by Gibco, and maintained at 37°C in a 5% CO_2 humidified incubator. The ND7/23 cell line was chosen as a sensorial PNS cell model and the NIH 3T3 cell line as a fibroblast model. Cells were routinely tested for mycoplasma by standard PCR.

DRG primary culture cells. Embryos (E17), obtained from euthanized pregnant Wistar rats, were placed in cold Hanks' balanced salt solution (HBSS, Sigma). The spinal cord with the DRGs attached was isolated from the ventral region of the embryos. The DRGs were gently detached and placed in HBSS. Subsequently, the DRGs were incubated in 0.1% (w/v) trypsin (Sigma) in HBSS without Ca^{2+} and Mg^{2+} (Sigma) for 15 min at 37ºC and collected by centrifugation (2 min at 1700 rpm). The DRGs were then dissociated in complete medium (DMEM/F12 high glucose, from Gibco), 10% (w/v) FBS and 1% (w/v) PS) using fired Pasteur pipettes. The obtained cell suspension was plated on a tissue culture polystyrene (TCPS) flask for 90 min, in order to purify the DRG dissociated culture (DRGc) from TCPS adherent cells. From the final cell suspension, 2x10^4 cells.cm^{-2} were seeded in a 24-well plate on glass coverslips treated with poly D-Lysine (PDL, 0.1 mg.ml^{-1}, Sigma) and laminin (10 μg.ml^{-1}, Sigma), in DMEM/F12 supplemented with 50 ng.ml^{-1} of nerve growth factor (NGF 7s; Invitrogen) and 5-fluoro-2'-deoxyuridine (60 μM, Sigma). Medium was supplemented with NGF (50 ng.ml^{-1}) every 2 days and renewed every week. This protocol allowed a neuron purity >80% in DRGc, at 24 hrs post-plating, as determined by immunocytochemistry. Briefly, DRGc were fixed with 4% (w/v)

paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at RT, permeabilized for 20 min with PBS containing 0.1% (v/v) Tween 20 and blocked for 30 min with PBS containing 3% BSA (w/v). Cells were incubated with rabbit polyclonal anti-200kDa Neurofilament (N200; 1:1000; Abcam, UK) in PBS with 3% (w/v) BSA overnight. After washing with PBS, cells were incubated with goat anti-rabbit Alexafluor 488 labeled antibody (1:500, Invitrogen) in a 1% (w/v) BSA solution on PBS for 1 hr. Finally, the cell nucleus were stained with DAPI (0.1µg.ml^{-1}, Sigma) and mounted using Vectashield (Vector, UK). The percentage of DRG cells was determined by counting 5 different fields per replicate (n=3).

HC binding studies

For this study the HC protein was labelled with a rhodamine fluorochrome. Briefly, 1 mg of rhodamine-NHS (ROX–5(6)-Carboxy-X-rhodamine N-succinimidyl ester, Sigma) dissolved in 500 µl of anydride dimethyl formamide (Sigma) was added drop wise to 6 mg of HC (1mg.ml^{-1}) in buffer A (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and let to react for 1 hr. The obtained solution was washed and concentrated using a 30 kDa cut-off filter (Millipore, Ireland). After filtration (0.22 µm, TPP), the rhodamine-labelled HC (HC-rox) concentration was assessed using the BCA assay (Pierce, USA), set to 1 mg.ml^{-1} with buffer A and stored at -20 ºC until further use.

To investigate the cell binding specificity of the HC fragment, cells were incubated with HC-rox. The ND7/23 and NIH 3T3 cell lines were seeded at a cell density of 2.0x10^4 and 2.5x10^4 cells.cm^{-2}, respectively, on glass coverslips coated with PDL placed in a 24-well plate. Briefly, 24 hrs post-seeding, cells were incubated at 4°C for 15 min and then washed twice with cold HBSS before a 1 hr incubation at 4°C with HC-rox at a concentration of 5 µg.ml^{-1} in HBSS containing 0.1% (w/v) BSA. Additionally, in the case of the ND7/23 cell line, a control was performed in which a 15-min incubation with 10 µg.ml^{-1} of unlabelled HC was performed prior to the incubation with HC-rox. After washing twice with HBSS, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at RT, stained with DAPI (1µg.ml^{-1}, Sigma) for 5 min and mounted on slides with Vectashield (Vector). Cells were analyzed using an inverted fluorescent microscope (Axiovert 200M, Zeiss, Germany).

Cellular internalization studies

ND7/23 and NIH 3T3 cells were sub-cultured in 24-well TCPS plates for 24 hrs as described in section 2.7. Cells were exposed, for 1 hour at 37ºC, to ternary complexes (2 µg of plasmid DNA/well) formulated with different HC-PEG amounts, respectively, 0, 1.25, 2.5, 5 and 7.5 µg per 2 µg of DNA. Plasmid DNA was labelled

with YOYO-1 (Invitrogen, USA) as indicated by the manufacturer (1 mole YOYO-1 per 100 bp of plasmid DNA). For the competition assays, prior addition of the ternary complexes, cells were incubated for 20 min at 4ºC with a 100-fold excess of free HC or a 5-fold excess of the correspondent unlabeled ternary complex. For complex internalization assessment, cells were incubated with a trypan blue solution (0.2 $mg.ml^{-1}$ in PBS, Sigma) for 5 min, trypsinized and processed for flow cytometry. Ten thousand gated events were taken for each replicate (n=3) using a FACSCalibur cytometer (BD Biosciences, USA) and analysed by histogram for positivity for YOYO-1 using the FlowJo software (version 8.3.7, USA).

Transfection studies

Cell lines. The ND7/23 and NIH 3T3 cells were sub-cultured for 24 hrs as described for the HC binding studies. Two hours prior transfection, medium was removed and replaced by 0.5 ml of fresh medium. Different N/P ratios of the core complexes were tested (2, 3, 6 and 12) using PEI and PEISH. For the ternary complexes different formulations (0, 1.25, 2.5, 5 and 7.5 µg of HC-PEG per 2 µg of plasmid DNA) were tested. Cells were exposed, for 6 hrs at 37ºC, to complexes containing 2 µg of plasmid DNA before the medium was renewed. Cells were incubated for an additional 48, 72 or 96-hour period and processed for transfection activity or efficiency assessment, using β-galactosidase or GFP as the reporter genes.

The transfection activity, corresponding to the β-Gal activity (ortho-nitrophenyl-β-galactoside (ONPG) hydrolyses), was determined by an enzymatic assay (Promega). All experiments were performed in triplicate and expressed in terms of specific activity (nmoles of ONPG hydrolyzed/min/mg total protein). BCA assay (Pierce) was used to quantify the total protein content. For transfection efficiency assessment, cells were analysed by flow cytometry for GFP expression. Twenty thousand gated events were taken for each replicate (n=3) as previously described.

Primary cultures. DRGc were seeded at a density of 2.0×10^4 $cells.cm^{-2}$ and 3 to 4 days post-seeding they were incubated with complexes for 6 hrs. Complexes based on PEISH prepared at an N/P=3, with or without 7.5 µg of HC-PEG moieties, were tested. 72 hrs post-transfection both luciferase, GFP or BDNF gene expression was determined, and 96 hrs post-transfection the expression of the first gene. The luciferase assay was performed according to the manufacturer instructions (Promega). In these conditions, 5 pg of Luciferase (Sigma) had a value of 703 Relative light units (RLU). RLU where collected for 10 seconds and normalized to the

cell extract total protein. Immunostaining was performed to assess the percentage of GFP positive cells. Discrimination between neurons and non-neuron cells was achieved by N200 staining. Briefly, DRGc were fixed, permeabilized for 20 min with PBS containing 0.1% (v/v) Tween 20 and blocked for 30 min with PBS containing 3% BSA (w/v). Biotin conjugated mouse monoclonal anti-GFP (1:200; Novus Biologicals, USA) and N200 rabbit polyclonal (1:1000; Abcam, UK) were incubated in PBS-3% BSA overnight. After washing with PBS, cells were incubated with PBS containing 1% (v/v) H_2O_2 for 10 min, then, with peroxidase coupled ABC complex (Vectastain Elite, Vector, UK) for 30 min and finally with DAB/Ni solution (Vector, UK). To reveal the N200 staining, the DRGc were incubated with goat anti-rabbit Alexafluor 488 labeled antibody in a 1% (w/v) BSA solution in PBS (1:500, Invitrogen) for 1 hr. Finally, the cells were stained with DAPI (0.1µg.ml^{-1}, Sigma) and mounted using Vectashield. The percentage of transfected cells, DAB/Ni positive, was determined by counting 5 different fields per replicate (n=3). Images were collected using an inverted fluorescence microscope (Axiovert 200M, Zeiss, Germany). BDNF protein secretion was determined in cell media (24-hr culture period), using a BDNF enzyme-linked immunosorbant assay (ELISA) kit (Chemikine, Chemicon) following the manufacturer instructions and expressed as pg.ml^{-1}.

Cytotoxicity assay

To assess cell viability after transfection, a resazurin based assay was used [26]. Briefly, 24 hrs post-transfection a solution of resazurin (0.1 mg.ml^{-1} in PBS, Sigma) was added to each well to a final 10% (v/v) concentration. After 4 hrs of incubation at 37ºC, 200 µL of the medium was transferred to a black-walled 96-well plate (Greiner) and fluorescence was measured (λexc= 530 nm, λem=590 nm, Spectra Max GeminiXS – Molecular Devices). Results were expressed as percentage of metabolic activity of treated cells relative to untreated cells.

Statistical analysis

Using the Graphpad Prism 5.0 software, statistically significant differences between several groups were analyzed by non-parametric Kruskal-Wallis test, followed by Dunns post-test. Non-parametric Man-Whitney test was used to compare two groups. A p value lower than 0.05 was considered statistically significant.

Results

Complex core characterization and transfection evaluation

As determined by the Ellman's assay [24], 7.8±0.2% of primary amines of PEI were substituted by thiol groups in the PEISH polymer.

Cationic polymers like PEI form self-assembled complexes with anionic plasmid DNA by electrostatic interactions. When DNA is loosely complexed or in a free form, it freely migrates through an agarose gel. When PEI and PEISH based complexes were applied in an agarose gel and submitted to an electric field, no free DNA was detected for all N/P molar ratios tested (Fig. S1, supplementary data (SD)). The complex size and zeta potential were determined as a function of the N/P molar ratio used in the preparation of the binary complexes. No significant differences were found in terms of polydispersity index (Pdi) between PEI and PEISH based complexes. The Pdi values ranged between 0.222 and 0.409 for all N/P molar ratios tested. PEI based complexes ranged from 55±3 to 130±5 nm in diameter (Fig. S2 A, SD) and -26±1 to 46±1 mV in terms of zeta potential (Fig. S2 B, SD), which was directly related with the N/P molar ratio. PEI thiolation enabled the formation of significantly smaller particles at N/P molar ratios of 1 and 2 (Fig. S2 A, SD), as well as with lower zeta potential values for N/P molar ratios ≥2 (Fig. S2 B, SD).

Transfection activity mediated by PEI and PEISH based complexes prepared with N/P molar ratios of 2, 3, 6 and 12 was assessed in the ND7/23 cell line, 48 and 72 hrs post transfection (Fig. S3, SD). β-Gal activity was significantly higher for PEISH based vectors for N/P=2, at both time points tested. For N/P=3, the transfection activity was found to be similar for both polymer systems. At higher N/P values, the transfection activity was significantly reduced when mediated by PEISH based complexes. Relative viability assessed 24 hrs post transfection showed significant cell toxicity of the complexes prepared at N/P molar ratios higher than 3 (Fig.S4, SD).

Taken together the results of the core complex characterization, the cytocompatibility and transfection profile, we selected the N/P molar ratio of 3 as the formulation for the development of the ternary complex. By FACS analysis, the transfection efficiency of the thiolated binary complexes prepared at an N/P=3 was found to be 13.3±0.5% and 12.8±0.8% on the ND7/23 cell population, at 48 and 72 hrs post-transfection, respectively (data not shown).

HC fragment cell specificity

The determination of the binding specificity of the purified HC protein to the ND7/23 cell line is an important step in order to validate this cell line as an *in vitro* model for

neurospecificity evaluation of the developed complexes. The HC fragment of the tetanus toxin was fluorescently labelled with rhodamine (HC-rox) and found to specifically bind to the ND7/23 cell line (Fig. 2A), as compared to the control NIH 3T3 cell line (Fig. 2C). When cells were pre-incubated with a 100-fold excess of unlabelled HC, a significant reduction of the fluorescent signal was observed (Fig. 2B).

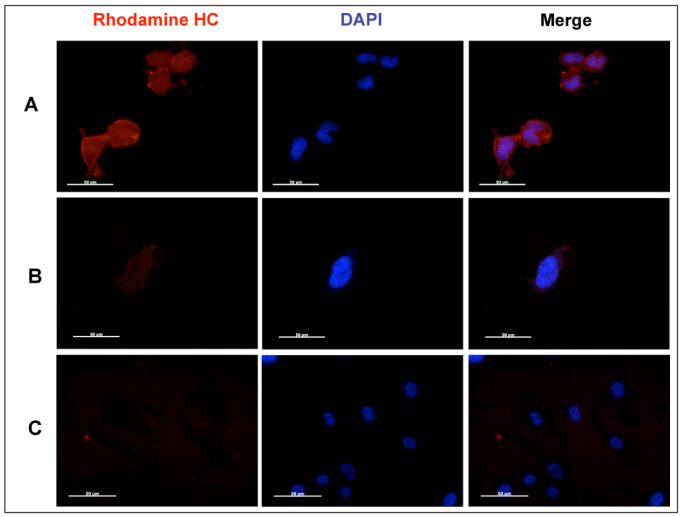


Figure 2- Microscope images corresponding to the binding affinity of the rhodamine-labelled tetanus toxin fragment (HC-rox). Lane A corresponds to the ND7/23 cell line, lane B to the ND7/23 cell line previously incubated with an excess of unlabelled tetanus toxin fragment and lane C to the NIH 3T3 cell line. Scale Bar = 50 μm.

Ternary complex formation

The strategy followed for the formation of a ternary polyplex considers two steps. In a first step the polymer was thiolated in order to obtain a thiol-decorated polyplex. The addition of the HC fragment was performed via a 5 kDa bifunctional poly(ethylene glycol) (PEG) linker reactive for the thiol groups present in the binary complexes (Fig 3).

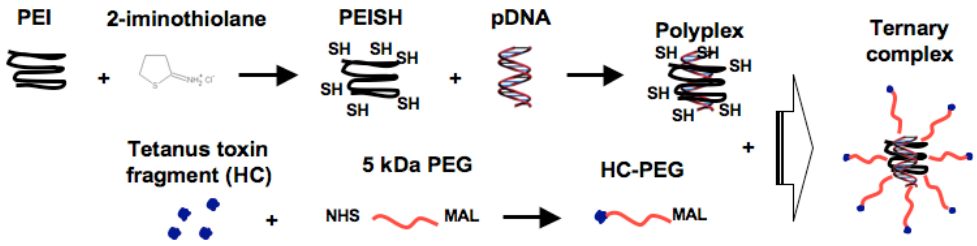
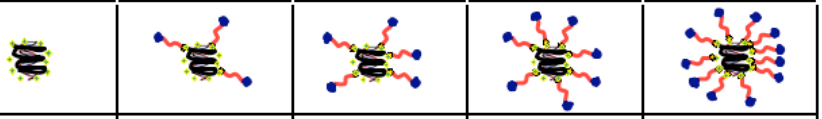


Figure 3- Proposed model for the formation of a ternary complex. Two steps are considered: the introduction of thiol moieties in the binary polyplex and a subsequential step in which the tetanus toxin fragment (HC) is coupled to the polyplex surface via a maleimide (MAL) grafted (thiol reactive) 5 kDa PEG linker.

The coupling efficiency of HC-PEG to the complexes, as determined by radiolabelling, ranged between 89±3% and 95±3% (Table 1).

Table 1- Theoretical thiol/HC-PEG fold content for each formulation tested and percentage of HC-PEG coupled to the complexes, as determined by radiolabelling.

	µg HC-PEG				
	0	1.25	2.5	5	7.5
					
Molar ratio of SH content of PEISH to HC-PEG	-	17.8	8.9	4.5	2.9
% bound HC-PEG	-	90±2	89±3	95±3	92±1

Footnotes: SH- thiol groups; HC- tetanus toxin fragment c; PEG- polyethylene glycol; PEISH- thiolated poly(ethylenimine).

The characterization of the ternary complexes by light scattering showed that the particle size peaked for the 1.25 µg HC-PEG formulation (Fig. 4A). For higher HC-PEG content, a reduction of the complex size was observed, which stabilized in the same size range of the binary complex (Fig. 4A). Additionally, no significant differences were found in terms of Pdi for the complex formulations tested (Fig. 4A). The addition of HC-PEG to the complexes, in the concentration range tested, showed to have no significant influence on the complex zeta potential (Fig. 4B), although a reduction tendency was observed for both ternary formulations prepared with the lowest HC-PEG content (i.e. 1.25 and 2.5 µg HC-PEG).

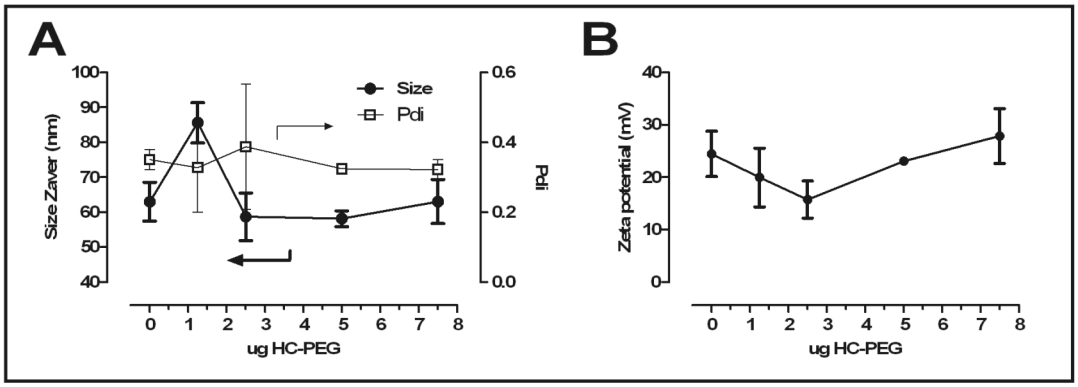


Figure 4- Characterization of ternary complexes based on PEISH (N/P=3) as a function of µg of HC-PEG per formulation (A) size and Pdi and (B) zeta potential (n=3,Aver±SD).

Ternary complex internalization

In the ND7/23 cell line, the extent of ternary complex cell-internalization was found to slightly decrease for the two formulations prepared with the lowest HC-PEG concentrations tested (1.25 and 2.5 µg of HC-PEG), when compared with the binary polyplexes (Fig. 5A). However, for the two highest concentrations of HC-PEG tested (5 and 7.5 µg of HC-PEG per formulation), the same extent of cell internalization values as for the binary complexes were observed (Fig. 5A). In contrast, the increase of the HC-PEG content in the complexes resulted in an opposite effect for the NIH 3T3 cell line. Although the two formulations prepared with the lowest HC-PEG concentration (1.25 and 2.5 µg of HC-PEG) had similar internalization pattern as the binary complexes, the formulations with the highest HC-PEG concentration were significantly less internalized by this fibroblast cell line (Fig. 5B).

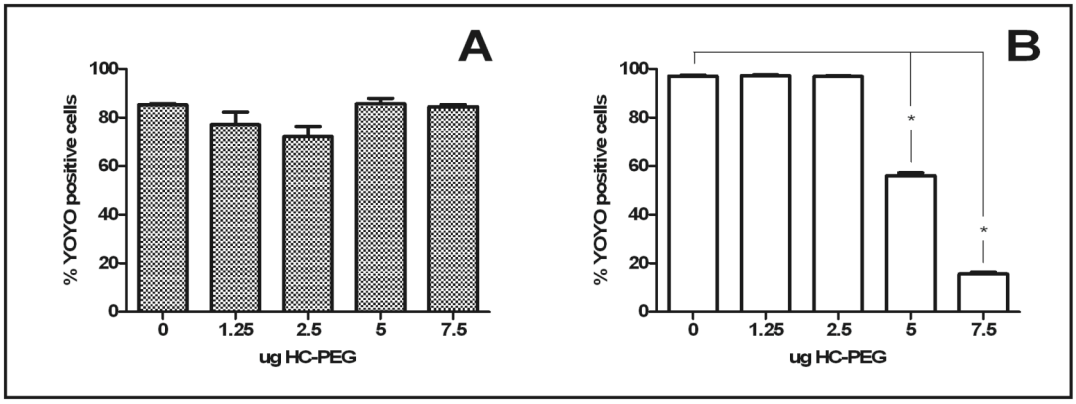


Figure 5 - Extent of cell internalization of ternary complexes prepared with PEISH (N/P=3) and with increasing amounts of HC-PEG in (A) ND7/23 and (B) NIH 3T3 cell lines, respectively (n=3, Aver±SD; * denotes statistically difference between groups and the binary formulation $p<0.05$).

The receptor-mediated internalization of the formulation prepared with 7.5 μg HC-PEG was further tested by means of competition tests. Following pre-incubation with a 100-fold excess of HC fragment, the ternary complex internalization was significantly reduced in the ND7/23 cells (Fig.6 B and C). When pre-incubated with a 5-fold excess of the correspondent unlabelled ternary complexes, a similar effect was observed, even though to a lower extent (Fig.6 B and D). Additional internalization studies with the binary formulation of PEISH (N/P=3) were performed in the ND7/23 cell line pre-incubated with a 100-fold excess of HC fragment. In this case the internalization levels were found not to significantly vary from the ones shown in Fig. 5A for this formulation.

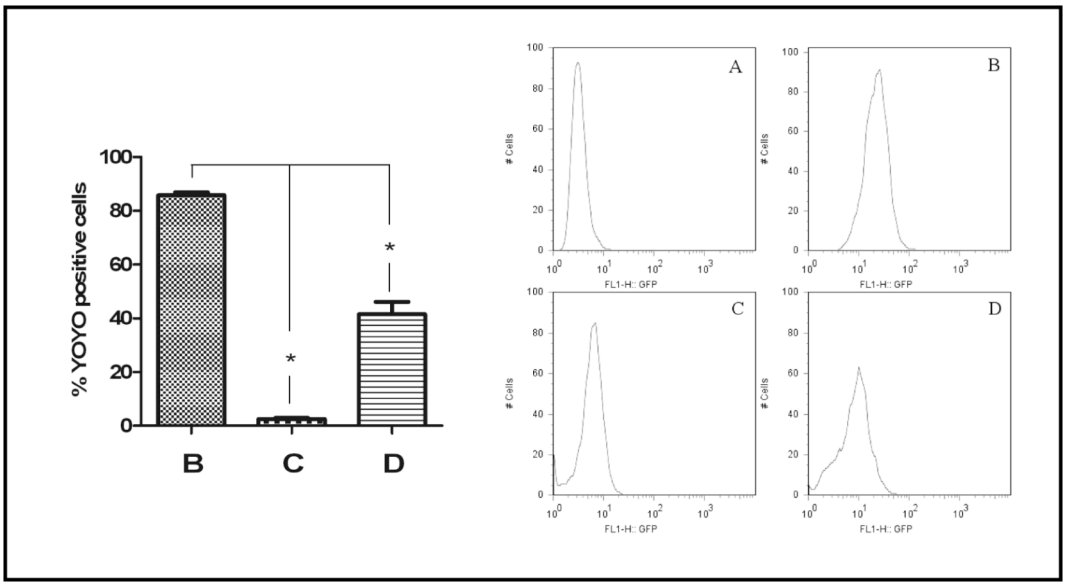


Figure 6 - Internalization in the ND7/23 cell line of ternary complexes based on PEISH N/P=3; (A) ND7/23 isotype; (B) 7.5 μg HC-PEG formulation; (C) competition with a 100-fold excess of free HC and (D) with a 5-fold excess of the correspondent unlabelled ternary complexes. (n=3, Aver±SD; * denotes statistically difference between groups, $p<0.05$).

Transfection efficiency of cell lines mediated by ternary complexes

In the ND7/23 cell line, at 48 hrs post transfection, approximately 10% of the cell population was positive for GFP after transfection with the binary complexes (Fig. 7A). The introduction of HC moieties at the complex surface initially leads to a reduction of transfection (formulations containing 1.25 and 2.5 μg of HC-PEG) at 48 hrs post transfection. Nevertheless, at higher HC protein content (formulations containing 5 and 7.5 μg of HC-PEG), the transfection efficiency was significantly increased when compared with the binary formulation (Fig. 7A). In the case of the 5 μg HC-PEG formulation a slight increase in transfection efficiency along time was observed, in contrast with the clear reduction in the case of the 7.5 μg HC-PEG formulation (Fig. 7A). For the NIH 3T3 cells, a decrease in transfection efficiency was seen with the increase of HC protein content on the complexes, which was significantly reduced for the two highest HC-PEG content complex formulations (Fig. 7B). A significant decrease in cell viability was observed in the ND7/23 cell line for the highest HC-PEG formulation at 24 hrs post-transfection, when compared with untreated cells (Fig. S5 A, SD).

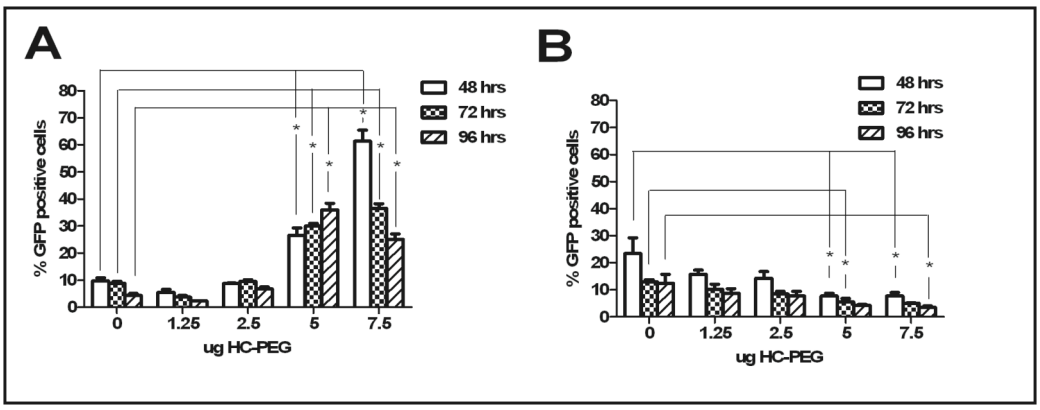


Figure 7- GFP positive cells at 48, 72 and 96 hrs post transfection of ternary complexes based on PEISH (N/P=3) as a function of HC-PEG in (A) ND7/23 and (B) NIH 3T3 cell lines. (n=3, Aver±SD; * denotes statistically difference between groups and control, $p<0.05$).

Ternary complex transfection in primary cultures

The 7.5 µg HC-PEG ternary complex formulation was tested in dissociated DRG cultures and the transfection mediated by these complexes evaluated in terms of luciferase expression, 72 and 96 hrs post-transfection. As seen in Fig. 8 A, 72 hrs post-transfection the transfection activity was significantly reduced for the HC-PEG grafted complexes, in comparison with the binary complexes. Nevertheless, 96 hrs post transfection no significant differences were observed between groups (Fig. 8A). GFP immunostaining revealed that at the early evaluation point no significant differences were observed in terms of the % of total transfected cells (Fig. 8B). Nevertheless, by discriminating the neuron from non-neuron cell populations, one could observe significant differences between the 0 and 7.5 µg of HC-PEG formulations, with $p=0.0044$ and $p=0.0349$, respectively (Fig. 8C).

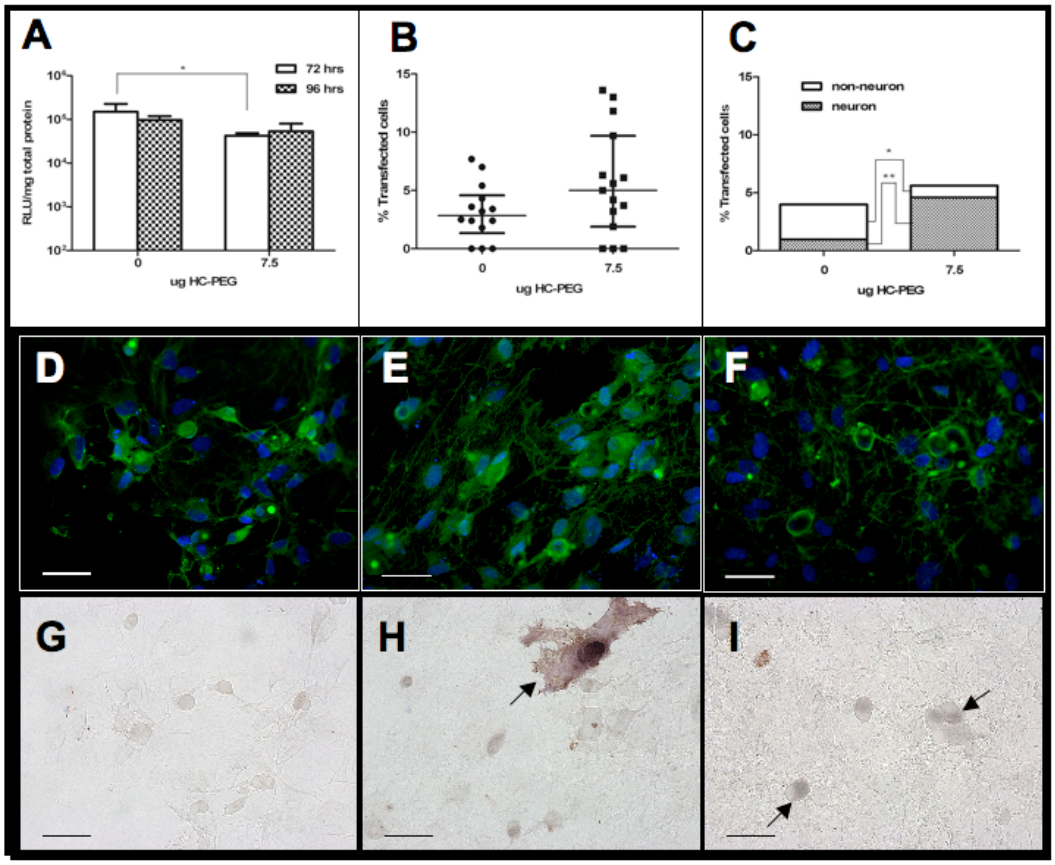


Figure 8- A) Transfection activity of complexes based on PEISH at N/P=3 with or without HC-PEG moieties, at 72 and 96 hrs post-transfection in dissociated dorsal root ganglia cultures (DRGc; Aver±SD; * $p<0.05$); **B**) transfection efficiency of the complexes at 72 hrs post-transfection in DRGc (median±interquartile range), **C**) classification of DRGc transfected cells as neuron and non-neuron cells; **D, E, F**) immunocitochemistry of DRGc with N200 and DAPI for untreated cells, and cultures treated with binary complexes (PEISH) and ternary complexes with 7.5 µg HC-PEG, respectively (bar=50um). **G, H, I**) immunocitochemistry of DRGc with anti-GFP (DAB/Ni) for untreated, PEISH and PEISH with 7.5 µg HC-PEG, respectively. Scale bar = 50 µm.

The non-neuron cell transfection was reduced while the neuron cell transfection was increased when the HC-PEG moiety was introduced to the complexes (Fig. 8 C-I). No significant toxicity was found in terms of cell metabolic activity for both PEISH and PEISH 7.5 µg HC-PEG (Fig. S7, SD).

Additionally, the ability of the PEISH based ternary complexes to mediate the transfection of the DRGc with a plasmid encoding for the BDNF was also tested. As seen in Figure 9 PEISH based complexes were able to elicit significantly higher BDNF expression (>100 $pg.ml^{-1}$) in relation to naked DNA.

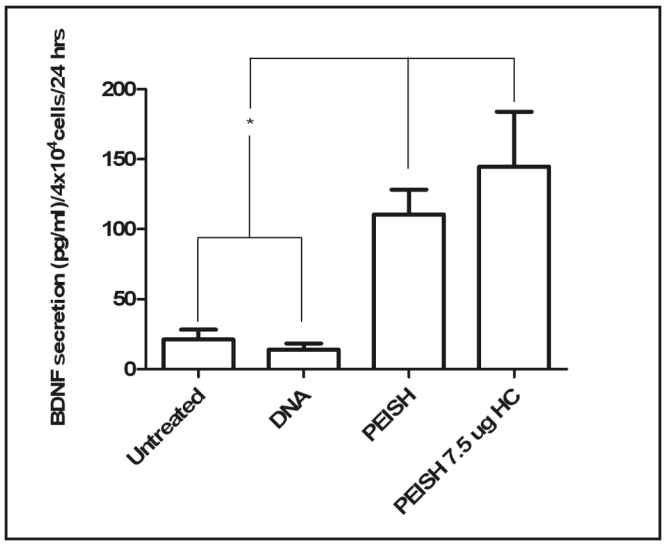


Figure 9- BDNF secretion (24-hr period) in dissociated dorsal root ganglia cultures (DRGc) mediated by complexes based on PEISH at N/P=3 with or without HC-PEG moieties, at 72 hrs post-transfection, the naked plasmid encoding for the BDNF was used as control (DNA; Aver±SD; * $p<0.05$).

In addition, BDNF levels of cells treated with vectors transporting the GFP gene were also tested in order to assess the effect of the vector alone in endogenous BDNF expression. The levels of BDNF were shown not to significantly vary from untreated cells (data not shown).

Discussion

A simple, safe and efficient system that can specifically transfect peripheral sensorial neurons can bring new answers to address peripheral neuropathies. Towards the achievement of such a system, our approach is based on the use of the cationic polymer PEI, which was shown to be an efficient transfection agent, both *in vitro* [6, 27] and *in vivo* [28-30]. However, the use of PEI based complexes can compromise the intended cellular targeting, due to their excessive positive charge [8].

In order to develop neuron specific complexes, the thiolation of PEI was performed to allow the subsequent functionalization of PEI:DNA complexes with a neuron targeting protein. Firstly, the N/P molar ratio of thiolated PEI based complexes was optimized aiming at establishing the required complex properties to attain efficient transfection of neuronal cells and, in a second stage a non-toxic fragment from the tetanus toxin (HC) was grafted to the surface of the nanocomplexes, in order to prone these specifically for neurons.

A binary complex based on PEISH prepared at an N/P ratio of 3 was found to be optimal in terms of size and zeta potential characteristics and ability to transfect the ND7/23 cell line. Moreover, complexes prepared at this N/P molar ratio were found to be non-toxic under the tested conditions. PEI thiolation has been previously explored in order to develop triggered complexes that could be stable in the oxidizing extracellular environment but unstable in the reducing intracellular environment [31]. Carlisle *et al*. [21] showed that thiolated PEI based complexes, when used at a total amine to phosphate molar ratio ≥ 10, inhibited complex transfection capacity, indicating an over stabilization of the complex. Our results are in agreement with this study, showing that for N/P molar ratios higher than 3 (corresponding to a total amines to phosphate molar ratio of 12) a significant reduction of transfection activity was observed in the ND7/23 cell line.

Having established the conditions for the preparation of the core of the complex, the grafting of the protein moieties was pursued. The use of a PEG spacer is expected to increase complex hydrophilicity and therefore to enhance complex stability in an aqueous environment, as well as increase the protein moieties exposure. The grafting of the bifunctional PEG to HC was optimized in order to obtain approximately one active PEG per protein. The amount of HC-PEG coupled to the complexes was found to be over 89% for all the prepared formulations. The ternary complex size, zeta potential and Pdi were shown not to vary significantly for the formulations prepared with HC-PEG content higher than 2.5 µg, when compared to the binary complex.

The binding specificity of the developed ternary complexes was tested both in the ND7/23 sensorial neuron cell model and NIH 3T3 control cell line. A significant decrease in the extent of complex internalization with increasing amounts of HC-PEG was found for the NIH 3T3 cells, clearly indicating the targeting potential of the ternary complexes. Additionally, one could observe that the high levels of ternary complex internalization were reduced both by the competition with free HC or the correspondent unlabeled ternary complexes (tested for the 7.5 µg HC-PEG formulation). Upon increase of HC-PEG moieties, the transfection efficiency was augmented in the ND7/23 cell line (up to 7 fold), whereas in the NIH 3T3 cells a significant decrease was observed, corroborating the results obtained in the internalization and competition studies. Our results indicate that although the overall levels of internalization are at constant levels in the ND7/23 cell line, the incorporation of HC-PEG moieties clearly triggers a specific internalization of the complexes.

Signs of toxicity were observed in the ND7/23 cells in the conditions that mediated the higher transfection levels (7.5 μg HC-PEG formulation). Considering that the levels of gene expression were also the highest for this formulation (see Fig. S6, SD), one can associate this toxic effect with the high levels of GFP protein, which has been described as cytotoxic when over-expressed [32-34].

Both transfection activity and efficiency mediated by the binary and ternary complexes (7.5 μg HC-PEG formulation) were assessed in a DRG primary culture, in order to evaluate the transfection specificity of the developed ternary vectors. Transfection activity, as evaluated by luciferase assay, was shown to be significantly lower or similar, at 72 and 96 hrs post-transfection, respectively, when cells were treated with targeted complexes with relation to the binary complexes. Furthermore, in terms of transfection efficiency, the % of total transfected cells was found not to significantly vary between the binary and the ternary complexes. However, when cell population discrimination was undertaken - neuron *versus* non-neuron cells - a significant increase in the % of transfected neurons together with an inversely proportional decrease in the % of transfected non-neuron cells was observed for the ternary complexes. Taken together, these findings indicate that upon functionalization of the complexes, transfection was promoted in primary neurons, thus reinforcing the targeting potential of the developed vectors. Moreover, we showed that the developed targeted vectors could transfect DRG primary cultures with a plasmid enconding for BDNF, leading to the efficient expression of this relevant neurotrophic factor.

Pun *et al.* [14] showed that targeted cellular binding to differentiated PC-12 and dissociated DRG cells can be obtained by grafting PEI with the tet-1 peptide, a peptide obtained by phage display exhibiting similar binding activities to tetanus toxin [35]. Nevertheless, no transfection was observed in fully differentiated PC-12 or dissociated DRG cultures [14]. We hypothesise that by retaining the retrograde transport ability [36], the HC fragment will enable an improved intracellular trafficking of the ternary complexes, thus leading to efficient transfection of neuron cells. Further studies are currently being performed to shade light over this process.

By using an integrative approach we have developed a multi-component cell-targeted gene delivery vector that can be formed by the simple mixture of the different components. To our knowledge this is the first report of a non-viral vector not only able to transfect DRG neuron cells, but also in a targeted fashion. The application of the developed ternary complexes *in vivo* in a regeneration model, following a minimally invasive application is currently being investigated. In addition, both the

stability of the ternary complexes in an *in vivo* environment and their ability to undergo retrograde transport will be studied.

Conclusions

Neuron targeted gene delivery systems encounter numerous barriers implying newer approaches in their development. In this study, we proposed a novel multi-component nanoparticle system that successfully targeted and transfected neuronal cell lines as well as DRG neuron primary cells. Being able to target the PNS, this system brings new perspectives for the *in vivo* application of gene therapy approaches towards PNS mediated by non-viral vectors.

Acknowledgements

This project was carried out under the Portuguese Foundation for Science and Technology (FCT) contract POCI/SAU-BMA/58170/2004. The authors would like to thank Manuela Brás (INEB) for the FTIR measurements, Patrícia Cardoso (INEB) and Susana Carrilho (IBMC) for the mycoplasma screening of the cell cultures and Raquel Gonçalves and Simon Monard for the precious help with flow cytometry. Hugo Oliveira and Liliana R. Pires acknowledge FCT for their PhD scholarships (SFRH/BD/22090/2005 and SFRH/BD/46015/2008, respectively).

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Supplementary data

Complexes based on PEI and PEISH were prepared with a range of N/P molar ratios, using pCMV-GFP. 20 µl of each complex solution or DNA alone, together with 4µl of loading buffer (Fermentas) were loaded in a 1% (w/v) agarose (Cambrex) gel, with 0.05 $\mu g.ml^{-1}$ of ethidium bromide (Q-BioGene). The electrophoresis was run in a 90V field for 45 min, using Tris-Acetate-EDTA buffer (pH 8) as the running buffer.

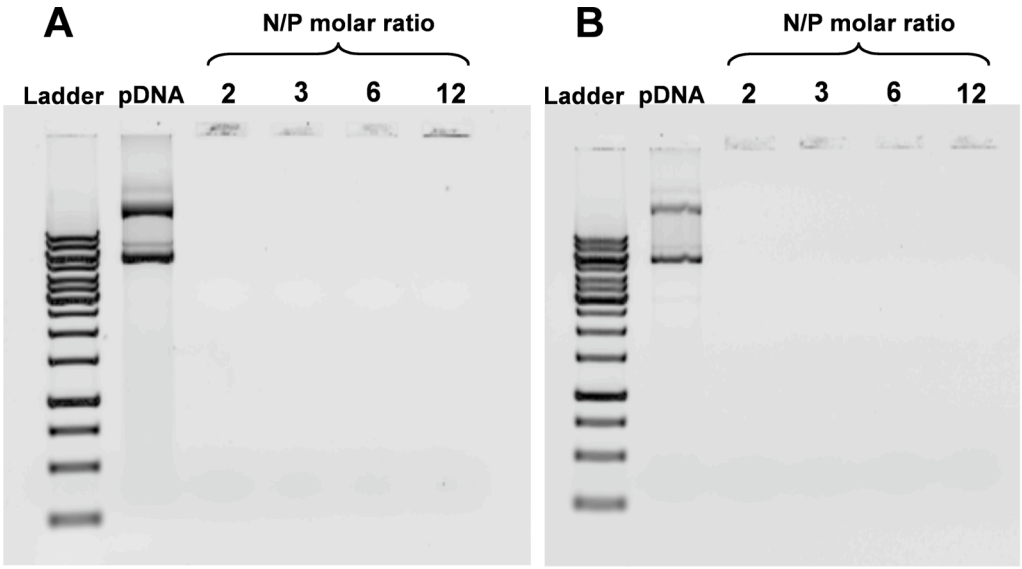

Figure S1- Agarose gel electrophoresis of complexes as a function of N/P molar ratio for (A) PEI and (B) PEISH.

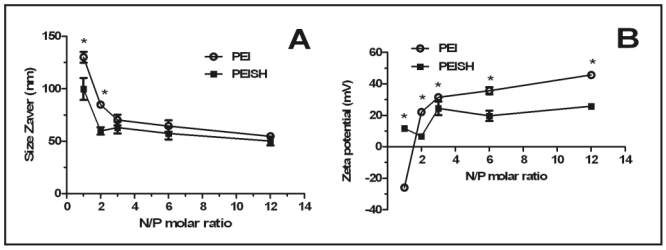

Figure S2- Characterization of binary complexes based on PEI and PEISH as a function of N/P molar ratio; (A) size and (B) zeta potential (n=3, Aver±SD; * denotes statistically difference between PEI and PEISH for the same N/P ratio, $p \leq 0.05$).

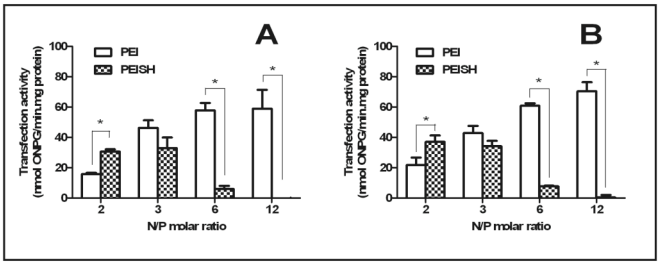

Figure S3 - Transfection activity evaluated (A) 48 hrs and (B) 72 hrs post-transfection in ND7/23 cells treated with binary complexes based on PEI and PEISH prepared at different N/P molar ratios. (n=3, Aver±SD; * denotes statistically difference between groups at the same N/P ratio, $p<0.05$).

Relative viability of the ND7/23 cells was assessed by means of a resazurin assay at 24 hrs post transfection with PEI and PEISH base complexes as a function of the N/P molar ratio.

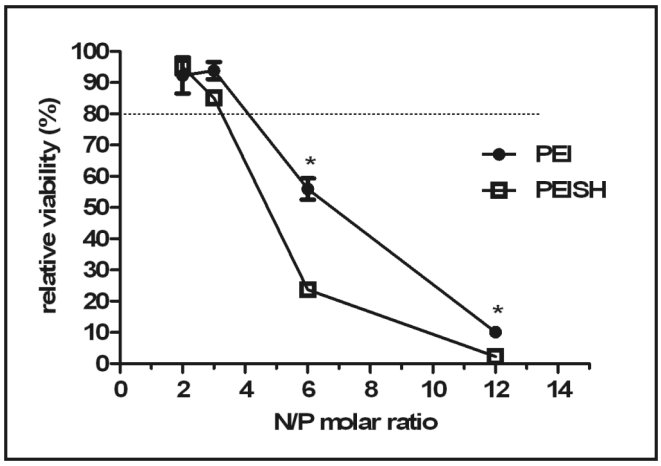

Figure S4- Relative viability at 24 hrs post transfection, of complexes based in PEI and PEISH as a function of the N/P molar ratio (n=3, Aver±SD; * denotes statistically difference between groups, $p<0.05$).

Relative viability of the ND7/23 and NIH 3T3 cells was assessed by means of a resazurin assay at 24 hrs post transfection with ternary complexes based in PEISH as a function of the amount of HC-PEG.

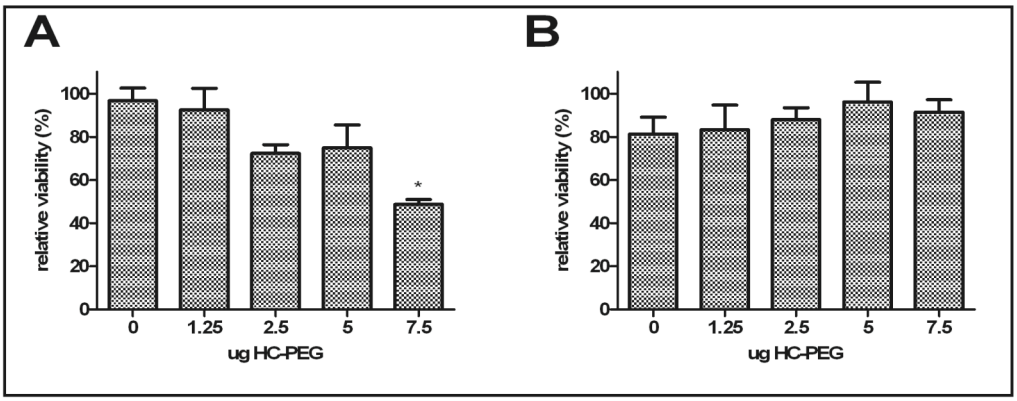

Figure S5- Relative viability at 24 hrs, post transfection, of ternary complexes based on PEISH (N/P=3) as a function of HC-PEG in (A) ND7/23 and (B) NIH 3T3 cell lines. (n=3, Aver±SD; * denotes statistically difference between group and untreated cells, $p<0.05$).

Transfection activity expressed as average mean fluorescence of ND7/23 cells after transfection with ternary complexes based in PEISH as a function of the amount of HC-PEG.

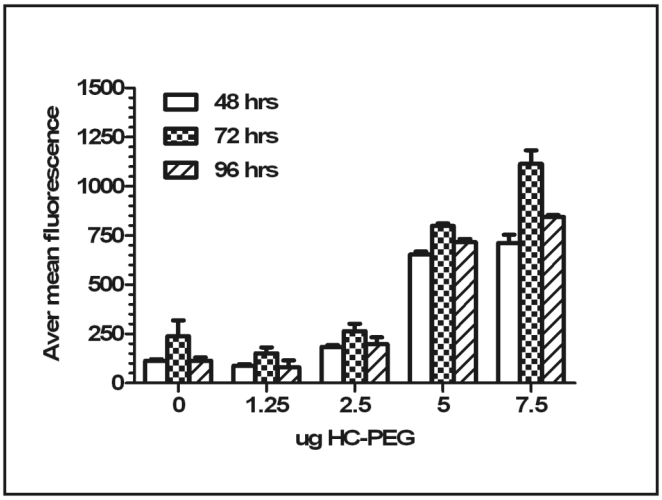

Figure S6- Average mean fluorescence at 48, 72 and 96 hrs post transfection, of ternary complexes based on PEISH (N/P=3) as a function of HC-PEG in ND7/23 cell line.

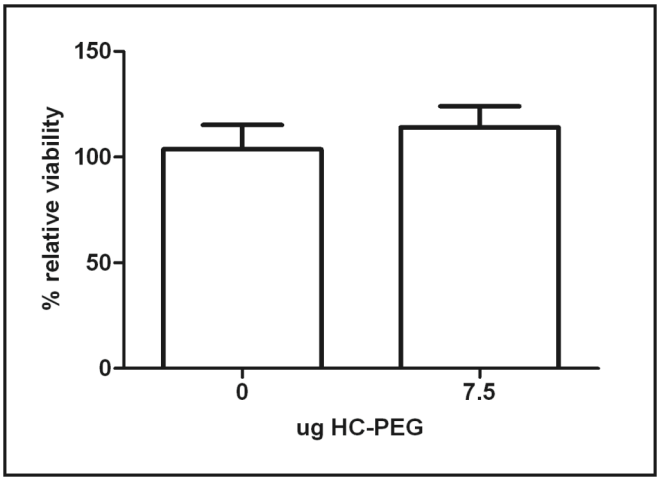

Figure S7- Relative viability at 24 hrs, post transfection, of ternary complexes based on PEISH at N/P=3 with or without HC-PEG moieties (n=3, Aver±SD).

Chapter IV

Tailoring targeted gene delivery nanoparticles with molecular recognition force spectroscopy *

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* Submitted

Abstract

Molecular recognition force spectroscopy is proposed as a novel screening-tool to optimize the targeting moieties density of functionalized nanoparticles towards attaining cell-specific interaction. By tailoring the nanoparticle formulation, the unbinding event probability between nanoparticles tethered to an atomic force microscopy tip and neuronal cells could be directly correlated to the nanoparticle gene vectorizing capacity. This novel approach can open new avenues in the field of nanomedicine.

Gene therapy can be defined as a method that provides to somatic cells the genetic information required for producing specific therapeutic proteins in order to correct or modulate determined pathologies.[1] For most of the clinically relevant conditions the expression of the delivered therapeutic gene should be constrained to a certain cell type. Extensive work has been developed in the recent years in order to optimize and achieve successful gene delivery to mammalian cells. While targeted systems have primarily to interact with specific cell surface molecules, most of the developed non-viral gene delivery systems associate to cells by non-specific interactions. In order to target non-viral gene delivery vectors to specific cell populations, efforts have been made to combine targeting moieties to the surface of nanoparticles (see [2] for a review). We have recently developed vectors targeted to the peripheral nervous system (PNS) and showed that the ligand moieties density plays an important role in the performance of the vectors.[3] However, the optimization of the targeting moieties density implies tedious and time-consuming *in vitro* studies. In this report we propose the use of molecular recognition force spectroscopy (MRFS), using atomic force microscopy (AFM), as a novel screening technique in order to optimize targeted gene delivery systems. For MRFS experiments, single molecules are bound to the AFM tip via flexible poly(ethylene glycol) (PEG) linkers.[4, 5] We used, as a model, a previously defined nanoparticle, poly(ethylene imine) (PEI)-based gene delivery system bearing different amounts of a 50 kDa tetanus toxin fragment (HC) as targeting moiety.[3] By using heterobifunctional PEG_{18} we were able to tether both the HC fragment, via an aldehyde group (according to [6]), or the ternary nanoparticles, via a maleimide group (Figure 1A and B, respectively; see support information (SI) for details) to the tip. The isolated trisialoganglioside GT1b receptor, which is reported to be specific for the HC fragment,[7] was grafted onto amino functionalized mica (with 3-aminopropyltriethoxysilane according to [8], Figure 1C, SI for details). Topographical AFM imaging of the cantilever chip was undertaken, using magnetic AC (MAC) mode, in order to visualize nanoparticle presence on the surface (Figure 1D). Single nanoparticle with an average diameter on the range of what previously determined by laser light scattering (63±6 nm) [3] could be observed (Figure 1E and see Figure S1 in SI for further details).

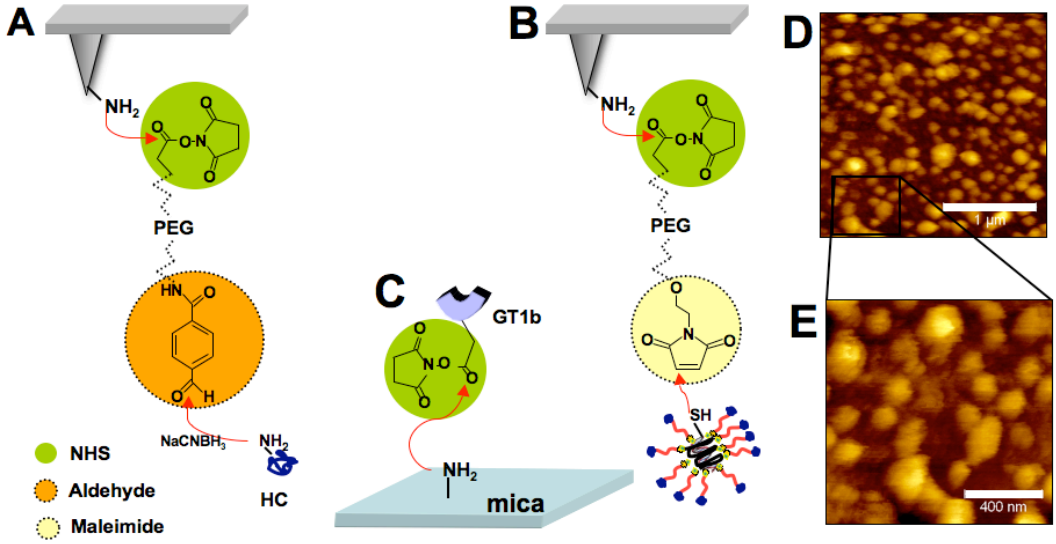


Figure 1- Schematic representation of the chemical tethering strategies used to bind the tetanus toxin fragment (HC fragment) (A) and the functionalized nanoparticles to the AFM tip via a heterobifunctional PEG_{18}. (C) NHS activated GT1b trisialoganglioside receptor was grafted on mica. Tip and mica were amino functionalized using 3-aminopropyltriethoxysilane. (D) The tethering of the nanoparticles was confirmed by imaging the surface of the cantilever using magnetic AC mode. (E) Single nanoparticles could be resolved by AFM imaging.

Force distance cycles were acquired by approaching the HC- or nanoparticle-modified tip to the GT1b modified mica or cells, followed by its retraction. Non-specific adhesion could be observed in the case of GT1b receptors on mica (Figure 2A and B). Nonetheless, the characteristic shape of the PEG linker stretching allows to distinguish the specific unbinding events from non-specific adhesion.[4] Indeed, the interaction between ligand and receptor showed a characteristic non-linear force signal for the four systems tested (Figure 2). The specific force signals were used to determine rupture forces and their accuracy for the previously described settings by calculating empirical probability density functions (PDFs), according to Baumgartner *et al.*.[9] PDFs are advantageous to histograms as data is weighted by its reliability (i.e. standard deviation). Representative PDFs for the different studied settings are presented in Figure 3. As observed in Figure 3A the PDF of interaction between the HC fragment and the GT1b receptor presented a monomodal distribution with an unbinding event probability (UEP) of 16.2% at a retraction velocity of 600 $nm.s^{-1}$. To prove that measured forces were due to specific interactions, surface blocking with free HC fragment was performed (see SI for details) resulting in a drop of the UEP to 0.4%. In the case of nanoparticle/GT1b receptor interaction, a monomodal peak was

also observed with a UEP of 22.0% at a retraction velocity of 500 nm.s^{-1} (Figure 3B). Again, after blocking the UEP dropped to 2.4% indicating the specificity of the measured forces.

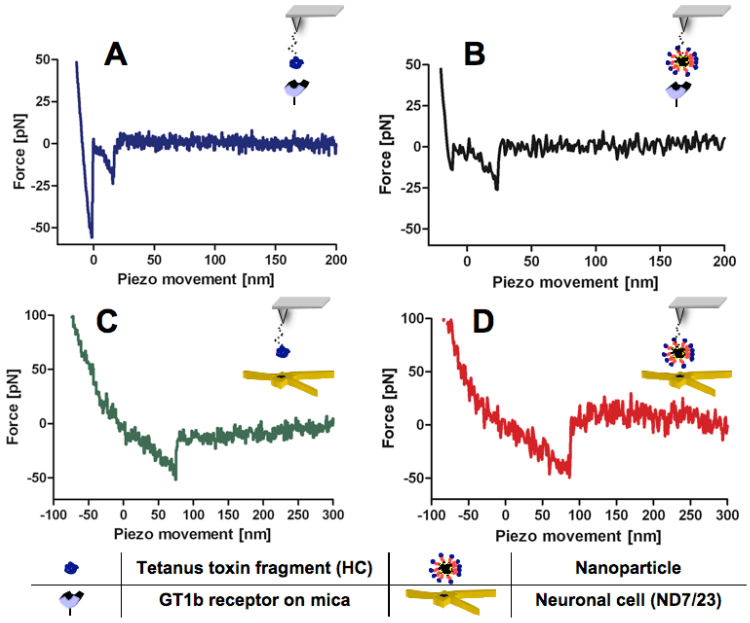


Figure 2- Force traces acquired with the AFM in force spectroscopy mode showing single molecular unbinding events. Typical force distance retraces considering the interaction between the isolated tetanus toxin fragment (HC fragment) and GT1b trisialoganglioside receptor grafted on mica (A), functionalized nanoparticle and GT1b trisialoganglioside receptor (B), HC fragment and neuronal cell (C) and functionalized nanoparticle and neuronal cell (D).

For the specific interactions of the HC fragment and ND7/23 cells (neuronal cell line), the PDF showed a monomodal peak with an UEP of 16.2% (Figure 3C). The blocking with the HC fragment as well as in the control cells (NIH 3T3, fibroblasts) showed reduced UEP, 1.4 and 1.5%, respectively (Figure 3C). A monomodal peak was again observed in the case of the interaction of functionalized nanoparticles with ND7/23 cells with an UEP of 16.1% at a retraction velocity of 1000 nm.s^{-1} (Figure 3D). Again the control experiments point to the specificity of the measured forces with a drop of the UEP being observed after blocking with free HC fragment, with the control cells and when naked nanoparticles were used, with UEP of 1.1%, 0.3% and 0.7%, respectively (Figure 3D). Because molecular interaction forces depend on time scale of the measurements,[10] unbinding forces were assessed at different retraction velocities (300 to 2000 nm.s^{-1}). The unbinding forces were determined and the peak maxima were plotted against the loading rate (r). The loading rate was calculated by multiplying the retraction velocity by the effective spring constant k_{eff}, being k_{eff} the

slope of the force distance curve at rupture. [11] According to the theory that a single energy barrier is crossed in the thermally activated regime, a linear relation between the unbinding force and the logarithm of the loading rate is expected.[10, 12] For the different conditions tested, a linear relation was observed (Figure 4A). From this plot and by fitting equation 1 (see SI) the separation of the energy barrier in the direction of the force, x_β, and the kinetic off rate, k_{off}, can be determined.[10] For all tested conditions the x_β values were shown not to vary significantly (Figure 4B), as determined by a one-way ANOVA test considering a 95% confidence interval. In regard to the k_{off} values, and using the same statistical test, non-significant differences were found between the k_{off} values on cells (HC and nanoparticles; Figure 4B). By evaluating the interaction of isolated protein on isolated receptor interaction versus the isolated HC on ND7/23 cells we determined that both the unbinding forces and the average of ligand-receptor lifetime (given by k_{off}^{-1}) is higher in cells (Figure 4).

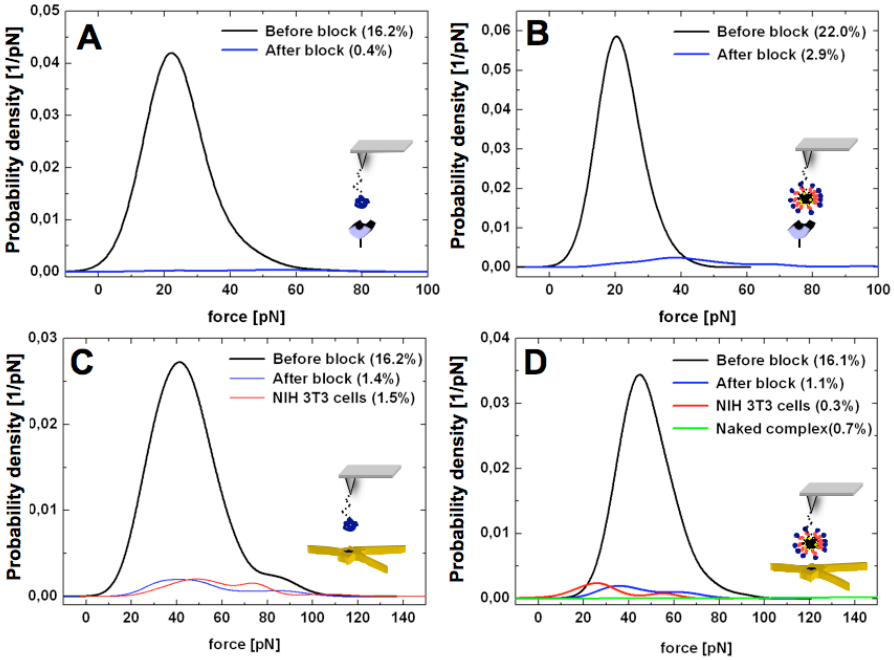
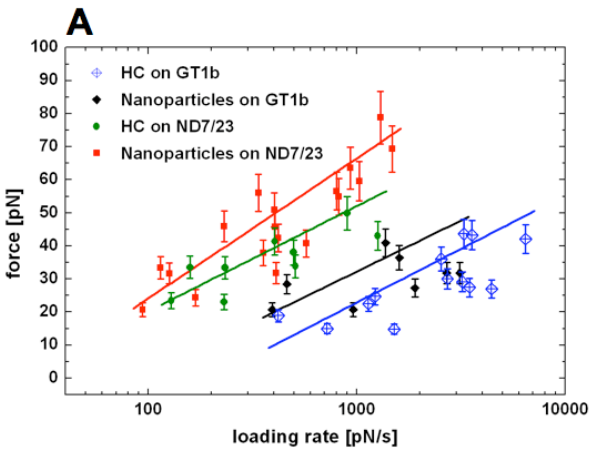


Figure 3- Probability density function (PDF) of rupture forces considering the interaction between the isolated tetanus toxin fragment (HC fragment) and GT1b trisialoganglioside receptor grafted on mica (A), functionalized nanoparticle and GT1b trisialoganglioside receptor (B), HC fragment and neuronal cells (C) and ternary nanoparticle and neuronal cell (D). In order to determine the specificity of the measured unbinding forces controls were performed by surface blocking with free HC fragment (0.1 mg.ml^{-1} for 1 hr; after block), in the control cell line (NIH 3T3 cells) or using nanoparticles lacking the HC moieties (naked complex). The unbinding probability, before and after block and in other controls are presented inside parenthesis.



B

	X_β (Å)	K_{off} (s^{-1})
HC on GT1b	2.91 ± 0.86	14 ± 5.53
Nanoparticles on GT1b	3.07 ± 1.42	6.71 ± 3.67
HC on ND7/23	2.97 ± 1.19	1.68 ± 1.05
Nanoparticles on ND7/23	2.24 ± 0.37	1.46 ± 0.42

Figure 4- Loading rate dependence of the measured unbinding forces considering the interaction between: isolated tetanus toxin fragment (HC fragment) and GT1b trisialoganglioside receptor grafted on mica (blue diamonds), ternary nanoparticle and GT1b trisialoganglioside receptor (black fill diamonds), HC fragment and neuronal cells (ND7/23; green filled circles) and ternary nanoparticle and neuronal cell (red filled squares). The resulting fitting (using Equation 1 in supporting information) is shown by straight lines with the same correspondent color (A). From the previous described fitting the position of the energy barrier along the direction of the force (x_β) and the kinetic off-rate constant for the dissociation of the complex in solution (k_{off}) could be determined (B).

In this work we used as targeting moieties the HC fragment which has been shown to interact specifically with peripheral neurons and to undergo retrograde transport.[13] It is well documented that GT1b gangliosides are required for interaction between HC and neuronal cell surface to occur. However, this interaction was found to be protease sensitive suggesting a dual receptor mechanism with an additional receptor being involved.[14] It is then expected that when the HC fragment interacts with the cell surface an association between the GT1b ganglioside receptor and the putative protein receptor occurs, explaining the higher forces measured and increased interaction lifetime (k_{off}^{-1}). Although to a lesser extent, significant differences were also found between k_{off} values of the HC or HC-functionalized nanoparticle interaction with the isolated GT1b receptor, despite the fact that the measured forces were of the same order of magnitude. This observation may be justified by the different steric environment that surrounds the HC protein when this is presented to the receptor thetered to the nanoparticles.

Although rare, multiple binding events could be observed in the case of the interaction between nanoparticles and the isolated GT1b receptor (See Figure S2 in

SI as illustration). In the case of the interaction between nanoparticles and the ND7/23 cells these were not observed indicating that the receptor density on cells could be encountered at a low density not enabling the occurrence of multiple HC fragment bind events. By using different nanoparticle formulations tethered on the tip and by performing force spectroscopy on the ND7/23 neuronal cell line we aimed to study the influence of the targeting moieties density on the binding probability. We could observe that the nanoparticle targeting moieties density influences the nanoparticle binding probability to the ND7/23 cells (Figure 5A), although not having an impact on the measured forces (Figure 5B). The 7.5 (µg of HC-PEG per 2 µg of plasmid DNA, see SI for details) nanoparticle formulation elicits the higher binding probability outcome (Figure 5A), and it was also responsible for the higher transfection efficiency in cells (Figure 5C).

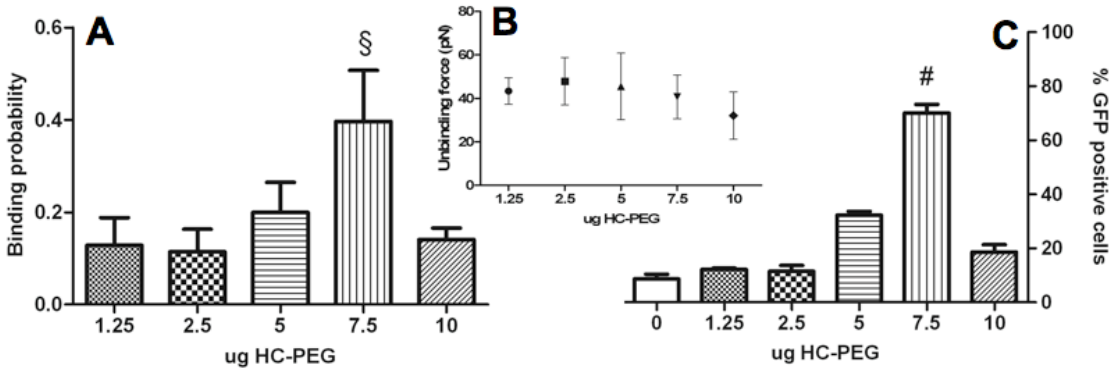


Figure 5- Influence of ligand density (expressed as µg of HC-PEG per 2 µg of plasmid DNA) on the binding probability on neuronal cells (ND7/23 cell line, A) and the correspondent force average values (B), percentage of green fluorescent protein (GFP) positive ND7/23 cells at 72 hrs post-transfection in relation to the nanoparticle formulation (average ± SD, C); (A) each formulation considers the average of 9 data sets, with one data set corresponding to 1000 curves performed with a retraction velocity of 1000 $nm.s^{-1}$; (C) each formulation considers 20 000 events in flow cytometry (n=3); § and # denotes statistical significant difference from other groups, $p<0.05$, as determined by one-way ANOVA test followed by a Bonferroni's multiple comparison test).

Our results show that for the aimed targeting outcome an optimal ligand density exists and, moreover, that surpassing this density a detrimental effect is obtained with the reduction of both the binding probability and the transfection efficiency (Figure 5A, B). The surface chain density and conformation of PEG are important factors that contribute to the 'stealth' characteristics of PEGylated nanoparticles. At

low density, most of the chains have higher mobility and are closer to the surface of the particle ('mushroom' conformation). However, as density increases, the range of motion decreases, and most of the chains are extended away from the surface ('brush' conformation).[15] It is accepted that an optimal PEG coverage would be in between the mushroom and brush like conformations, leading to an optimal surface steric hindrance.[16] In the present case, and although a decrease of binding probability was observed for the highest formulation, the measured forces did not suffer a significant variation. We hypothesize that while the ligand density increases also the associated PEG conformation changes, assuming a brush like structure which can inhibit the spatial access of the cell receptors to the HC fragments, explaining the reduction of binding probability and transfection levels. Gu et al. have also reported that a narrow defined targeting moieties density with optimal outcomes *in vitro* and *in vivo* could be obtained for aptamer-targeted nanoparticles for cancer applications.[17] In this study we were able to get new insights on the ligand-receptor mechanism and to determine the optimal targeted nanoparticle formulation regarding cells. The optimal ligand density allowing maximal cell-specific interaction is a critical issue in the tailoring of targeted systems, however, new tools are awaited in order to assist in their efficient design. AFM has become a widely used tool for measuring both intra- and intermolecular interactions. In this work we propose AFM as a screening tool to the efficient design of targeted nanoparticle systems. This new approach can find application in the growing field of nanomedicine.

Acknowledgements

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Support information

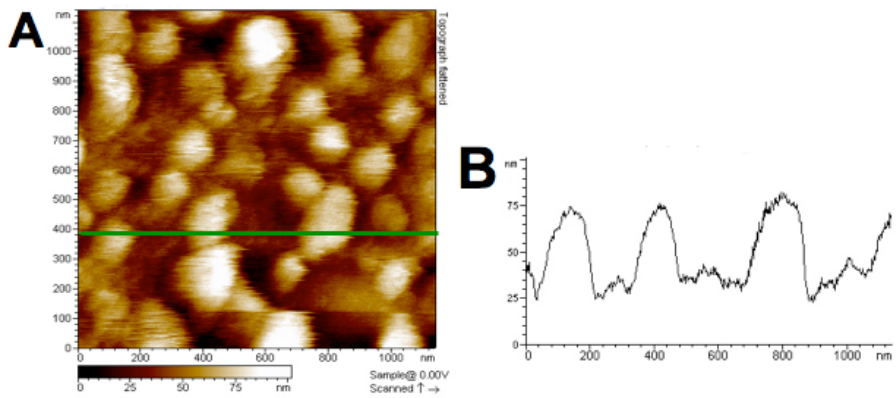

Figure S1- (A) The tethering of the nanoparticles was tested by imaging the surface of modified cantilever chip using the magnetic AC (MAC) mode, (B) Surface topography show nanoparticles with average diameter in the range of what has been previously described by our team (63±6 nm). [1]

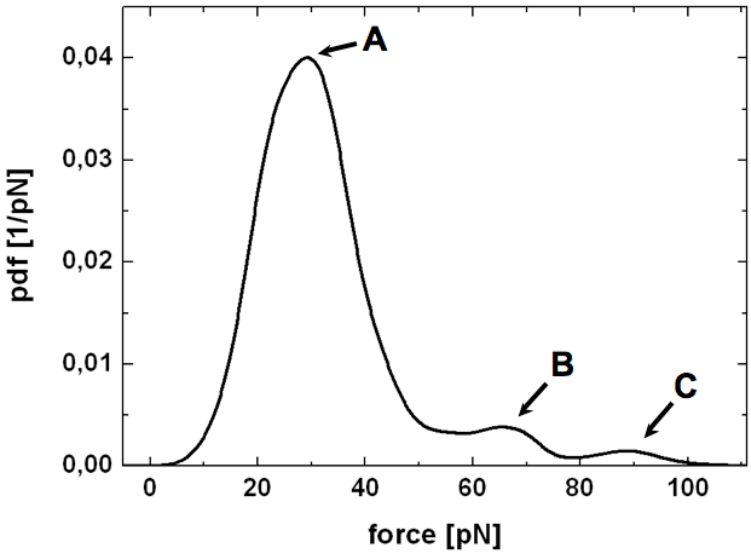

Figure S2- Illustrative example of a probability density function (PDF) of rupture forces between functionalized nanoparticle and GT1b ganglioside receptor. Although rare, multiple binding events were occasionally observed. In this example the highest peak presented a force at 29 pN corresponding to single unbinding (A), two smaller peaks could be observed at 65 and 88 pN (B and C, respectively) that can be associated with double and triple unbinding events (measured at a retraction velocity of 500 $nm.s^{-1}$).

Experimental section

Polymer

Branched PEI (25 kDa, Sigma) was thiolated with 2-iminothiolane (Sigma) and purified as previously described.[1] The thiolated PEI (PEISH) was dissolved at 1 $mg.ml^{-1}$ in a 5% (w/v) glucose solution (pH 7.4) and stored at -80 ºC until further use.

Plasmid DNA

The plasmid DNA used encoded for the green fluorescent protein (GFP; pCMV-GFP, 7.4 kb). Plasmid was produced in the DH5α competent *E. coli* strain transformed with the respective plasmid. Subsequently, DNA purification was performed using an endotoxin-free Maxiprep kit following the manufacture's instruction (GenElute, Sigma).

Tetanus toxin production and modification

The non-toxic fragment of the tetanus toxin (HC) was produced recombinantly using the BL21 *E. coli* strain. The plasmid encoding for the HC fragment was a kind offer from Prof. Neil Fairweather (King's College, UK). The HC production in the BL21 *E. coli* strain and purification was performed as previously described.[2] The obtained HC fragment was covalently linked to a poly(ethylene glycol) (PEG) spacer. Briefly, a heterobifunctional 5 kDa PEG (JenkemUSA, China) bearing an N-hydroxysuccinimide (NHS) and a maleimide (MAL) end group was used as indicated by the manufacturer, at a 2.5 PEG/HC protein molar ratio.[1]

Ternary nanoparticle formation

DNA-polymer complexes were prepared as described elsewhere [1] by mixing, while vortexing, equal volumes of plasmid DNA and PEISH solutions. The core complexes were formed using PEISH at an N/P molar ratio of 3 and let to stabilize for 15 min. Subsequently, at a final concentration ranging from 1.25 to 10 µg per 2 µg of plasmid DNA, HC-PEG was added to the complex mixture and used immediately.

Gt1b ganglioside modification

The carboxylic moieties of the Gt1b ganglioside were activated using O-(N-Succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) as follows. Half a milligram of GT1b (Sigma) was dissolved in 0.5 ml of dimethylformamide (DMF) and mixed with 0.5 ml of an equimolar solution of TSTU in DMF. Then, 0.5 ml of pyridine was added and the mixture was let to react during 3 hrs in an argon atmosphere, at

room temperature (RT) with constant stirring. Pyridine was then evaporated (rotavap) and, subsequently, DMF was evaporated in liquid nitrogen for 1.5 hrs. The obtained product was dissolved in a 5:1 (v/v) chloroform:methanol solution and contaminates were extracted with Buffer A (100 mM NaCl, 50 mM NaH_2PO_4, 1mM EDTA.Na_2, pH 7.5). The organic phase was dried and the obtained product was dissolved in DMF (0.5 $mg.ml^{-1}$) and kept at -80ºC until further use.

Tip chemistry

Commercially available silicon nitride atomic force microscopy (AFM) tips (Veeco Instruments) were amino functionalized using a gas phase method with (3-aminopropyl)triethoxysilane (APTES, Sigma), as previously described.[3] The attachment of the HC fragment to the tip surface was achieved via a heterobifunctional PEG spacer, aldehyde-PEG-NHS (PEG_{18}), as previously described.[4] Briefly, the amino functionalized tip was incubated in a 0.5 ml correspondent linker solution (6.6 $mg.ml^{-1}$) in chloroform (Sigma) with 2% (v/v) triethylamine (Sigma) for 2 hrs at RT. After rinsing twice in chloroform and dried in a N_2 flux, the tip was incubated for 1 hr, at 4ºC, in a 100µl solution of HC in phosphate buffered saline (PBS) (0.2 $mg.ml^{-1}$) with 2 µl of a 1 M $NaCNBH_3$ solutions (prepared by adding 32 mg of $NaCNBH_3$ to 500 µl of NaOH 10 mM). Then, 5 µl of a 1 M ethanolamine hydrochloride solution (pH 9.6) was added and incubation proceeded for 10 min at RT, in order block unreacted aldehyde groups. The tips were then washed 3 times with PBS and kept in PBS at 4ºC until further use. The attachment of the complexes to the tip surface was achieved via a heterobifunctional PEG spacer, MAL-PEG-NHS (PEG_{18}), as indicated. The amino functionalized tip was incubated in a 0.5 ml correspondent linker solution (2 $mg.ml^{-1}$) in chloroform (Sigma) with 6% (v/v) triethylamine (Sigma) for 2 hrs at RT. After rinsing twice in chloroform and dried in a N_2 flux, the tip was incubated for 24 hrs, at RT, in a 100µl dispersion of complexes freshly prepared as described above. The tips were then washed in PBS for 3 times and kept in PBS at 4ºC until further use.

Surface chemistry

Freshly cleaved sheets of mica were amino functionalized by a protocol analogous to that described above. To each amino functionalized mica sheet 30 µl of the modified Gt1b solution was applied and 3 µl of triethylamine were added before incubating 2 hrs at RT. After extensive washing with methanol, the sheets of mica were dried and kept in an N_2 atmosphere until further use.

Cell lines

ND7/23 (mouse neuroblastoma (N18 tg 2) x rat dorsal root ganglion neurone hybrid) or NIH 3T3 (mouse embryonic fibroblast) cell lines (both obtained from ECACC) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, supplemented with 10% (w/v) foetal bovine serum (FBS) (heat inactivated at 57ºC for 30 min.) and 1% PS (10,000 units.ml^{-1} penicillin and 10,000 μg.ml^{-1} streptomycin), all supplied by Gibco, and maintained at 37°C in a 5% CO_2 humidified incubator. The ND7/23 cell line was chosen as a sensorial peripheral nervous system cell model and the NIH 3T3 cell line as a fibroblast model. ND7/23 and NIH 3T3 cell lines were seeded at a cell density of 2.0x10^4 and 2.5x10^4 cells.cm^{-2}, respectively, on glass coverslips coated with poly(D-lysine) (PDL, 0.1 mg.ml^{-1}, Sigma) placed in a 24-well plate. Prior AFM measurements, cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min at RT and washed twice with PBS.

The evaluation of transfection efficiency mediated by the functionalized nanoparticles in the proposed *in vitro* model, was of critical importance in order to better assess the impact of their targeting potential on their ability to promote higher levels of transfection in neuronal cells, as compared to naked nanoparticles. For transfection efficiency assessment, cells were transfected and 72 hrs post-transfection analysed by flow cytometry for GFP expression with twenty thousand gated events taken for each replicate (n=3) as previously described.[1]

Force measurements

All measurements were carried out in PBS using a PicoPlus (Agilent) AFM. Force distance cycles were performed at RT by using HC fragment- or complexes-coated tips with 0.01–0.03 N.m^{-1} nominal spring constants and mica-immobilized Gt1b receptor or glass-immobilized cells. Force–distance cycles were recorded at 0.5, 1 or 2 Hz vertical sweeping frequency and 300 nm z-range for measurements between HC fragment-coated tips and isolated receptors or 500 nm z-range for HC fragment- or complexes-coated tips on isolated receptors or cells (see Figure S3 for an illustration).

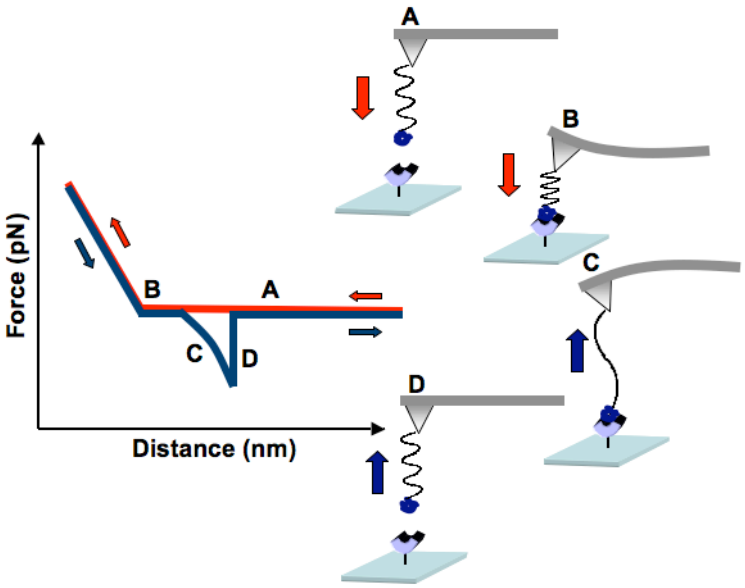

Figure S3- Schematic representation of a force distance cycle (FDC). The AFM tip approaches the sample surface (A). The ligand tethered to the tip encounters a receptor on the surface and, as the tip applies a constant and default force upon the surface, thus resulting in cantilever deflection (B). Subsequently, the tip retracts from the surface leading to the stretching of the linker (non-linear curve on the graph, C). Finally, enough force is applied to break the specific ligand/receptor bond, leading to a typical unbinding event (D).

For studies performed with complexes with different formulations, a 1 Hz vertical sweeping frequency at a 500 nm z-range were used. During one data set of 1000 force distance curves, the tip position was changed for a few 100 nm for several times to assure that the binding events were statistically reasonable. The specificity of unbinding events was performed via the surface blockage with free HC-fragment (0.1 mg.ml^{-1} for 1 h). The spring constants of the cantilevers were determined by using the thermal noise method.[5, 6] Empirical force distributions of the rupture forces of the last unbinding event (PDF) were calculated as previously described.[7] The loading rates were determined by multiplying the pulling velocity by the effective spring constant, i.e., the mean slope at rupture. In the single-barrier model, [8] the most probable rupture force F* is given as function of the loading rate (r)

$$F^* = \frac{k_B T}{x_b} \ln\left(\frac{r}{k_{off}\left(\frac{k_B T}{x_b}\right)}\right), \qquad \text{(Equation 1)}$$

where k_B is the Boltzmann constant, T the absolute temperature, k_{off} the rate of dissociation of a ligand-receptor complex and x_β being the projection of the transition state along the direction of the force. The parameters x_β and k_{off} were determined by fitting F* against ln r. The accuracy of the parameters was calculated by using propagation of errors assuming that the standard error of F* is ≈15% (10% accounting for the spring constant determination and 5% to account for the uncertainty in determining the most probable rupture force) as previously described.[9]

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Chapter V

Improving chitosan-mediated gene transfer by the introduction of intracellular buffering moieties into the chitosan backbone *

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Abstract

Chitosan was functionalized with imidazole moieties (CHimi) with the aim of improving its buffering capacity and promoting the endosomal escape ability of chitosan-DNA complexes, ultimately increasing their transfection efficiency. 5.6, 12.9 and 22.1% of the glucosamine residues of chitosan were substituted. Complexes with different molar ratios of primary amines to DNA phosphate anion (N/P) were prepared by a coacervation method. For an N/P>3 CHimi polymers are able to complex electrostatically with DNA and condense it into positively charged nanostructures (average size 260 nm and zeta potential +16 mV at pH 5.5). In the concentration range of 2.5-100 µg/ml the modified polymers had no cytotoxic effect on 293T cells. CHimi polymers with the highest degree of substitution were found to enhance β-gal expression in 293T and HepG2 cells. Bafilomycin A1 inhibited transfection, indicating that the protonation of the imidazole groups in the endolysosome pathway favors the escape of the complexes from the endosomes, increasing the amount of transgene that can reach the cell nucleus.

Introduction

Gene delivery has been proposed as a powerful tool in the field of regenerative medicine either as a therapeutic strategy by itself or in combination with cellular therapies and/or tissue engineering [1]. 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. However, the expression of these molecules should be strictly tuned and limited in time in order to avoid protein over-production and abnormal cell behavior.

A number of systems based on synthetic molecules have been developed to serve as vehicles for genetic material, with promising results in terms of transfection efficiency, namely cationic liposomes [2] and poly(ethylene imine) (PEI) based polymers [3]. While synthetic vectors assure transient transfection, the cytotoxicity of some of these systems has been limiting their application in a regenerative medicine scenario [4-6]. Chitosan (CH), a natural polyheterosaccharide derived from chitin by N-deacetylation, is a biodegradable polymer with well-established biocompatibility. Presently CH is under investigation for a wide number of biomedical applications, including gene delivery. CH has been described as a promising gene carrier [7] having as main advantages for its application in regenerative medicine to induce no toxic effects on cells and, as a non-viral vector, to mediate a transient gene expression. However, CH low transfection efficiency under physiological conditions has so far hampered its widespread application.

The capacity of CH to complex and condense polynucleotides, to form polyplexes of submicron dimension, as well as being able to protect the genetic material from endonuclease degradation is well established [7]. It is accepted that endocytosis constitutes the main pathway for polyplex internalization [8], thus the escape from the endosomal pathway is of crucial importance for the efficacy of a gene delivery system. In this regard, it should be emphasized that the discussion on the endosomal escape of polyplexes has been mainly focused on branched PEI. The high efficiency of this polymer as a transfection agent has been attributed to its buffering capacity in the endosomal pH range (7.4-5). According to the so-called “proton sponge” hypothesis proposed by Behr [9], cationic polymers with the ability to buffer endosomal pH induce osmotic swelling of the endosomes due to excessive proton and chloride accumulation with secondary water movement. Independently of the degree of N-acetylation (DA), CH has a limited buffering capacity [10], particularly when involved in the formation of complexes since part of the protonated primary

amines of the polymer are involved in the electrostatic interaction with polynucleotides.

The aim of this study was to investigate if the introduction of imidazole moieties into the CH backbone would enhance the buffering capacity of the polymer leading to an improvement of transfection efficiency. The imidazole ring is the functional unit of the histidine amino acid. Midoux et. al. have firstly proposed the grafting of imidazole moieties to a polymeric backbone, describing significant improvement in poly-L-lysine mediated transfection efficiency after grafting histidine to the polymer [11]. A similar approach was explored by other authors for a range of polycations, with different degrees of success [11-17]. The only reported study using a non-toxic and biodegradable starting material, describes the grafting of urocanic acid units (pKa=6.65 [18]) into the CH backbone [16]. However, with this system the authors were only able to transfect one out of the four cell lines tested. Furthermore, it is unclear if the single positive results obtained were due to an increase of the endosomal escape of the complexes or due to differences in size and stability of the complexes [16]. In the present study, CH was modified via an amidation reaction with 4-imidazole acetic acid sodium salt (pKa=7.5 [19]), a naturally occurring compound resultant of the metabolization of histidine. The compound selection was based on the search of a group that could have a higher pKa than CH, in order to improve the CH buffering capacity at physiological pHs. The pKa of CH primary amine groups is dependent on the DA of the polymer. Reported values range from 6.0 to 6.8, as the DA increases [20]. The ability of the modified polymers to complex, condense and protect DNA was assessed. Transfection studies were performed in 293T and HepG2 cells.

Materials and Methods

2.1. Materials

Technical grade CH (Chimarin™, DA 13%, apparent viscosity 8 mPas) was supplied by Medicarb, Sweden. CH endotoxin levels were assessed according to Nakagawa et. al [21] using the Limulus Amebocyte Lysate (LAL) QCL-1000® assay (Cambrex) and found to be below 0.1 EU/ml (an EU corresponding to a unit of measurement for endotoxin activity), respecting the US Department of Health and Human Services guidelines for implantable devices [22]. Imidazole-4-acetic acid sodium salt (ImiAcOH), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) 98% and N-hydroxysuccinimide (NHS) 97% were purchased from Sigma-Aldrich. Unless mentioned otherwise all solvents and reagents were of analytical grade.

A plasmid encoding for the β-galactosidase enzyme under the cytomegalovirus (CMV) early promoter was used in this study as reporter gene (pCMV-Sport β Gal, Invitrogen, 7853 bp). The plasmid was amplified in DH5α *E.coli* strand and isolated using the QIAGEN Plasmid Maxi Kit. The concentration and purity of the plasmid was assessed by spectrophotometric analysis. The ratio between optical density at 260 nm and 280 nm was > 1.8 and < 2.0.

2.2. Synthesis

CH was purified by filtration of a CH acidic solution and subsequent alkali (NaOH 1M) precipitation. Weight average molecular weight ($\overline{M}_w$), polydispersity index (PDI, given by the ratio $\overline{M}_w/\overline{M}_n$) and [η] of the purified polymer were determined by high performance size exclusion chromatography using the system 0.2 M $NaCH_3COO$/0.5 M CH_3COOH as eluent, according to Terbojevich *et. al*. [23]. All determinations were performed at RT. The $\overline{M}_w$, PDI and the [η] found for the starting material were, respectively, $1.21x10^5$, 2.12 and 4.80 $dl \cdot g^{-1}$. CH was modified by amidation of the glucosamine residues of CH by using an EDC/NHS condensation system [24]. Briefly, CH was dissolved at room temperature (RT) in 0.1 M HCl solution and the pH of the obtained solution adjusted to 6.5 with 1 M NaOH. 2-Morpholinoethanesulfonic acid (MES) was added to the obtained solution to a final concentration of 0.1 M (the final concentration in CH was 0.56 wt/vol %). NHS was dissolved in 0.1 M MES buffer (pH 6.5) (1 mol/mol of ImiAcOH) and was immediately added to the polymer solution. In a similar manner, EDC was dissolved in MES buffer and added to the reaction mix (2 mol/mol NHS). Finally, different ratios of ImiAcOH per glucosamine residue of CH were added to the CH solution in 0.1 M MES buffer (pH 6.5): 0.05, 0.15 and 0.30 mol/mol of glucosamine residue. From this point onwards, the obtained polymers will be identified by the abbreviations CHimi1, CHimi2 and CHimi3, respectively. After 24 h, the reaction mixture was poured into a solution of water/ammonia 7:3 vol:vol. The precipitated polymer was recovered (70-90% yield) by centrifugation and neutralized by washing with distilled and deionized water, freeze dried (Labconco) and, subsequently, vacuum dried at 60°C for 24 h.

2.3. Polymer characterization

Fourier Transformed – Infrared Spectroscopy (FT-IR) was performed using the KBr technique. Each pellet was prepared by blending 2 mg of the polymer (vacuum dried 24 h at 60°C) with 200 mg of KBr (dried 24 h at 105°C). The IR spectra were immediately recorded, after a 5-minute purge of the sample chamber with N_2, in a

FT-IR system 2000 from Perkin-Elmer, by accumulation of 200 interferograms, at a 4 cm^{-1} spectral resolution. Elemental analyses (carbon, hydrogen and nitrogen) of vacuum dried (24 h at 60ºC) were performed with a Carlo/Erba EA 1108 automated analyzer. 300 MHz ^{1}H-Nuclear Magnetic Resonance (NMR) spectra were acquired on a Bruker AMX 300 spectrometer using polymer solutions in 1.14 M DCl solution in D_2O (99.9% D)). The polymer solutions were prepared by stirring overnight at RT in a N_2 chamber vacuum dried polymer (24 h at 60°C). In the case of the ImiAcOH the solution was prepared in a similar way with the exception that the DCl was added only prior to measurement. The experiments were run at 60°C. The sample tube was inserted in the magnet and allowed to reach thermal equilibrium by waiting 10 min before performing the experiment.

pH titration. CH and CHimi3 were dissolved in HCl 0.1 M overnight. Subsequently, NaCl was added to a final concentration of 150 mM. The pH of equimolar CH and CHimi3 solutions ($8.26x10^{-6}$ M) was monitored (654 pH-meter, Metrohm) as 5 µl aliquots of 0.1 M NaOH were added.

2.4. Preparation of complexes

DNA-polymer complexes were formed by mixing, while vortexing, equal volumes of pre-heated (55°C for 10 min.) plasmid DNA pCMV-Sport-β. gal solutions (in 25 mM Na_2SO_4) and CH or CHimi solutions in 5 mM $NaCH_3COO$ buffer pH 5.5 [25]. Complexes were allowed to form for 15 min. at RT before further use. Complexes with different polymer:DNA ratios were prepared. The ratio value - N/P molar ratio - is expressed in terms of moles of primary amine groups (N) of CH or CHimi to moles of DNA phosphate groups (P).

2.5. Characterization of complexes

DNA retardation assay. Complexes were prepared at various N/P molar ratios as previously described. 2.5 µg of plasmid DNA was used for the preparation of the complexes in a final volume of 50 µl. 20 µl of each complex solution, together with 4µl of loading buffer (Fermentas) were loaded in a 1% agarose (Cambrex) gel, with 0.05 µg/ml of ethidium bromide (Q-BioGene). The electrophoresis was run in a 90V field for 45 min., using Tris-Acetate-EDTA buffer (pH 8) as the running buffer.

Complex size and zeta potential determination. Complexes were prepared at various N/P molar ratios as previously described. 10 µg of plasmid DNA was used for the preparation of the complexes. After stabilization complexes were diluted to 1 ml, using 5 mM $NaCH_3COO$ buffer (pH 5.5). Zeta potential and mean hydrodynamic size of the polyplexes were assessed using a Zetasizer Nano ZS (Malvern, UK). The

Smoluchowski model was applied for zeta potential determination and cumulant analysis was used for mean particle size determination. All measurements were performed in triplicate, at 25°C.

DNase protection assay. The DNase protection assay was performed as described elsewhere [26]. Complexes were prepared using 20 µg of plasmid DNA at various N/P molar ratios as previously described. After stabilization the complexes were suspended in 10 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.4, in a final volume of 975 µl. 25 µl of DNase I (1 U/µl, Fermentas) was added and the absorbance values at 260 nm were registered at 37°C, for 15 min., using a UV-1201 Shimadzu spectrophotometer (Japan).

2.6. *In vitro* tests

Cell culture. The cell line 293T, derived from human embryonic kidney cells was a kind offer from Simone Niclou (Netherlands Institute for Brain Research, Amsterdam, Netherlands). Human Caucasian hepatocyte carcinoma cells (HepG2) were obtained from ECACC. Mycoplasma free (assessed by PCR [27, 28]) cells were cultured in tissue culture dishes of 10 cm of diameter (Greiner Bio-one, CellStar) and maintained in complete medium: Dulbecco's MEM medium (glucose 4500 mg/l) with glutamax (DMEM), supplemented with 10% inactivated (60°C, 30 min.) fetal bovine serum (FBS) and 1% PS (10,000 units/ml penicillin and 10,000 mg/ml streptomycin) (all supplied by Gibco), at 37°C in a 5% CO_2, humidified incubator.

Cytotoxicity assay. To evaluate the influence of polymer concentration on cellular viability, 293T cells were exposed to increasing polymer concentrations (CH, CHimi1, CHimi2, CHimi3) and Escort V® (Sigma), a commercially available transfection reagent based on a specially processed PEI at a concentration of 1.3 mg/ml. 293T cells were seeded at a density of $5.0x10^4$ viable cells/cm^2 in complete culture medium in 96-well cell culture plates (TPP), and incubated for 24 h. 2 h prior contact, medium was refreshed (180 µl). Subsequently, the polymer solutions, phenol (positive control, 64 mg/ml according to norm NF EN 30993), or 5 mM $NaCH_3COO$ buffer (pH 5.5; negative control) were added to the culture medium (20 µl). The CH based polymers final concentration in the medium ranged from 2.5 to 100 µg/ml and for Escort V from 2.5 to 50 µg/ml. After 24 h of contact, cell metabolic activity was assessed by the MTT assay [29]. Briefly, at the end of the incubation period the culture medium was removed, and the cells rinsed with PBS. Cells were then incubated with MTT (0,5 mg/ml in supplemented DMEM without phenol red) at 37°C for 3 h. Subsequently, the medium was discarded and 100 µl of DMSO were added

to dissolve the formed formazan crystals. The optical density of the supernatant was read at 540 nm with normalization of interference at 690 nm using a microplate spectrophotometer (SLT Labinstruments, Austria). The results are expressed as relative viability (%) using the negative control as a reference. The results found for the cell viability of the negative control – cells treated with 5 mM $NaCH_3COO$ buffer (pH 5.5) – were similar to the ones found for non-treated cells (only in contact with complete culture medium) (data not shown). Six replicates of each incubation condition were performed.

Transfection. Cells were seeded 24 h prior transfection, at a density of $2.7x10^4$ viable cells/cm^2 on poly-D-Lysine hydrobromide (PDL, Sigma) coated 24-well tissue culture plates (Greiner Bio-one, CellStar). For staining procedures, cells were seeded on glass slides (Menzel-Gläser, Germany) coated with PDL. Two h prior transfection cell culture medium was removed and replaced with 500 µl of complete fresh medium. Complexes were prepared as previously described and added to the cells. The final DNA concentration used was 1.3 µg/cm^2. Escort V® was used as positive control, according to the manufacturer instructions (corresponding to 0.4 µg/cm^2 of plasmid DNA). Naked DNA (1.3 µg/cm^2) was also tested. The cells were incubated in the presence of the complexes/naked DNA for 24 h and gene expression was assessed at 48 and 72 h after transfection. Cell culture medium was refreshed daily. All transfections were performed at least at 3 independent times. Presented results are representative of each experiment. Cell viability of cells transfected with CH-based nanoparticles (N/P=18) and ESCORT V was assessed by means of the MTT assay, as described in the previous paragraph, 24 h post-transfection.

β-Galactosidase expression evaluation. At the selected evaluation time points, cells were rinsed with pre-warmed (37°C) PBS and suspended in lysis buffer (0.25 M Tris, pH 8.0). The plate was frozen at –20°C and thawed at 37°C in an incubator (1-2 cycles). Insoluble cell material was pelleted by centrifugation and the supernatant was collected. To determine the β-galactosidase (β-gal) activity of transfected cells the β-Gal assay protocol (Invitrogen, UK) was used in accordance with the manufacturer's instructions. Lysates of non-transfected cells were used as blank. The total protein content of the cell lysates was determined using the bicinchoninic acid assay (BCA, Pierce, USA) according to manufacturer's instructions. The transfection activity is expressed in terms of specific activity of β-gal (nmoles ONPG hydrolyzed per min. per mg of total protein). Transfection efficiency, defined as the percentage of

treated cells that express the transgene (β-Gal positive), was assessed by means of the staining of β-Gal expressing cells. Cells seeded on PDL coated glass slides and transfected as previously described were treated according to the β-Gal staining assay protocol (Invitrogen, UK), using X-Gal as substrate (Eppendorf). Reaction time was set to 3 h. In order to count the total number of cells on the glass slide, propidium iodide (PI) counter-staining was performed. The percentage of positive cells was determined by counting the total number of cells (PI positive cells) and β-Gal positive cells, using an epifluorescence microscope (CK2, Olympus, Japan) at a 400x magnification. The number of β-Gal positive cells, as well as the total cell number were estimated, independently, by two persons, who counted 5 fields each in 2 glass slides per condition tested.

Transfection in the presence of bafilomycin A1. To determine the role of the imidazole moieties introduced into the CH backbone in enhancing the escape of the DNA from the endosomes, 293T cells were transfected with CHimi3-based complexes and treated at different time periods of the procedure with the vacuolar type H^+-ATPases inhibitor, bafilomycin A1 (BafA1, 50 nM final concentration in the culture medium [10]). To assess the influence of BafA1 at the selected concentration on cellular viability, 293T cells were exposed to the drug. Cells were seeded at a density of $2.7x10^4$ cells/cm^2 (in 500 µl) in 24-well cell culture plates and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. 10 µl of BafA1 solution was added to each well. Cells were incubated with the drug either 2 or 24 h and the MTT assay was performed as previously described. Cell viability was not affected when the incubation time was limited to 2 h. For incubation periods of 24 h, cell viability was reduced to 50% in relation to untreated cells (data not shown).
CHimi3 based complexes were prepared at a N/P molar ratio of 18 as previously described**.** BafA1 or ethanol (control; as it is the solvent used to dissolve BafA1) were added to the cells either 30 min. prior the complex addition (t_{0-2}), or 2 h (t_{2-4}) and 4 h (t_{4-6}) post-transfection. The incubation period with BafA1 lasted for 2 h in complete medium after which the medium was refreshed. In parallel, cells were incubated with BafA1 or ethanol for 24 h after which the medium was refreshed. In all cases β-gal activity was assessed 48 h post-transfection. Results are presented as transfection activity inhibition in relation to the control (cells treated with ethanol).

2.8. Statistical data analysis

Data are given as mean ± standard deviation (SD). When data distribution obeyed a normal distribution the results were analyzed using one way analysis of variance (ANOVA) followed by Tukey's t-test for multiple comparisons. For non-normal distributions the results were statistically treated using nonparametric Mann-Whitney U-test. Results were considered statistically significant when $p<0.05$. Calculations were performed using SPSS® software for Windows (version 12.0).

Results

3.1. Polymer synthesis and characterization

Prior to chemical modification the purified CH was characterized in terms of DA by infrared spectroscopy according to Brugnerotto et. al. [30]. The average value (n=3) found for the DA of the purified CH was 16.4±0.6%. The synthesis of the imidazole grafted CH with different degrees of substitution (DS) was accomplished through the EDC/NHS mediated amidation of the glucosamine residue of the polymer. The infrared spectra of the obtained polymers, as well as of the unmodified CH are presented in Figure 1.

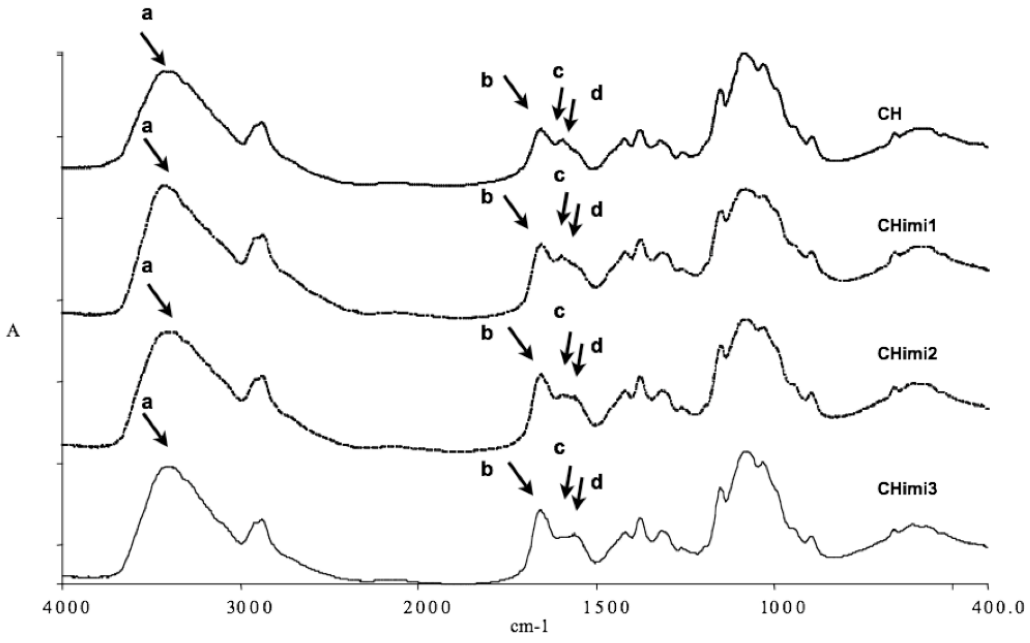

Figure 1 - Fourier transformed infrared spectra of the obtained materials. a) –OH (3450 cm^{-1}); b) Amide I (1654 cm^{-1}); c) $–NH^{+}$ deformation (1600 cm^{-1}) and d) Amide II (1560 cm^{-1}).

A spectrum analysis points to an increase of the Amide I peak at 1654 cm^{-1} and the Amide II peak at 1560 cm^{-1}, with the concomitant decrease of the amine $–NH^{+}$ deformation absorption peak near 1600 cm^{-1}. The DS of the glucosamine residues of the original CH attained for each polymer prepared was determined based on the increase of Amide I peak (1654 cm^{-1}) after modification, using the peak of O-H stretching at 3450 cm^{-1} as internal reference, as follows:

$$DS(\%) = \frac{\left(\frac{A_{1654}}{A_{3450}}\right)_{CHimi} - \left(\frac{A_{1654}}{A_{3450}}\right)_{CH}}{(1 - 0.164)} \times 100 \qquad \text{(Equation 1)}$$

A_{1654} and A_{3450} refer to the peak heights determined using a baseline set between 800-1900 and 1900-3850 cm^{-1}, respectively. The DS results are presented in Table I.

Table I - Degree of imidazole substitution of the modified polymers as determined by FTIR and elemental analysis.

Polymer	$DS_{theoretical}$[a] (%)	$DS_{calculated\ by\ FTIR}$ (%)	$DS_{calculated\ by\ elemental\ analysis}$ (%)
CH	-	-	-
CHimi1	5	5.2 ± 3.7	7.1
CHimi2	15	12.9 ± 4.1	16.1
CHimi3	30	22.1 ± 2.8	24.1

[a] Based on the mol of ImiAcOH/mol of glucosamine residue charged

The NMR analysis corroborated the findings of the FTIR spectroscopy. In the spectra of the polymers CHimi2 and CHimi3 two resonances were identified that can be assigned to protons of the imidazole ring (see Figure 2 for an example).

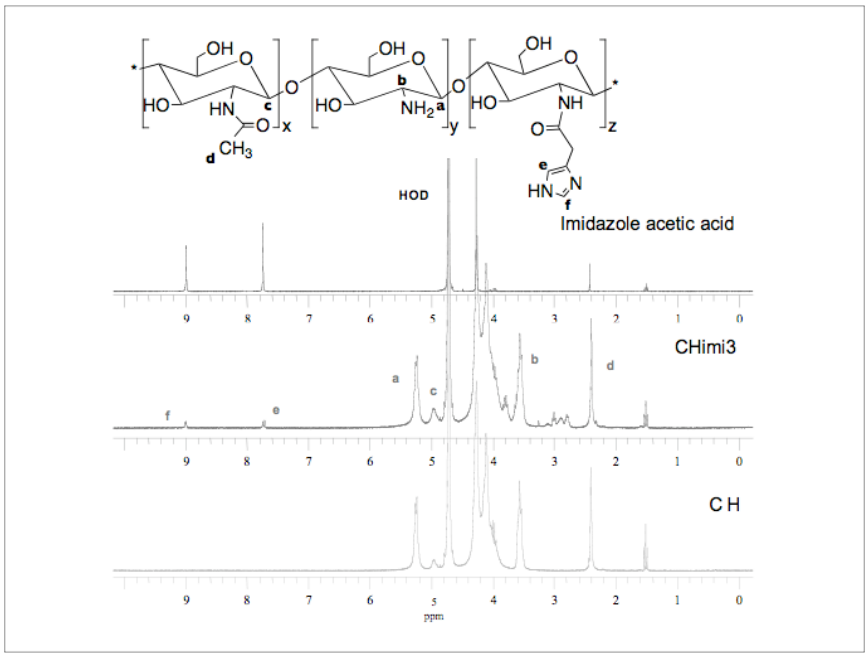

Figure 2 - ^{1}H-NMR spectra (300 MHz) in DCl/D_2O of the unmodified ImiAcOH, CHimi3 and CH.

The DS was also determined by elemental analysis (Table I) confirming the results obtained by FTIR. In general, the yield of the reaction is in accordance with the expected values. The fact that the reaction yield of the modification performed with the higher imidazole concentration (CHimi3) was lower than the expected value may be explained by a decrease of solubility of the polymer with the increase of the DS, that may hinder the introduction of new imidazole moieties into the polymer backbone. In order to evaluate if the introduction of imidazole moieties into the CH backbone resulted in any change in terms of cytotoxicity behavior of the parental polymer, the viability of 293T cells was assessed upon contact with increasing concentrations of modified polymer using the MTT assay. The cytotoxicity of unmodified CH and PEI (ESCORT V) was also evaluated. Figure 3 shows the relative cell viability in percentage, which was defined as the ratio between the metabolic activity of 293T cells exposed to polymer and untreated cells.

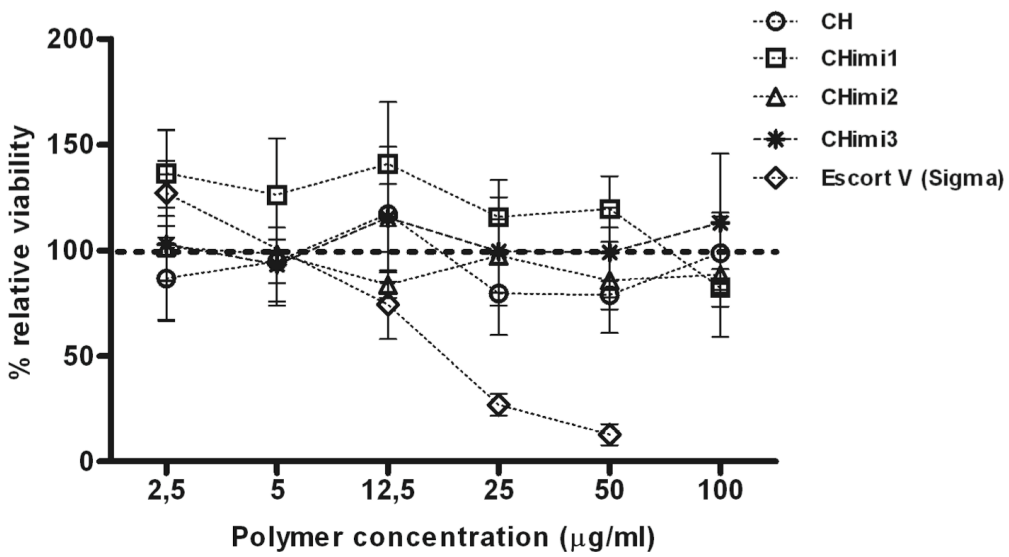

Figure 3 – Relative viability of 293T cells exposed to increasing concentrations of CH, CHimi1, CHimi2, CHimi3 and ESCORT V (average ± SD, n=6).

For all polymers, in the range of tested concentrations, cell viability was significantly higher than the viability of cells in contact with phenol, which was (4 ± 0.4%). The introduction of the imidazole groups into the CH backbone did not reveal a cytotoxic effect up to polymer concentrations of 100 μg/ml. No major differences were found among the CH-based polymer behaviors. When cells were incubated in medium containing increasing concentrations of PEI based solution, cell viability was drastically reduced down to 13% (50 μg/ml), in accordance to previous reports [31].

3.2. Polyplex characterization

To determine the minimum amount of CHimi polymers required to fully complex plasmid DNA, varying amounts of polymer were mixed with DNA solutions with a fixed plasmid DNA amount and the resulting complexes evaluated for their electrophoretic mobility. Independently of the degree of substitution, the modified polymers halt DNA mobility at the same N/P molar ratio as for the non-modified CH, which was found to be one (see Figure 12 in supporting information).

Upon proving the ability of the modified polymers to complex DNA, it was important to evaluate their ability to condense the genetic material into particles that could shield DNA from enzymatic attack and enable cellular uptake of the complexes. The effect of the degree of substitution and the N/P molar ratio on the particle size was determined by light scattering. Particles with N/P molar ratios ranging from 1 to 24 were evaluated (Figure 4).

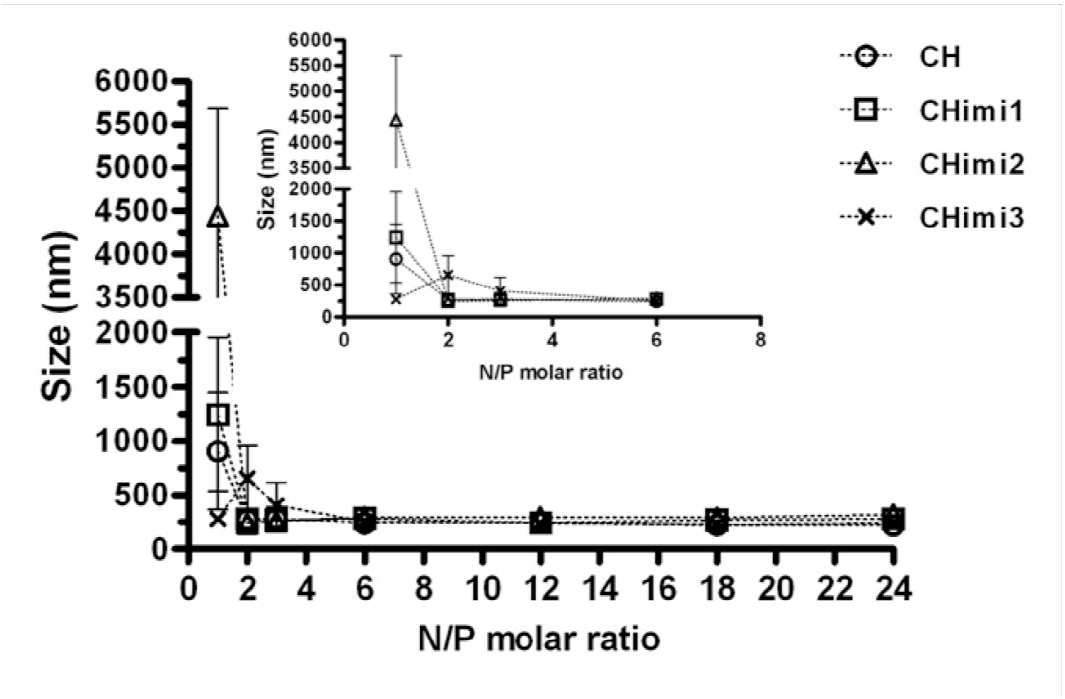

Figure 4 - Mean particle size of CH based complexes as a function of N/P molar ratio (measurements performed at 25°C, pH 5.5; average ± SD, n=3). A magnification of the graph is presented showing the results for the complexes prepared with the lower N/P ratio.

No differences were observed in the behavior of imidazole-grafted CH and unmodified CH. For N/P molar ratios above 3, complexes with mean particle size of approximately 260 nm were obtained, independently of the polymer used. For lower N/P molar ratios the size of the particles was in the micrometer scale.

The ability of the complex to protect the integrity of the plasmid DNA is a paramount condition in the overall transfection process, namely if *in vivo* application is foreseen. To determine if the developed polymer was able to protect plasmid DNA from nucleases, a DNase assay was performed. CH and CHimi based complexes were tested for N/P molar ratios of 1, 3 and 18, using naked DNA as control. This assay is based on the fact that intact DNA molecules possess hypochromicity and the absorbance at 260 nm increases by about 30% upon enzymatic digestion [26]. When higher N/P molar ratios were used, DNA protection was improved, both for CH and CHimi based complexes, when compared with naked DNA. See Figure 5 for an illustration of results obtained for CH and CHimi3, N/P molar ratio of 3 and 18. The results found for CHimi1 and CHimi2 based complexes followed a similar trend (data not shown).

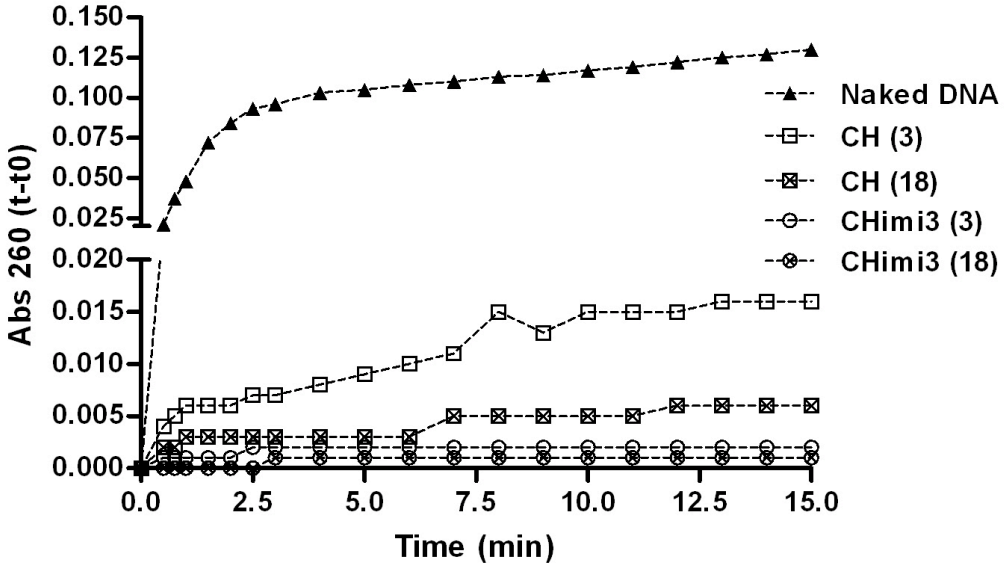

Figure 5 – Variation of absorbance values at 260 nm ($t-t_0$) as a function of time after addition of DNase I (pH 7.4, 37°C). The N/P ratio of the complexes is presented in parenthesis. Naked DNA was used as control.

Complex dispersions were also characterized in terms of zeta potential. The obtained results are presented in Figure 6.

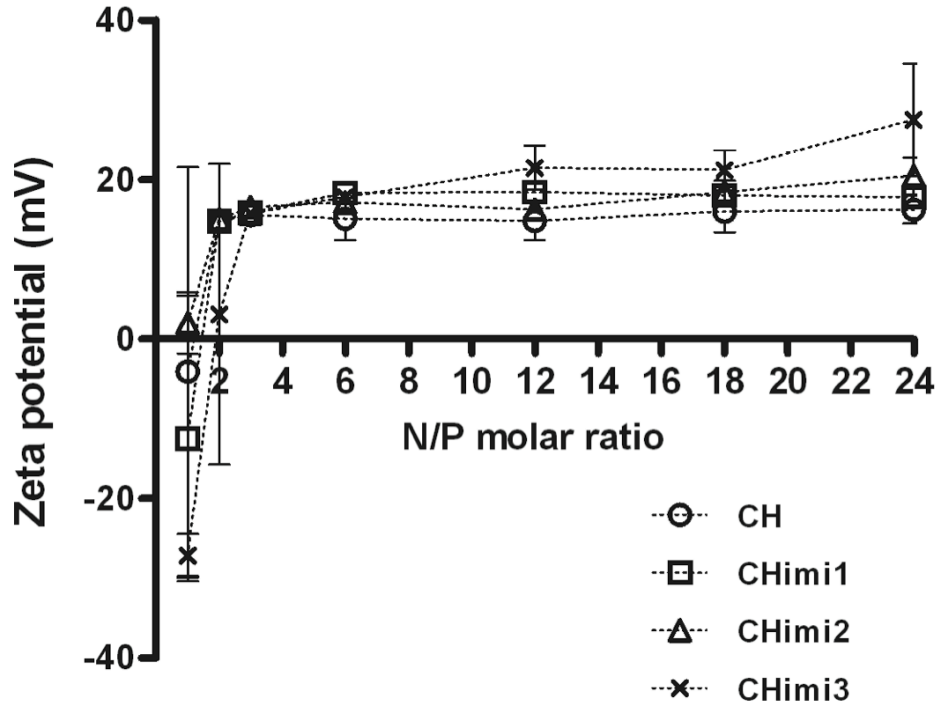

Figure 6 - Zeta potential of CH based complexes as a function of N/P molar ratio (measurements performed at 25°C, pH 5.5; average ± SD, n=3).

According to Pouton *et. al* the zeta potential has a determinant role in the *in vitro* transfection process, where only positively charged particles are active [32]. In all cases, zeta potential determinations have shown that an excess of polymer allowed the assembly of particles with a positive global net charge that stabilizes at a value of +16 mV, which is in agreement with the values found in literature for complexes based on CH [33].

3.3. Assessment of transfection mediated by imidazole-grafted CH

To evaluate the effect of the degree of substitution of CH on the ability of the resulting complexes to mediate gene delivery, 293T cells maintained in complete culture medium were transfected with complexes prepared with CH with increasing degrees of substitution. For each polymer the effect of polyplex composition – molar ratio of primary amine groups (N) to phosphate (P) – was studied by means of the assessment of the β-galactosidase activity upon gene delivery. The N/P molar ratios tested were 3, 12, 18 and 24. Transfection mediated by the PEI-based transfection agent (ESCORT V) was also assessed. Gene expression was assessed 48 and 72 h post-transfection. Representative results of 293T cell transfection are presented in Figure 7A and 7B, respectively.

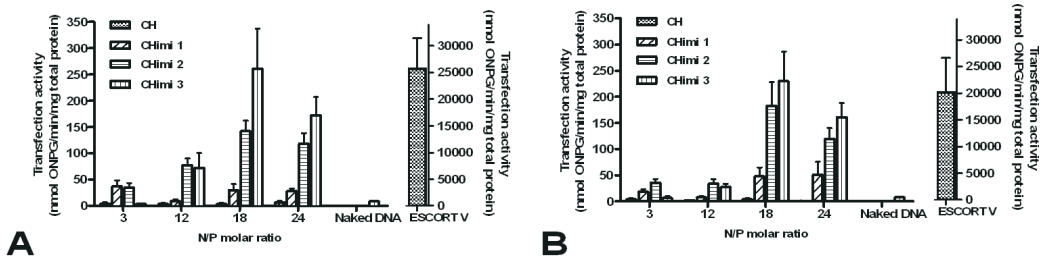

Figure 7 – Transfection activity evaluated 48 h (A) and 72 h (B) post-transfection of 293T cells treated with CH or CHimi based complexes as well as with naked plasmid DNA and ESCORT V (average ± SD, n=3).

An increase in transfection activity with increasing imidazole content can be observed. The higher the degree of modification of CH, the higher the transfection activity achieved. For N/P molar ratios higher than 3, transfection activity attained with CHimi2 and CHimi3 based vectors was significantly higher than that observed with naked DNA, non-modified CH and CHimi1. No effect of the N/P molar ratio on transfection activity was observed for both non-modified CH and CHimi1. However,

for both CHimi2 and CHimi3 (polymers with the highest imidazole content) an enhancement of transfection activity was observed with increasing polymer content up to an N/P molar ratio of 18, after which a plateau is reached. No significant differences were found between CHimi2 and CHimi3 based complexes. Transfection activity showed to be stable in the time frame of this study. For both evaluation time points, cells transfected with ESCORT V have shown higher transfection activities - $2.6x10^4$ and $2.0x10^4$ nmol ONPG/min./mg of protein at 48 and 72 h, respectively. In terms of cell viability, 24 h post-transfection all cells treated with CH-based materials showed viability values above 80% in relation to non-transfected cells (Figure 8). In contrast, cells transfected with ESCORT V presented only 39% of cell viability in relation to the latter (Figure 8).

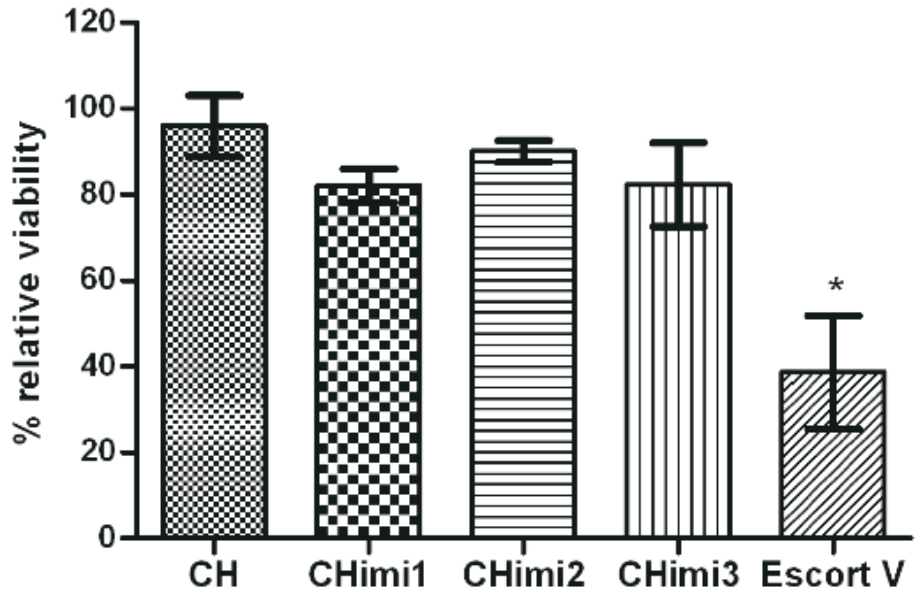

Figure 8 – Relative viability of 293T cells 24 h after transfection mediated by CH-based polyplexes (N/P molar ration = 18) and ESCORT V (average ± SD, n=3). * significantly different ($p<0.001$).

In a similar manner to what was observed in terms of the transfection activity results, the number of β-gal expressing cells increased when cultures were transfected with the modified CH (see Figure 13.A in supporting information). A similar trend was observed when the N/P molar ratio of the complexes was increased for each composition tested (see Figure 13.A in supporting information). Cells transfected with CH and CHimi (any degree of modification and any ratio) were well spread and attached, while cell cultures transfected with PEI showed clear signs of cytotoxicity,

with the cell layer presenting rounded shape cells and cellular debris (see Figure 13.B in supporting information).

Besides the qualitative evaluation, staining and counterstaining of glass slides was performed in order to determine the total number of cells and β-gal positive cells. This allowed the calculation of the transfection efficiency, which is an important parameter in gene delivery appraisal. The transfection efficiency results assessed 48 h post-transfection are presented in Figure 9.

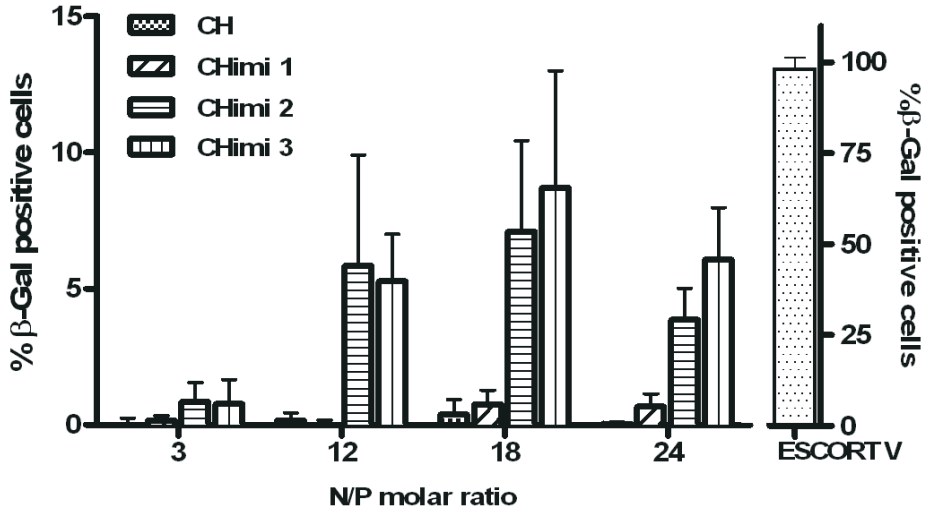

Figure 9 – Percentage of β-Gal positive cells 48 h post-transfection as a function of N/P ratio for 293T cells transfected with CH based vectors and for 293T cells treated with ESCORT V (average ± SD, n=3).

For 72 h post-transfection this parameter was not determined as, due to the confluence state of the cultures, an accurate determination of positive and total number of cells was not possible. For an N/P molar ratio of 3 there were no differences between vectors based on non-modified and imidazole-grafted CH, for all the degrees of modification tested. Significant differences in the number of positive cells were found for cultures transfected with CH or CHimi1 and CHimi2 or CHimi3 for N/P molar ratios equal or higher than 12. Transfection efficiency for PEI based system was also assessed with results found to be above 95%.

The developed CHimi based systems were also tested for their ability to transfect HepG2 cells (N/P molar ratios tested were 12, 18 and 24). The obtained results (Figure 10) show that the developed vectors successfully transfect these cells, in a

manner dependent on the degree of modification of the polymer and on the N/P molar ratio of the complexes.

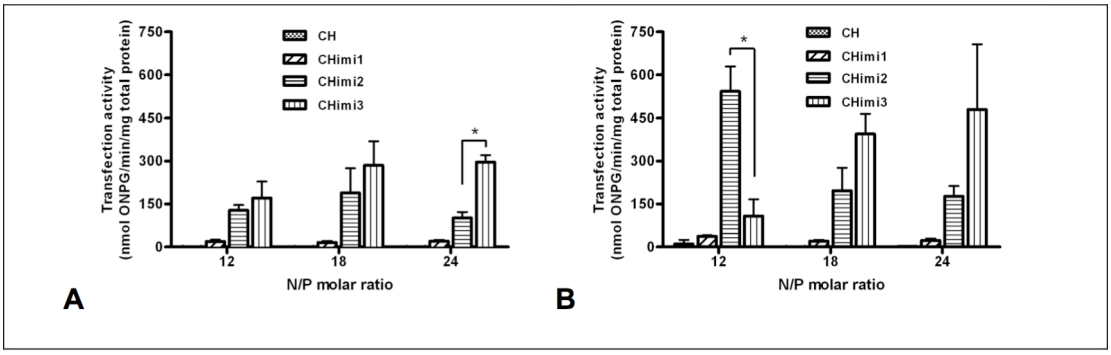

Figure 10 – Transfection activity evaluated 48 h (A) and 72 h (B) post-transfection of HepG2 cells treated with CH or CHimi based complexes (average ± SD, n=3). * significantly different ($p<0.05$).

As for the 293T cells, transfection was higher when mediated by the polymers with the higher degree of modification. Although not always statistically significant, a tendency was observed for higher transfection activities when cells were treated with complexes with a higher N/P molar ratio.

3.4. Effect of bafilomycin A1 on CHimi3-mediated transfection activity

In the current study it is proposed that the introduction of imidazole moieties into the CH backbone would lead to an enhancement of transfection activity mediated by the polyplexes, by facilitating endosomal escape and consequent release into the cytoplasm. To test this hypothesis, cells were transfected with CHimi3 and Escort V in the presence or in the absence of BafA1. BafA1 was selected in this study over other agents that can interfere with the acidification process of endosomes [34] due to its specificity of action at the endosome level. BafA1 is a selective potent inhibitor of vacuolar type H^+ ATPases [35], which are responsible for the acidification that occurs in the internal space of several organelles of the vacuolar system. Although this drug does not interfere with endocytosis and recycling, it is well established that a reduction on the extent of the degradation of internalized proteins from endosome to lysosomes can be observed [36].

The results presented in Figure 11 show that when BafA1 is present in the culture medium during the 24 h of contact between cells and the complexes, transfection is inhibited up to 75% in the case of CHimi3 complexes and 94 % in the case of Escort V (values significantly different from 100%, $p<0.01$).

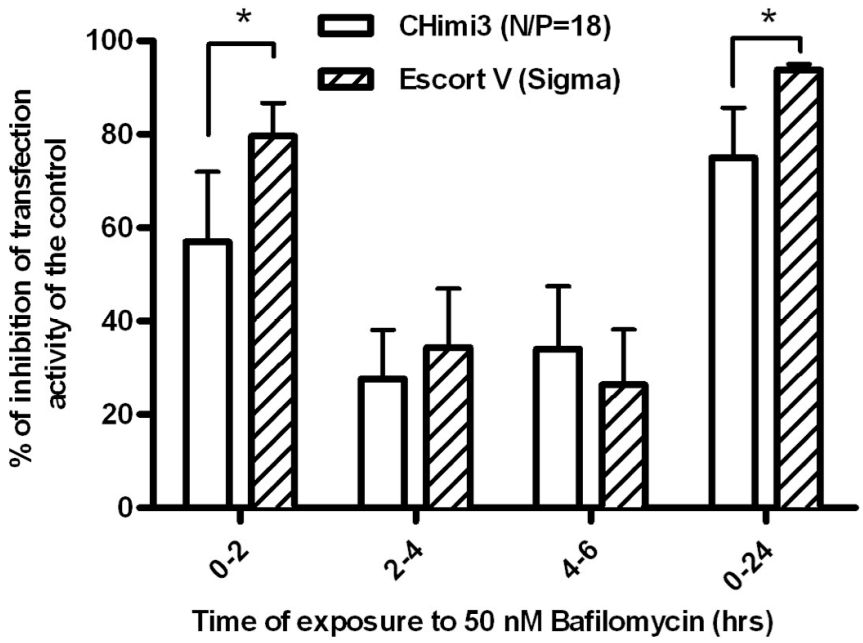

Figure 11 – Influence of bafilomycin A1 (Baf A1) on the transfection activity mediated by CHimi3 (N/P molar ration = 18) and ESCORT V. BafA1 was added to the cells either 30 min before transfection (t_{0-2}), or 2 h (t_{2-4}) and 4 h (t_{4-6}) post-transfection. For these conditions the exposure time of the cultures to BafA1 post-transfection was 2 h. Simultaneously, cells were incubated with BafA1 for 24 h post-transfection. β-gal activity was assessed 48 h post-transfection (average ± SD, n=6). * significantly different ($p<0.05$).

Furthermore the effect of the presence of BafA1 for 2 h in the culture medium at different periods after transfection was also evaluated. As illustrated in Figure 11, a reduction on transfection activity was observed, this effect being dependent on the incubation time of the drug with the cells. For both systems tested, the % of inhibition of the levels of gene expression is higher when BafA1 is present in the first 2 h post-transfection. However, the impact is less pronounced for the CHimi3 based complexes. For the treatments performed 2 h and 4 h post-transfection the % of inhibition was below 40% for both systems.

Discussion

CH is a natural polymer, with a cationic character under lightly acid conditions that due to its low cytotoxicity is emerging as an alternative non-viral vector for gene therapy. However, the efficacy presented by CH-based vectors is insufficient [37, 38], therefore better performing strategies are needed. In the present study the

improvement of the endosomal escape capability of CH was the strategy under investigation. For this purpose, imidazole moieties where introduced into the CH backbone, in order to mimic the hypothesized mechanism of action of PEI and improve CH efficiency as a gene delivery vector. The aim of this work was to assess if the proposed modification of CH would not interfere with the basic properties of CH as a vector, namely the ability of complex and condense DNA, while improving its gene delivery efficiency.

Electrophoretic retention of DNA was assessed for complexes prepared with increasing amounts of polymer. It was observed that the capacity of CH to retain the plasmid DNA has not been altered by the introduction of imidazole moieties. Independently of the degree of substitution, the modified polymer halts DNA mobility at the same N/P molar ratio (primary amines to plasmid DNA phosphate groups ratio) as non-modified CH, which was found to be one. Upon proving the ability of the modified polymers to complex DNA it was important to evaluate their ability to condense the genetic material into particles that could shield DNA from enzymatic attack and enable cellular internalization. For N/P molar ratios above 3 no differences were observed in the behavior of imidazole-grafted CH, in terms of mean particle size, charge and ability to protect DNA from endonuclease degradation. Large particles and polydisperse populations were only observed at lower N/P molar ratios. Other authors have also reported increased size at molar ratio 1 (condition at which the zeta potential of the complexes is close to neutrality), which could be explained by instability of the colloidal system. Under these conditions, the particle population is very heterogeneous and the charge and size characteristics of the complexes favor the formation of large aggregates [39, 33]. The dimension range referred in literature for CH-based complexes with DNA is quite wide, and found to be strongly dependent on preparation conditions [25, 33]. Some authors have reported particles with sizes ranging from 150 to 250 nm [33, 40], while others have reported sizes ranging from 5 μm to 8 μm [41]. For particles prepared by the coacervation method the size range reported was between 150 and 500 nm [33]. The results obtained with our systems are therefore, in close agreement with these findings.

The ability of the different CH-modified polymers developed in this work to mediate gene transfer in physiological conditions (pH 7.4 and presence of serum) into cultured cells was evaluated at 48 and 72 hours post-transfection. When compared to non-modified polymer, the use of polyplexes with imidazole residues resulted in an improvement of both transfection efficiency and activity, without compromising cell viability. This improvement depended both on the imidazole content, and also on the N/P molar ratio used. However, it was not observed a linear relationship between the

imidazole content of CH and reporter gene expression, as no significant differences were found for CHimi2 and CHimi3, though the latter showed a better performance. In this study the levels of transfection mediated by unmodified CH are residual. Other studies report similar results for CH with identical characteristics [42]. Although we are aware that one can improve this result by altering the transfection conditions, for example by lowering the pH of the cell culture medium at transfection [42], this was not the subject of the present study.

The size of the polyplex species is a critical parameter that affects transgene expression and it is generally believed that small size of particles and lack of aggregation enhances transfection by facilitating the entry of the polyplexes into the cells by endocytosis [43]. Considering the fact that the size of the complexes prepared with un- and modified CH is in the same order of magnitude and also that no major differences were observed in terms of the zeta potential of the complexes (for N/P molar ratios >3), it is reasonable to assume that the contribution of these physical parameters can be excluded as a reason to explain the gene delivery enhancement of the modified systems. The hypothesis behind this study is that the introduction of imidazole moieties into the CH backbone will lead to a better endosome escaping capacity of the polymer via membrane destabilization of the acidified endosomes, thus resulting in an improvement of transfection. The reduction on the levels of β-gal expression observed upon transfection of the cells in the presence of BafA1 clearly demonstrate that the introduction of imidazole moieties into the CH backbone plays a major role in facilitating the escape of the complexes from the endosome in a process dependent on the acidification of the latter. The results found for the commercial transfection agent are in agreement to the ones previously reported in literature for other cell lines [44]. For PEI based vectors it is hypothesized that an increase of the osmotic pressure caused by the strong buffering capacity of PEI polymers - a process know as “proton sponge effect” [9] – leads to the complex release from the endosomes as a consequence of their lyses. Similarly, the presented results suggest that CHimi3 can undergo protonation during the endosome maturation process, which in turn may lead to the buffering of the endosomes and to their destabilization. The buffering capacity of CHimi3 was compared to the one of unmodified CH by means of an acid-base titration. The obtained results (see Figure 14 in supporting material) show an improvement of the buffering capacity of CHimi3 in relation to CH, within the pH range of 6.5 to 5.5. Consequently, complexes can escape from the endosomes to the cytoplasm, increasing the chances of reaching the nucleus and mediate a successful delivery of the transgene. The enhancement of transfection observed for CHimi vectors as

compared to unmodified CH clearly support this theory. Whether the increased gene delivery efficiency is due to an improvement in the polymer buffering capacity that leads to complex swelling and consequent release from the endosomes [41] and/or to inoperative endosomal/lysosomal enzymes [45] is a subject of real significance still to be established, not only to explain the obtained results but also to contribute to further improvements of these vectors. From the time-profile of inhibition in the presence of BafA1 it can be concluded that for both systems (CH-derivatives and PEI-based complexes) the escape of the endosome occurs predominantly in the first hours post-contact.

While it was observed that transfection was impaired in the presence of a vacuolar type H+ ATPase inhibitor, it is noteworthy to refer that for both vectors tested no complete inhibition of transfection could be observed. For CHimi3 based vectors up to 25% of the transfection cannot be explained by the above-mentioned process. This observation supposes the existence of an alternative route for the complexes other than the endolysosomal pathway. In caveolar-mediated endocytosis, particles are internalized in primary endocytic vesicles that fuse, to larger, more complex tubular membrane organelles – “caveosomes” – that maintain a neutral pH [46]. 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 [47] and generally considered small in size [47, 48]. However, it is important to notice that caveolae constitutes 10-20% of cell surface in endothelial cells [48], where this endocytic pathway could be a relevant portal to enter into the cell [47].

In view of the obtained results, imidazole functionalized CH can be considered as a very promising candidate for application as a vector of therapeutic genes in a tissue regeneration scenario. Considering the time span of gene expression, CHimi2 and CHimi3 were found to mediate gene delivery that resulted in an improved and sustained transgene expression over the time frame of this study. Cell cultures grow with a typical morphology, with a regular increase in protein content (data not shown), while specific activity suffered a slight increase. The latter observation was previously documented for CH-based systems [39, 49]. Although, the introduction of imidazole moieties resulted in an enhanced gene expression, PEI-based polymers remain a more efficient model. However, their application in a clinic scenario has been limited due to its toxicity [37], non-degradability and limited efficiency in the presence of serum [50]. An important finding of this study is the absence of cytotoxicity by imidazole-grafted CH even for long incubation times, as assessed

from different experimental approaches (including evaluation of cell morphology by optical microscopy).

Conclusions

In the present study CH was functionalized with imidazole moieties with the aim of improving its buffering capacity and promote the endosomal escape capability of CH-DNA complexes, ultimately increasing the gene delivery efficiency of these systems. It was shown that the capacity of CH to complex, condense and protect DNA has not been altered by the introduction of imidazole moieties. Transfection studies were performed in 293T and HepG2 cells and proof-of-principle for the buffering capacity of modified materials with consequent improvement of transfection efficiency while maintaining cell viability was demonstrated. The fact that the developed imidazole-grafted CH are biodegradable, non toxic, mediate efficient gene transfer into mammalian cells, and exhibit adequate properties for iv administration render these type of complexes as promising tools for *in vivo* gene therapy applications in the field of regenerative medicine.

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Supplementary data

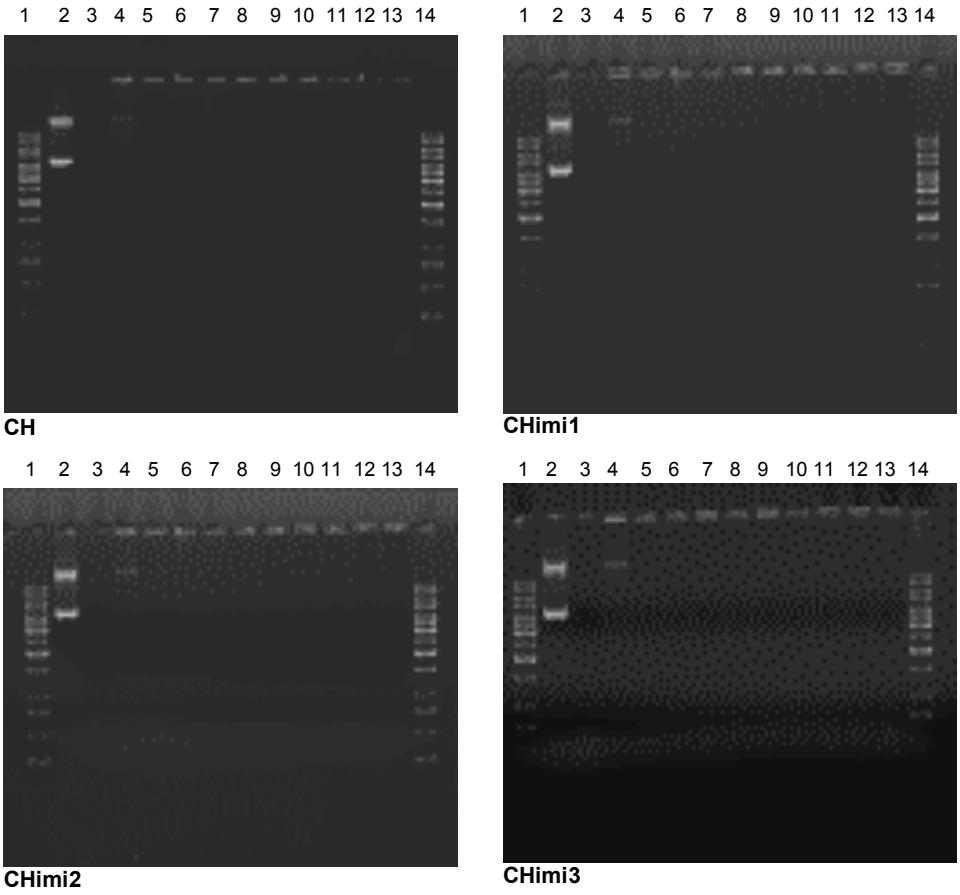

Figure 12 – Electrophoretic retention of DNA by CH, CHimi1, CHimi2 and CHimi3. Unless otherwise mentioned, lane assignments correspond to N/P molar ratios tested and are as follows: Lanes 1 and 14 - gene ruler 1 Kb DNA ladder; lane 2 – plasmid DNA solution; lane 3 – polymer solution; lane 4 - 0.5; lane 5 - 1; lane 6 - 1.5; lane 7 - 2; lane 8 - 2.5; lane 9 - 3; lane 10 - 3.5; lane 11 - 4; lane 12 – 5 and lane 13 – 6.

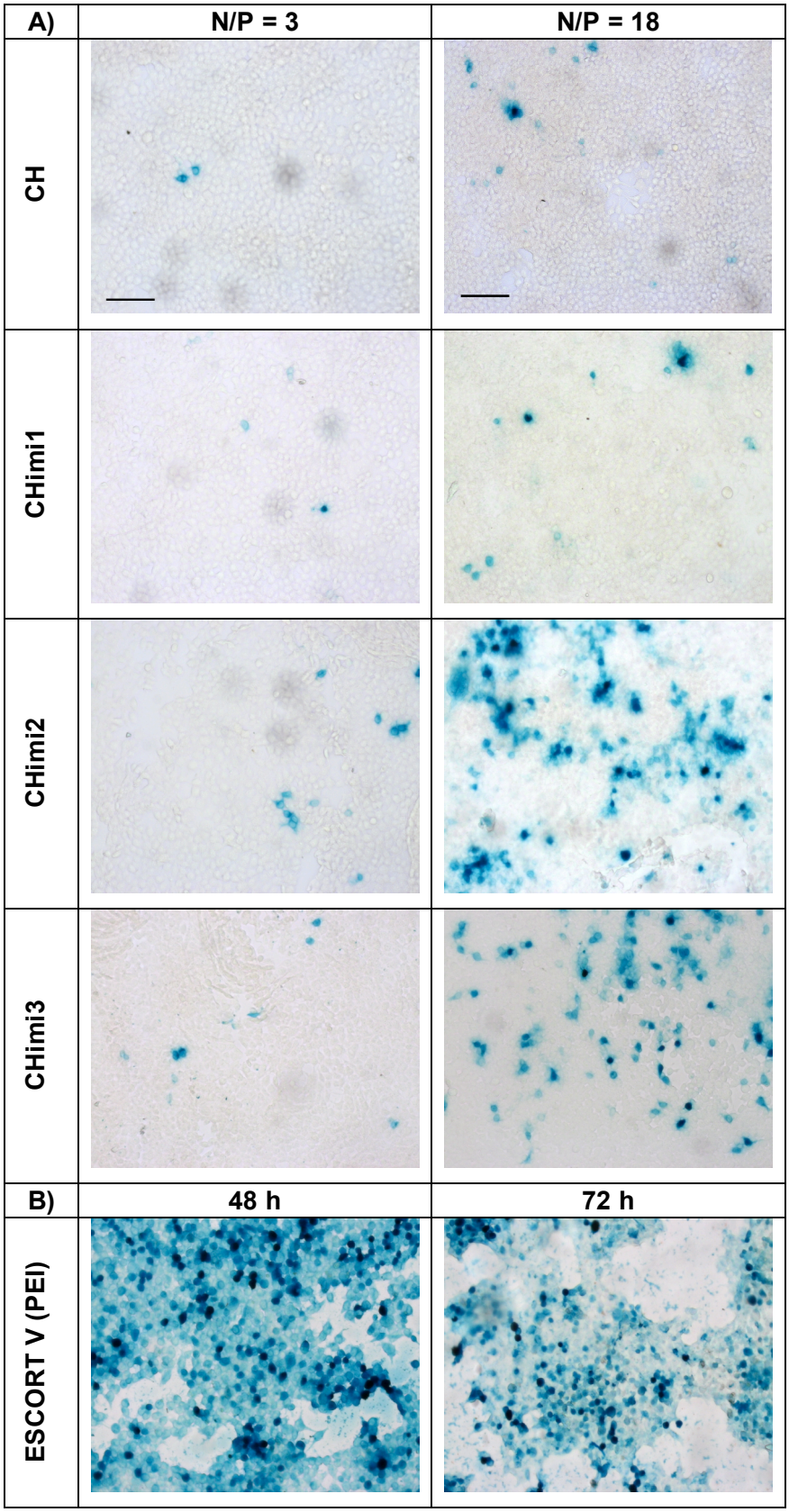

Figure 13 – β-Gal staining images of cells transfected with (A) CH based complexes at different N/P molar ratios 48 h post-transfection and (B) ESCORT V (PEI) 48 and 72 h post-transfection (Scale bar = 100 μm).

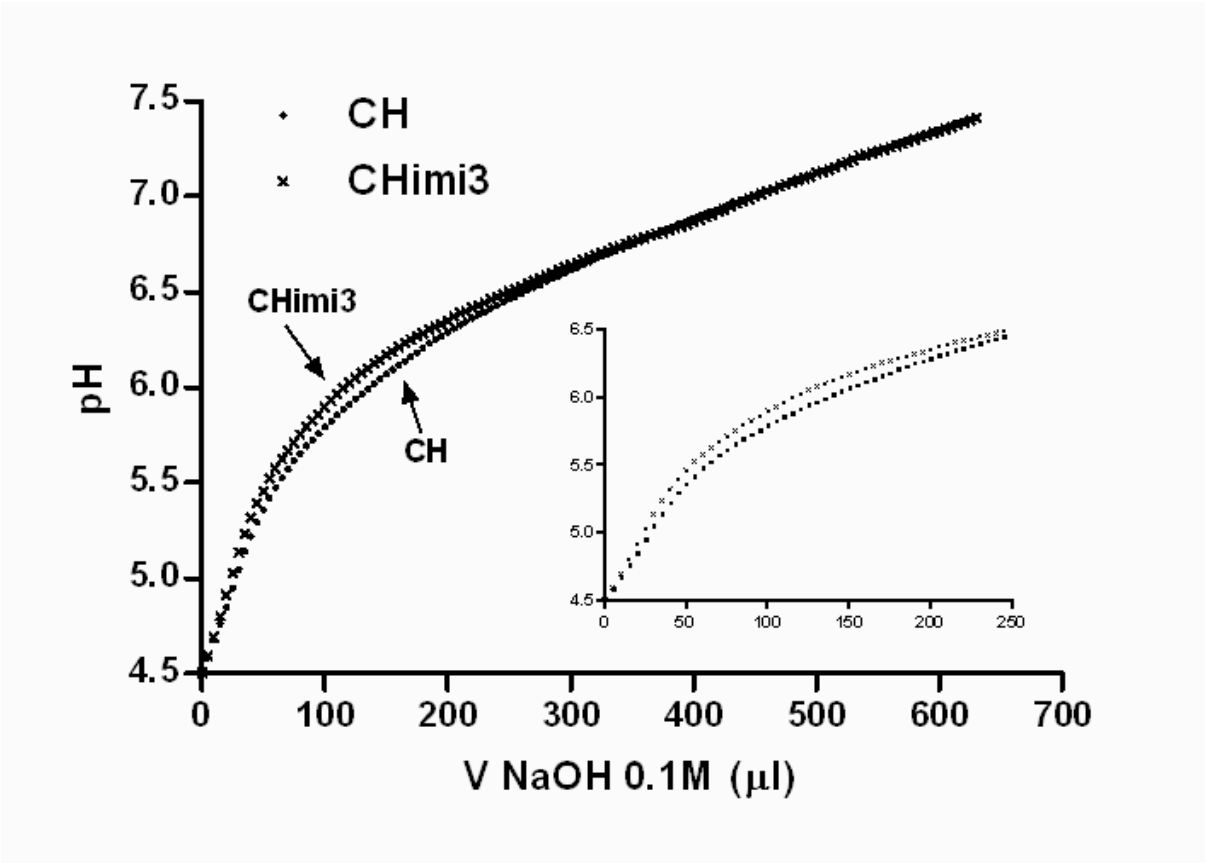

Figure 14 – CH and CHimi3 titration with 0.1 M NaOH. Each polymer was diluted in 150 mM NaCl to a final molar concentration of 8.26×10^{-6} M.

Chapter VI

Chitosan-based gene delivery vectors targeted to the peripheral nervous system *

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Abstract

A non-toxic, targeted, simple and efficient system that can specifically transfect peripheral sensorial neurons can pave the way towards the development of new therapeutics for the treatment of peripheral neuropathies. In this study chitosan (CH), a biodegradable polymer, was used as the starting material in the design of a multicomponent vector targeted to the peripheral nervous system (PNS). Polycation-DNA complexes were optimized using imidazole- and thiol-grafted CH (CHimiSH), in order to increase transfection efficiency and allow the formation of a ternary complex, respectively. The 50 kDa non-toxic fragment from the tetanus toxin (HC), shown to interact specifically with peripheral neurons and undergo retrograde transport, was grafted to the binary complex via a bi-functional poly(ethylene glycol) (HC-PEG) reactive for the thiol moieties present in the complex surface. The targeting of the developed ternary complexes was assessed by means of internalization and transfection studies in the ND7/23 (neuronal) vs. NIH 3T3 (fibroblast) cell lines. Targeted transfection was further confirmed in dorsal root ganglion dissociated primary cultures. A versatile, multi-component nanoparticle system that successfully targets and transfects neuronal cell lines, as well as DRG primary neuron cultures was obtained for the 1.0 (w/w) HC-PEG/DNA ternary complex formulation.

Introduction

The peripheral nervous system may be subject of damage by a variety of insults including traumatic injuries, diseases, tumors or iatrogenic lesions, that may result in a partial or total loss of motor, sensory and autonomic functions in the involved segments of the body [1]. The peripheral neuropathies have challenged the conventional approaches of treatment, and perhaps more importantly the used therapies have primarily been palliative rather than curative. With the enormous progress that has been made in our understanding of the biology of neurotrophic factors, and their application in neurologic diseases, new therapeutic approaches have arisen with the promise to arrest or reverse the disease process [2]. Several studies have demonstrated that a number of neurotrophic factors, including nerve growth factor, neurotrophin-3, insulin-like growth factors and vascular epithelial growth factor can prevent the degeneration process of peripheral sensory axons [2]. In spite of this, these short-lived factors cannot be administrated to patients for long term, due to unwanted systemic effects [3]. The local expression of these neurotrophic factors by specific cell populations, achieved by means of gene transfer, could be used to attain desired outcomes, thus avoiding unwanted adverse systemic effects and rapid *in vivo* clearance [4].

Neurotoxins have been for long explored to target the nervous system. Such an example is the non-toxic carboxylic terminal fragment from the tetanus toxin (HC). This fragment undergoes cell-specific internalization and neuronal retrograde transport [5,6], and previous *in vivo* studies have shown that systemic administration of enzyme-HC conjugates resulted in their delivery to the brain stem, motor neurons of the spinal cord and to a lesser extent the dorsal root ganglia (DRG) [7-9]. Additionally, Fairweather and co-workers have reported [10] a 10-fold increase of transfection activity in the neuronal cell lines N18 RE 105 (neuroblastoma x glioma mouse/rat hybrid) and F98 (glioma) by grafting the HC fragment to poly-lysine vectors. In contrast, the transfection of epithelial cell lines (CaCo-2 and HeLa cells) showed only a two-fold increase.

Recently, we have developed poly(ethylene imine) (PEI) based vectors targeted towards the peripheral nervous system (PNS) [11]. PEI is one of the gold standards of non-viral gene delivery systems [12]. However, when aiming at an application in a regeneration scenario, its low biodegradability and harmful systemic effects [13,14] are a major cause of concern. Aware of this hurdle, but inspired in the promising outcome obtained with the targeted PEI-based vectors, we pursued the development of such systems using chitosan (CH) as a starting material. CH, a co-polymer of glucosamine

and N-acetyl-D-glucosamine, is a biodegradable material with a well established biocompatibility [15]. CH has been proposed as a good candidate for gene delivery, as when positively charged it can effectively complex nucleic acids and protect them from nuclease degradation [16]. However, CH presents a low transfection efficiency under physiological conditions, which has held back its widespread use. We have previously shown that the incorporation of imidazole moieties into the CH backbone resulted in an increase in transfection efficiency of this vector [17].

The main objective of the present work was the development of a non-toxic and effective gene delivery system, based on imidazole-grafted chitosan (CHimi), which can be used for the delivery of therapeutic genes specifically to the sensory neuron population [11]. Imidazole grafting of CH was performed in order to increase the buffering capacity of the polymer, by improving the endosomal escape [17]. Subsequently, CHimi thiolation was pursued as a mean to neutralize the charge of the complex and downsize unspecific cell interactions [18], as well as obtaining a thiol-functionalized binary complex. The grafting of the targeting moieties - HC fragment - was performed by thiol chemistry via a bifunctional poly(ethylene glycol) (PEG) spacer, optimizing the accessibility of the protein moieties to cell interaction.

Envisaging a clinical scenario we believe that a successful system has to include several features, acting synergistically, in order to circumvent the natural cell barriers. In addition, considering that a gene delivery system has to incorporate a number of characteristics that enable the gene transport to the cell nucleus, we developed a step-by-step strategy in which the various cellular barriers were addressed systematically. An efficient CH-based system was successfully developed residing in physical and chemical self-assembly, optimized for targeted transfection of the sensorial neuron cell population.

Materials and Methods

Chitosan Purification and modification

Technical grade CH (Chimarin™, degree acetylation (DA) 13%, apparent viscosity 8 mPas) was supplied by Medicarb, Sweden. The polymer was further purified by precipitation as previously described [17]. The purified polymer was characterized by gel permeation chromatography (GPC) and Fourier Transform-Infrared Spectroscopy (FT-IR) [17]. The average weight molecular weight of the starting material was found to be 7.8±0.5 x 10^4 (GPC in 0.5 M CH_3COOH - 0.2 M CH_3COONa, 25ºC). The DA determined by FT-IR according to Brugnerotto *et al*. [19] was found to be 11.5±1.6%. Endotoxin levels of the purified CH extracts were assessed using the Limulus Amebocyte Lysate Assay (QCL-1000, Cambrex), following the manufacturer

instructions. Endotoxin levels were found to be below 0.1 EU.ml^{-1}, respecting the US Department of Health and Human Services guidelines [20] for implantable devices. Modified CH carrying imidazole moieties (CHimi, see Fig. 1) was obtained by the amidation of the glucosamine residues of CH using an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC)/N-hydroxysuccinimide (NHS) condensation system [17]. CHimi was further modified with 2-iminothiolane (Sigma, 0.5 mol/mol of free amine groups of CHimi), as previously described [21]. Briefly, 35 mg of CHimi were dissolved in 22.5 ml of acetic acid 1% (v/v) and the pH of the solution adjusted to 5.0. Afterwards, 7.5 mg of 2-iminothiolane (Sigma) was added and the reaction let to occur for 6 hrs at room temperature (RT) under agitation. The resulting polymer solution was subsequently dialyzed for 3 days in the dark at 4ºC, using a 3.5 kDa membrane (Spectrum labs, USA), against a 5 mM HCl solution. The dialized solution was then freeze-dried and the resulting powder (CHimiSH) stored at -80ºC until further use. CH, CHimi and CHimiSH solutions were prepared as follows: 10 mg of polymer was dissolved overnight in 4 ml of acetic acid 1% (v/v). Afterwards, 4 ml of 5 mM acetate buffer pH 5.5 was added and the pH of the solution adjusted to 5.5 with NaOH 1 M. The volume was completed to 10 ml with 5 mM acetate buffer pH 5.5 and the resulting solutions were stored at -80ºC until further use.

Plasmid DNA

The plasmid DNAs used encoded for the β-Galactosidase (β-Gal; pCMV-Sport-βGal, 7.8kb, Invitrogen), green fluorescent protein (GFP; pCMV-GFP, 7.4kb, a kind offer of Dr Luigi Naldini) or Luciferase (Luc; pCMV-LUC, 6.4kb, BD) genes. Plasmids were produced in a DH5α competent *E. coli* strain transformed with the respective plasmid. Subsequently, DNA purification was performed using an endotoxin-free Maxiprep kit following the manufacturer instructions (GenElute, Sigma). Plasmid concentration and purity were assessed by UV spectroscopy. Plasmid solutions with an absorbance (260/280 nm) ratio comprised between 1.7 and 2.0 were used in all studies.

Chitosan:DNA complex preparation (binary complexes)

DNA-polymer complexes were formed by mixing, while vortexing, equal volumes of pre-heated (55°C for 10 min) plasmid DNA solutions (in 25mM Na_2SO_4) and either CH, CHimi or CHimiSH solutions in 5mM CH_3COONa buffer pH 5.5 [17,22]. CH- and CHimi-based complexes were allowed to form for 15 min at RT before further use. In the case of CHimiSH-based complexes, these were let to form and oxidize for 24 hrs at RT. CHimi-based complexes with different molar ratios of primary amine groups

(N) to moles of DNA phosphate groups (P) – N/P molar ratio – were prepared. The same weight ratio used in the CHimi complex preparation was applied in the preparation of the CHimiSH-based complexes.

HC-PEG grafted nanoparticle formation

The non-toxic fragment of tetanus toxin (HC) was produced recombinantly using the BL21 *E. coli* strain, as previously described [11]. The HC fragment was covalently linked to a PEG spacer bearing a maleimide end group (MAL), to a final molar ratio of PEG/HC of 1.5±0.4 [11]. Hereafter, the PEG-modified HC will be designated as HC-PEG. To produce HC-PEG grafted nanoparticles, CHimiSH was complexed with plasmid DNA, as described in section 2.3, with an weight ratio equivalent to CHimi at N/P=6. After 15 min of incubation at RT, HC-PEG was added to the binary complex mixture and let to react for 24 hrs at RT. Complexes with different HC-PEG concentrations were prepared and tested, i.e. 0.3, 0.5, 1.0 and 1.6, expressed as w/w ratio of HC-PEG per plasmid DNA. The efficiency of HC-PEG binding to the complexes was assessed by means of Iodo-gen radiolabelling, as previously described [11], using acetate buffer for nanoparticle washing.

Complexes size and zeta potential determination

Complexes were prepared as described in the previous sections. Ten μg of plasmid DNA (pCMV-GFP) was used for each formulation and the complex dispersion diluted to 1 ml, using 5 mM CH_3COONa buffer (pH 5.5). The particle zeta potential and size were assessed using a Zetasizer Nano Zs (Malvern) following the manufacturer instructions. The Smoluchowski model was applied for zeta potential determination and cumulant analysis was used for mean particle size determination. All measurements were performed in triplicate, at 25°C.

Cell culture

Cell lines. ND7/23 (mouse neuroblastoma (N18 tg 2) x rat dorsal root ganglion neurone hybrid) or NIH 3T3 (mouse embryonic fibroblast) cell lines (both obtained from ECACC) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax, supplemented with 10% (v/v) foetal bovine serum (FBS) (heat inactivated at 56ºC for 30 min) and 1% (v/v) PS (10 000 units.ml^{-1} penicillin and 10 000 μg.ml^{-1} streptomycin) all supplied by Gibco, and maintained at 37°C in a 5% CO_2 humidified incubator. The ND7/23 cell line was chosen as a sensorial PNS cell model

and the NIH 3T3 cell line as a fibroblast model. Cells were routinely tested for mycoplasma by standard PCR.

DRG primary culture cells. Embryos (E17), obtained from euthanized pregnant Wistar rats, were placed in cold Hanks' balanced salt solution (HBSS) (Sigma). The spinal cord with the DRGs attached was isolated from the ventral region of the embryos. The DRGs were gently detached, incubated in 0.1% (w/v) trypsin (Sigma) in HBSS without Ca^{2+} and Mg^{2+} (Sigma) for 15 min at 37ºC for 15 min at 37ºC and collected by centrifugation (2 min at 1700 rpm). The DRGs were subsequently dissociated in complete medium (DMEM/F12 (1:1), high glucose, from Gibco, 10% (v/v) FBS, 1% (v/v) PS), using fired Pasteur pipettes. The obtained cell suspension was plated on a tissue culture polystyrene (TCPS, Greiner) flask for 90 min, in order to purify the DRG dissociated culture (DRGc) from TCPS adherent cells. From the final cell suspension, $2x10^4$ cells.cm^{-2} were seeded in a 24-well plate on glass coverslips (Sofdan) treated with poly(D-Lysine) (PDL, 0.1 mg.ml^{-1}, Sigma) and laminin (10 μg.ml^{-1}, Sigma), in DMEM/F12 supplemented with 50 ng.ml^{-1} of nerve growth factor (NGF 7s; Invitrogen) and 5-fluoro-2'-deoxyuridine (60 μM, Sigma). Medium was supplemented with NGF (50 ng.ml^{-1}) every 2 days and renewed every week. This protocol allowed a neuron purity >80% in DRGc, at 24 hrs post-plating, as determined by immunocytochemistry. Briefly, DRGc were fixed, permeabilized for 20 min with PBS containing 0.1% (v/v) Tween 20 and blocked for 30 min with PBS containing 3% (w/v) bovine serum albumin (BSA). Cultures were incubated overnight with rabbit polyclonal anti-200kDa Neurofilament (N200; 1:1000; Abcam) in 3% (w/v) BSA in PBS. After washing with PBS, cells were incubated with goat anti-rabbit Alexafluor 488 labeled antibody (1:500, Invitrogen) in 1% BSA (w/v) PBS for 1 hr. Finally, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 0.1 μg.ml^{-1}, Sigma) and mounted using Vectashield (Vector). The percentage of neuron cells was determined by counting 5 different fields (630x magnification) per replicate (n=4).

Internalization studies

ND7/23 or NIH 3T3 cells were subcultured 24 hrs prior to transfection in supplemented DMEM at a cell density of $2.0x10^4$ or $2.5x10^4$ cells.cm^{-2}, respectively, in 24-well TCPS plates (Greiner) coated with PDL. Cells were exposed, for 1 hr at 37ºC, to HC-PEG grafted nanoparticles (4.8 μg of plasmid DNA/well, pCMV-GFP) formulated with different HC-PEG amounts. Plasmid DNA was labelled with YOYO-1 (Invitrogen) as indicated by the manufacturer (1 mole YOYO-1 per 200 bp of plasmid DNA). For the competition assay, prior addition of the HC-PEG grafted nanoparticles,

cells were incubated for 20 min at 4ºC with a 100-fold excess of free HC. For complex internalization assessment, cells were incubated with a trypan blue solution (0.2 $mg.ml^{-1}$ in PBS, Sigma) for 5 min (in order to quench extracellular fluorescence [23]), trypsinized and processed for flow cytometry. Ten thousand gated events were taken for each replicate (n=3) using a FACSCalibur cytometer (BD Biosciences) and analysed by histogram for positivity for YOYO-1 using the FlowJo software (version 8.3.7).

Transfection studies

Cell lines. ND7/23 or NIH 3T3 cells were subcultured 24 hrs prior to transfection as described in section 2.7. Two hours prior transfection the medium was removed and replaced by 0.5 ml of supplemented DMEM medium. The transfection mix was prepared as described above. 4.8 μg of plasmid DNA was used for each well. Different N/P molar ratios were tested - 3, 6, 12 and 18 - using CH and CHimi. CHimiSH-based formulations were prepared with the same weight ratio as CHimi-based complexes. For HC-PEG grafted nanoparticle different formulations (0.0, 0.3, 0.5, 1.0 and 1.6 μg of HC-PEG/DNA (w/w)) were tested, using CHimiSH-based binary complexes with a weight ratio equivalent to CHimi at N/P=6. 24 hrs post-transfection media was removed and 1 ml of fresh complete DMEM medium was added to each well. Cells were incubated for an additional 24, 48 or 72 hr period post-transfection, with daily renewing of the media. At the defined time points, cells were processed for transfection activity or efficiency assessment. The transfection activity, corresponding to the β-Gal activity (ortho-nitrophenyl-β-galactoside (ONPG) hydrolyses), was measured by an enzymatic assay (Invitrogen). All experiments were performed in triplicate and expressed in terms of specific transfection activity (nmoles of ONPG hydrolyzed/min/mg total protein). BCA assay (Pierce) was used to quantify the total protein content. For transfection efficiency assessment, cells were analysed by flow cytometry for GFP expression, as described in section 2.7, omitting the trypan blue incubation step. Twenty thousand gated events were taken for each replicate (n=3) and analyzed as previously described.

Primary cultures. DRGs were obtained as previously described, seeded at a density of 2.0×10^4 $cells.cm^{-2}$ on PDL coated glass slides. Three to 4 days post-seeding the cultures were incubated with complexes for 24 hrs. Complexes based on CHimiSH prepared at an N/P=6 with or without 1.0 μg of HC-PEG moieties were tested. 72 hrs post-transfection, both GFP and luciferase gene expression were determined, and 96

hrs post-transfection the expression of the latter gene. The luciferase assay was performed according to the manufacturer instructions (Promega). RLU's where collected for 10 seconds and normalized to the cell extract total protein. Immunostaining was performed to assess the percentage of GFP positive cells. Discrimination between neurons and non-neuron cells was achieved by the N200 staining. Briefly, DRGc were fixed, permeabilized for 20 min with PBS containing 0.1% (v/v) Tween 20 and blocked for 30 min with PBS containing 3% (w/v) BSA. Cells were incubated overnight with biotin conjugated mouse monoclonal anti-GFP (1:200; Novus Biologicals) and N200 rabbit polyclonal (1:1000) in 3% (w/v) BSA in PBS. After washing with PBS, cells were incubated for 10 min with PBS containing 1% (v/v) H_2O_2, then, with peroxidase coupled ABC complex (Vectastain Elite, Vector) for 30 min and finally with DAB/Ni solution (Vector). To reveal the N200 staining, the DRGc were incubated with goat anti-rabbit Alexafluor 488 labeled antibody in 1% (w/v) BSA in PBS (1:500, Invitrogen) for 1 hr. Finally, the cells were stained with DAPI (0.1 $\mu g.ml^{-1}$) and mounted using Vectashield. The percentage of transfected cells (DAB/Ni positive) was determined by counting 5 different fields (630x magnification) per replicate (n=4). Images were collected using an inverted fluorescence microscope (Axiovert 200M, Zeiss).

Cytotoxicity assay

In order to determine cell viability 24 hrs post-transfection, a resazurin-based assay was used [24]. Briefly, a sterile solution of resazurin (0.1 $mg.ml^{-1}$ in PBS, Sigma) was added to each well to a final 10% (v/v) concentration. After 4 hrs of incubation at 37ºC, 200 µl of the medium was transferred to a black-walled 96-well plate (Greiner) and fluorescence was measured (λexc= 530 nm, λem=590 nm, Spectra Max GeminiXS – Molecular Devices). Results were expressed as percentage of metabolic activity of treated cells relative to untreated cells.

Statistical analysis

Using the Graphpad Prism 5.0 software the D'Agostino and Pearson omnibus normality test was used in order to test if data obeyed to a Gaussian distribution. Statistically significant differences between several groups were analyzed by the non-parametric Kruskal-Wallis test, followed by Dunns post-test. The non-parametric Mann-Whitney test was used to compare two groups. A p value lower than 0.05 was considered statistically significant.

Results and Discussion

Polymer characterization

In this work we studied the effect of introducing two different functionalities into the CH backbone on its efficacy as a gene delivery vector. Imidazole grafting of CH was performed by EDC/NHS mediated amidation. 22.8% of primary amines of the original CH were substituted, as determined by FT-IR [17]. Subsequently, thiol moieties were introduced into the CHimi structure (CHimiSH, Fig. 1). Upon complexation of CHimiSH with DNA, the presence of thiol pending groups will allow the cross-linking of the polycation and concomitant functionalization of the complexes with protein moieties via thiol chemistry. The total amount of thiol groups grafted to the polymer was 62±5 μmol.g^{-1} of polymer, as determined by the Ellman's assay [25], corresponding to 3.6% substitution of the CH primary amines by thiol groups.

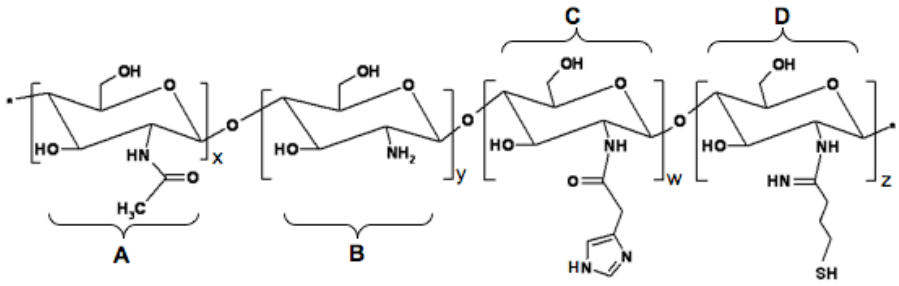


Figure 1 - Chemical structure of imidazole and 2-iminothiolane grafted chitosan. The repeating units include: A) N-acetyl glucosamine; B) glucosamine; C) imidazole grafted monomer and D) 2-iminothiolane grafted monomer.

Binary complex characterization

To assess the impact of the proposed modifications of CH on the complex characteristics as a function of their formulation, binary complexes (polymer-DNA) were prepared with variable amounts of polymer. Complexes based on CHimiSH were prepared using the same polymer:DNA weight ratio as CHimi, to allow the evaluation of the effect of the presence of thiol moieties on the properties of the complexes. When complexes were applied in an agarose gel and submitted to an electric field, no free DNA was detected for all formulations tested (Fig.S1, supplementary information (SI)), demonstrating that CHimi thiolation does not impair the polymer ability to complex DNA. For the N/P molar ratios tested, CHimi-based complexes exhibit an average diameter and zeta potential ranging from 174 to 197 nm and 15 to 18 mV, respectively (Fig. S2, SI). Unmodified CH-based complexes were also characterized, for the same N/P molar ratios, with no significant differences being observed in relation to the latter, neither in terms of particle size nor zeta

potential (data not shown). CHimi thiolation enabled the formation of significantly smaller particles for N/P≥6 correspondent formulation (Fig.S2, SI). Moreover, no significant differences were found in terms of polydispersity index (Pdi) between CHimi- and CHimiSH-based complexes, parameter that ranged from 0.126 to 0.279 for the different complex type (data not shown), indicating that the presence of the thiol functionalities did not compromise the complex dispersion stability. The characterization of the CHimiSH-based particles in terms of zeta potential showed that CHimi thiolation dramatically influences the complex overall charge, with CHimiSH-based complexes attaining zeta potential values near neutrality (Fig. S2, B in SI).

It has been shown that CH thiolation can influence polyplex characteristics. Lee et al. have shown that by thiolating a 33 kDa CH using thioglycolic acid, and obtaining a degree of substitution of 360±34 μmol of thioglycolic acid per gram of polymer, a decrease in the oxidized complex size was observed [26]. The obtained results are in line with this previous study. As expected, the charge and size of the CHimiSH-DNA complexes is significantly reduced, for the high polymer content formulations, due to the decrease in free primary amine groups result of the 2-iminothiolane grafting into the CH backbone. Moreover, the formation of disulfide cross-links in the polymer network, consequence of the excess of thiol groups, may be occurring as well and contributing to this effect.

Transfection mediated by binary complexes

In order to evaluate the influence of the polymer to DNA ratio on the ability of these systems to transfect the neuronal cell line ND7/23, the transfection activity and efficiency of these vectors were determined. In the case of unmodified CH-based vectors, an increase in the polymer content of the complexes was found to have no effect on the transfection activity, which remains at vestigial levels (Fig.2). In the case of the CHimi-based vectors an increase in the polymer content of the complexes leads to an increase in transfection activity, which peaks for N/P molar ratios of 3 (Fig.2). CHimiSH-based vectors show the same behavior as CHimi-based complexes (Fig.2). Additionally, the effect of thiolation alone was also tested using unmodified CH with a similar thiolation degree (data not shown). In this case no significant transfection improvement was detected in the ND7/23 cell line in relation with CH alone, showing that for the conditions tested, polymer thiolation does not influence transfection.

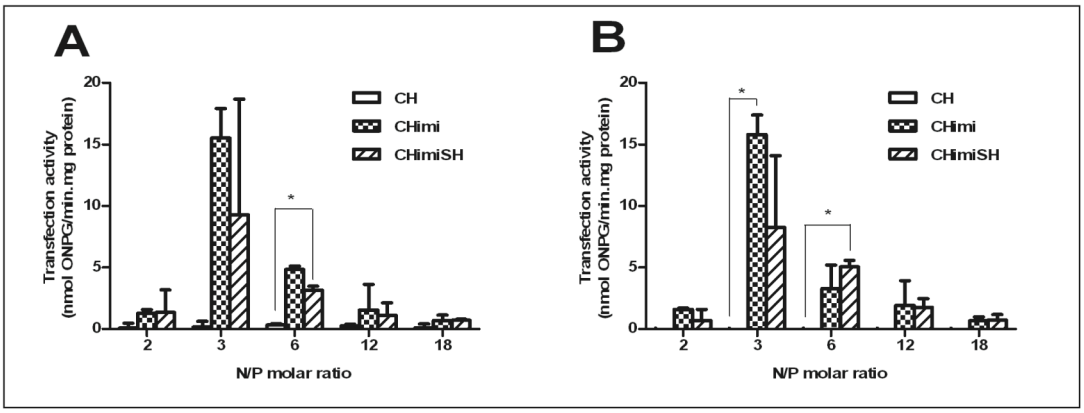


Figure 2 - Transfection activity of complexes based on CH, CHimi or CHimiSH at (A) 48 and (B) 72 hrs post-transfection (n=3, Aver±SD; * denotes statistically difference between the three groups at an N/P molar ratio, $p<0.05$; representative data of 3 independent experiments).

The transfection efficiency of CHimi- and CHimiSH-based complexes was also assessed by means of flow cytometry 48 and 72 hrs post-transfection. This parameter was also dependent on the polymer ratio, with the higher levels of transfection being attained, both at 48 and 72 hrs, for the complexes with an N/P molar ratio of 6 (Fig.S3, SI). No statistically significant differences were found in terms of transfection efficiency between CHimi- and CHimiSH-based complexes (Fig.S3, SI), showing that, although CHimi thiolation has a significant effect on complex physical properties (size and zeta potential), it does not influence transfection efficiency in the conditions tested. Cell viability was assessed 24 hrs post-transfection using a resazurin-based assay, and for none of the formulations tested significant signs of cytotoxicity were observed (Fig.S4, SI).

It has been previously shown that the thiolation of CH improves the transfection efficiency of CH-based vectors [26,27]. Lee *et al*. showed increased transfection efficiency in HEK 293, HEP-2 and MDCK cell lines when using thiolated CH-based vectors, comparatively with unmodified CH [26]. Loretz *et al*. also showed improved transfection in the Caco-2 cell line using a thiolated CH [27]. In this study, the aim of thiolating CHimi was not to improve transfection, but rather to reduce the zeta potential of the complexes and at the same time to explore this chemistry for the design of a HC-PEG grafted nanoparticle.

By evaluating both transfection activity and efficiency of the binary complexes we aimed at determining the optimal polymer to DNA ratio of the CHimiSH-based binary complex to be used as the basis for the development of HC-PEG grafted nanoparticles. As the transfection activity was found to be not significantly different

for the tested ratios 3 and 6 and, additionally, the highest levels of transfection efficiency were observed for the latter formulation, a correspondent N/P ratio of 6 was chosen as the basis for the formation of HC-PEG grafted nanoparticles.

Formation of HC-PEG grafted nanoparticles

The strategy followed for the formation of a HC-PEG grafted nanoparticles considered two steps. In a first step the polymer was thiolated in order to decrease complex charge. Furthermore, thiolation of the polymer provides the complex with thiol moieties that here were used for further complex functionalization with the HC moieties. This was explored in a second stage of complex preparation, where the addition of the HC fragment is performed via a 5 kDa bifunctional PEG linker bearing NHS and maleimide end groups, being the latter reactive towards thiol moieties (Fig. 3).

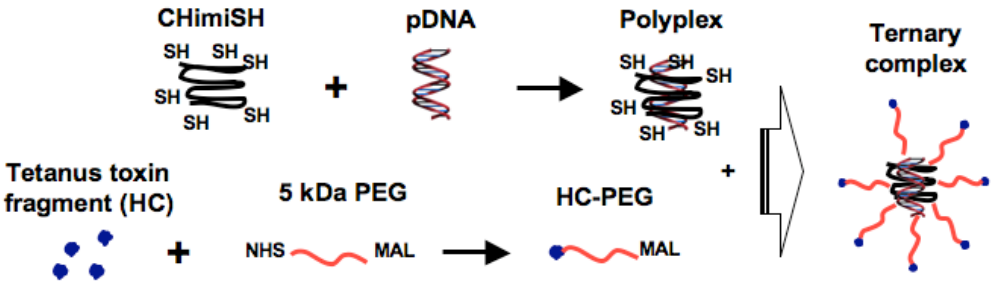
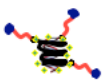
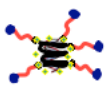
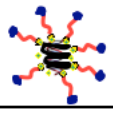
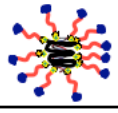


Figure 3 - Proposed model for the formation of a HC-PEG grafted nanoparticle. The formation of the complex is considered in two steps, the formation of the core of the complex (binary complex) and the grafting of the tetanus toxin fragment (HC) in the complex surface via a maleimide (MAL) grafted (thiol reactive) 5 kDa PEG linker.

The coupling of HC-PEG to the complexes, quantified by radiolabelling, ranged between 59.7% and 73.9% (Table 1). Higher coupling efficiency was observed for the two highest HC-PEG formulations tested. However, for all prepared complexes the % of bound HC-PEG was found to be not significantly different from 100%, as tested by Wilcoxon signed-rank test.

Table 1 - Theoretical thiol/HC-PEG ratio content for each prepared formulation and percentage of HC-PEG coupled to the complexes, as determined by radiolabelling.

	HC-PEG/DNA (w/w)				
	0	0.3	0.5	1	1.6
					
Molar ratio of SH content of CHimiSH to HC-PEG	-	51	26	13	9
% bound HC-PEG	-	59.7 ±10.4	65.2 ±22.4	73.7 ±7.0	73.9 ±0.2

Although in a non-statistically significant manner, the size of the complexes increased slightly for the two initial HC-PEG grafted nanoparticle formulations (as compared to controls), followed by a reduction in the two formulations with the highest HC-PEG content (Fig.4, A). Additionally, no significant differences were found in terms of Pdi for the tested formulations (Fig.4, A).

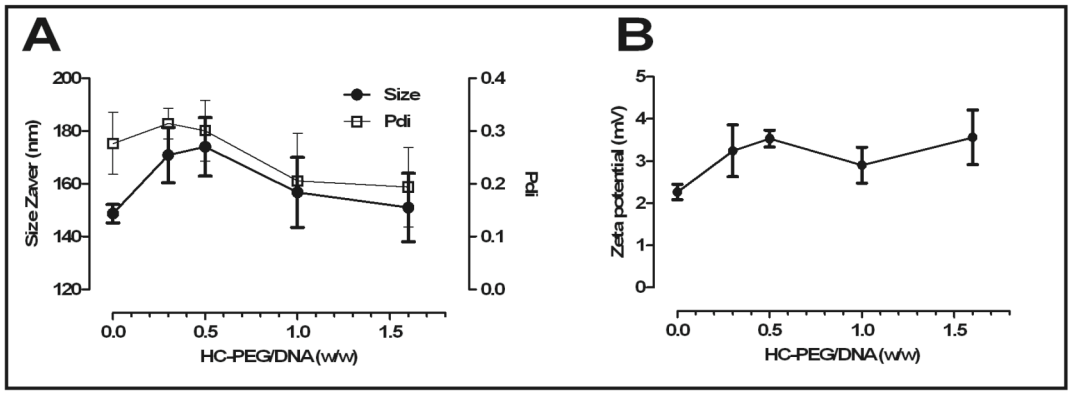


Figure 4 - Characterization of HC-PEG grafted nanoparticles based on CHimiSH and pCMV-GFP, as a function of HC-PEG/DNA (w/w) ratio; (A) size and Pdi and (B) zeta potential (n=3, Aver±SD).

The obtained results indicate that the functionalization of the complexes with HC-PEG not only does not compromise the complex stability in dispersion as it further contributes to its stabilization, which is indicated by the slight decrease in the Pdi value associated with the increase in modification degree (Fig.4, A). We hypothesize that the pegylation the complex surface may contribute to the improvement of the dispersion stability in an aqueous environment.

In terms of complex charge the functionalization with HC-PEG lead to a modest increase of complex zeta potential values. This could be anticipated as HC presents a positive zeta potential of 12.1±6.1 mV, under the tested conditions (5 mM acetate buffer, pH 5.5).

Internalization studies

The internalization behavior of the developed HC-PEG grafted nanoparticles was assessed in the two proposed cells lines - ND7/23 and NIH 3T3 - by means of FACS analysis. In the neuronal cell line the extent of internalization was found to increase when crescent amounts of HC-PEG are associated to the complexes (Fig.5, A). The internalization of the HC-PEG grafted nanoparticle formulation containing an HC-PEG/DNA ratio of 1.0 and 1.6 was significantly higher, almost doubling in relation to the other ratios tested (Fig.5, A). In contrast, the formulation with an HC-PEG/DNA ratio of 1.0 exhibited a different behavior with the NIH 3T3 cell line, with a significant decrease in the extent of internalization being observed when compared with the binary complexes (Fig. 5, B).

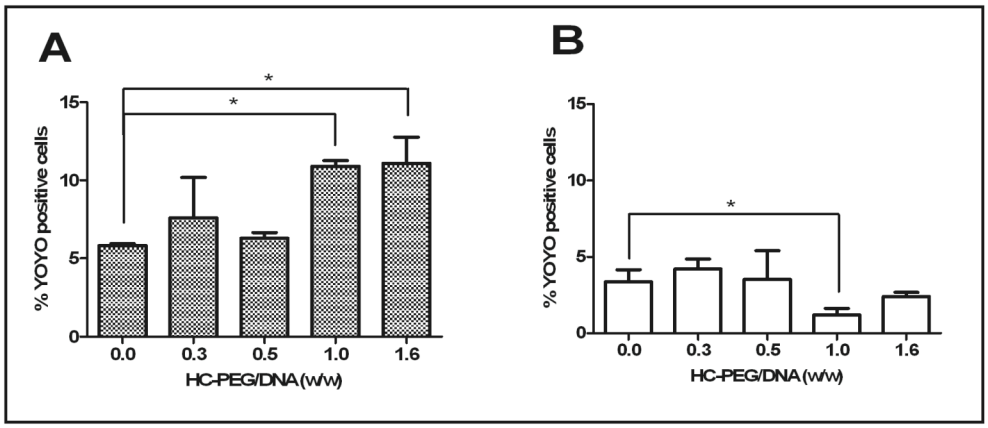


Figure 5 - Internalization of HC-PEG grafted nanoparticles based on CHimiSH and pCMV-GFP, functionalized with increasing amounts of HC-PEG in (A) ND7/23 and (B) NIH 3T3 cell lines, respectively. (n=3, Aver±SD; * denotes statistically difference between groups of ternary complexes and the binary complex, $p<0.05$; representative data of 3 independent experiments).

In parallel, cellular internalization studies were performed by incubating the HC-PEG grafted nanoparticles prepared at a HC-PEG/DNA ratio of 1.0 with ND7/23 cells previously treated with a 100-fold excess of free HC protein with. A statistically significant reduction of the number of ND7/23 cells internalizing the HC-PEG grafted nanoparticles was observed (Fig.6), confirming the targeting potential of the

developed nanoparticles. As controls, additional internalization studies were performed by incubating the ND7/23 cells pre-treated with a 100-fold excess of HC fragment with the binary formulation. In this case the internalization levels were found not to significantly vary from the ones shown in Fig. 5A for this formulation (data not shown).

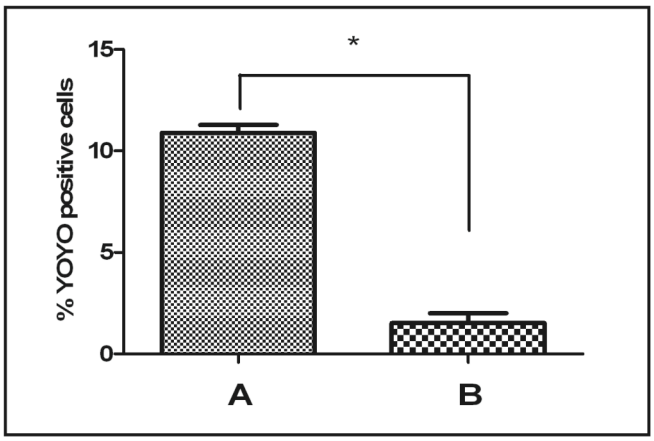


Figure 6 – Extent of cellular internalization of HC-PEG grafted nanoparticles based on CHimiSH and pCMV-GFP, functionalized with 1.0 (w/w) HC-PEG/DNA. ND7/23 cells were incubated with the ternary complexes in A) the absence or B) the presence of 100-fold free HC (n=3, Aver±SD; * denotes statistically difference between groups, $p<0.05$; representative data of 3 independent experiments).

Transfection studies mediated by HC-PEG grafted nanoparticles

The evaluation of transfection efficiency mediated by the developed complexes in the proposed *in vitro* model, was of critical importance in order to better assess the impact of their targeting potential on their ability to promote higher levels of transfection in neuronal cells, as compared to binary complexes. In the ND7/23 cell line, the transfection is initially impaired with the increase of HC-PEG amount, decreasing significantly for the 0.5 formulation (Fig.7 A). However, with the increase of HC-PEG content the opposite effect is observed, with transfection values regaining the same levels of transfection observed for the binary complexes, as seen for the 1.0 formulation (Fig.7, A). Additionally, the transfection of the ND7/3 cell line was found to be stable for the time period tested – up to 96 hrs post-transfection (Fig.7, A). Furthermore, no significant toxicity was found for all formulations studied, as assessed in terms of differences in cellular metabolic activity between treated and untreated cells (Fig.S5, SI).

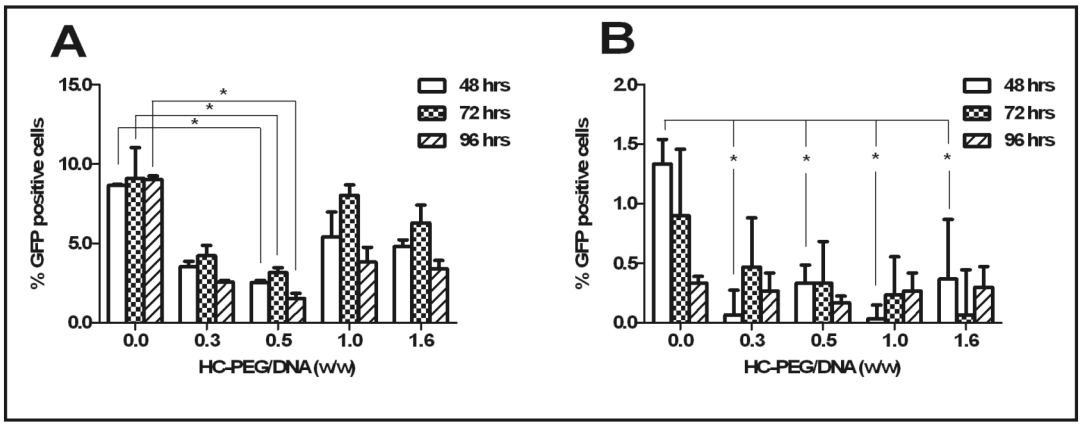


Figure 7 - Transfection efficiency of HC-PEG grafted nanoparticles based on CHimiSH and pCMV-GFP, functionalized with various amounts of HC-PEG in (A) ND7/23 and (B) NIH 3T3 cell lines, respectively, at 48, 72 and 96 hrs post-transfection (n=3, Aver±SD; * denotes statistically difference between groups of ternary complexes and the binary complexes at the same time point, $p<0.05$; representative data of 3 independent experiments).

The targeting potential of the developed formulations becomes more evident when comparing the transfection efficiency results obtained in ND7/23 *versus* NIH 3T3 cell cultures. With the inclusion of HC protein moieties in the complex formulation, a significant decrease in transfection efficiency was observed at 48 hrs for the NIH 3T3 cells for all formulations tested (Fig.7, B).

In this study, a direct correlation between the extent of cell targeting and transfection efficiency (% of transfected cells) could not be established. Although a significant increase in terms of cell internalization was observed in the ND7/23 cell line with a concomitant decrease of internalization in NIH3T3 cells for the 1.0 formulation *versus* the binary complexes, this was not translated in terms of enhancement of transfection in the former cells. We have previously shown for a PEI-based system that upon functionalization of the complexes with HC moieties, although complex internalization levels in the ND7/27 cell line are not significantly affected they are significantly reduced in fibroblast cultures (NIH 3T3) and a large increase of transfection efficiency in the ND7/23 cells is observed [11]. It is likely that in the present system other factors, downstream from complex uptake, are conditioning the overall transfection ability. We demonstrated that the imidazole moieties lead to an increase of the buffering capacity of the CH vectors, mimicking the “proton sponge effect” of PEI [17]. Nevertheless, the limited efficacy of the CH- in relation with PEI-based vectors

to escape the endosomal degradation pathway can be limiting the overall transfection efficiency.

The potential of the developed HC-PEG grafted nanoparticles to mediate a targeted gene delivery was further tested in a primary sensorial neuron culture model. Transfection mediated by the 1.0 formulation was tested in dissociated DRG cells and evaluated in terms of luciferase expression, 72 and 96 hrs post-transfection, and compared with that mediated by the binary complexes. As seen in Fig.8, the transfection activity was significantly increased 72 hrs post-transfection for the HC-PEG grafted complexes in comparison with the binary complexes. Moreover, the transfection levels were found to be stable in the time points tested (Fig.8).

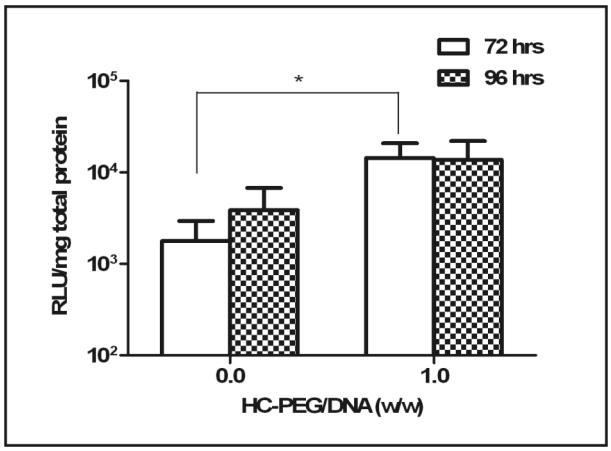


Figure 8 - Transfection activity of complexes based on CHimiSH and pCMV-Luc, with or without HC-PEG moieties, at 72 and 96 hrs post-transfection of dissociated dorsal root ganglia cultures (DRGc; n=4, Aver±SD; * denotes statistically difference between groups at same time point, $p<0.05$; representative data of 3 independent experiments).

In order to assess which cells were eliciting these transfection levels, a categorization of the transfected DRG culture was performed. GFP immunostaining revealed that, at 72 hrs post-transfection, no significant differences were observed in terms of the % of total transfected cells (Fig.9, A, C). However, by discriminating the neuron and non-neuron cell populations, a significant decrease was observed for the non-neuron population when the HC-PEG grafted nanoparticles were used (Fig.9, B). In addition, and although not significant, a slight increase in transfection of the neuron population was also observed (Fig.9, B, G, H, I).

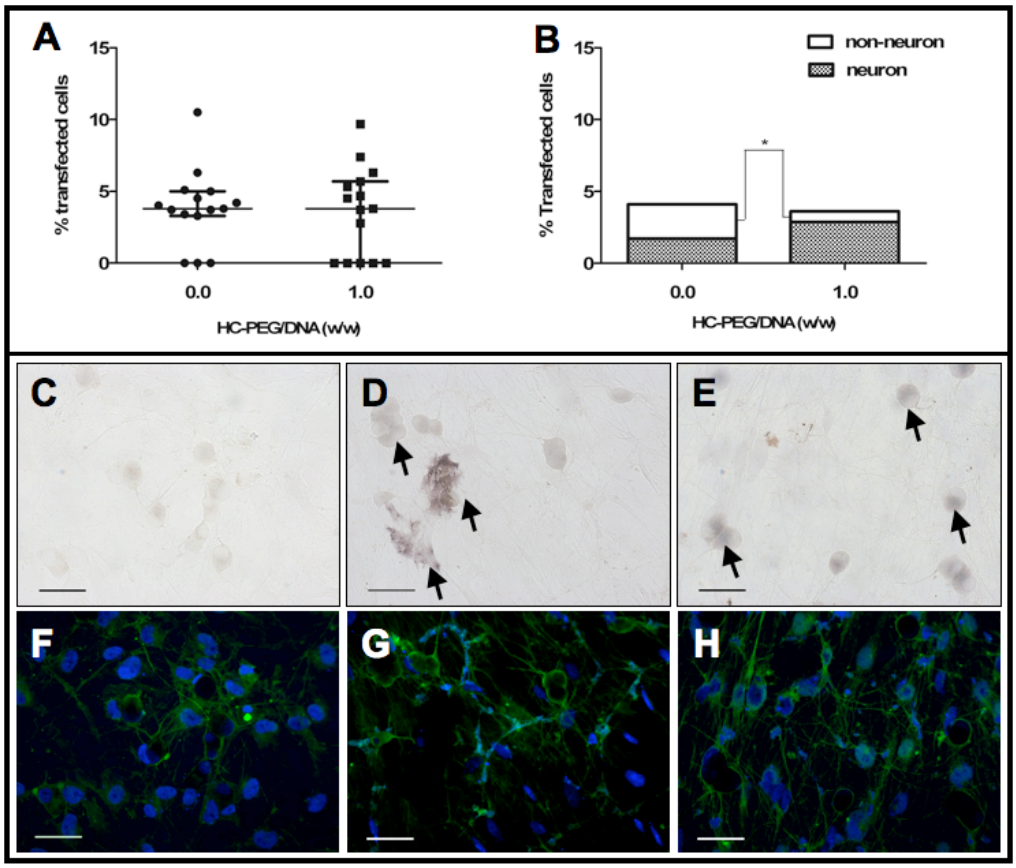


Figure 9 - A) Transfection efficiency of complexes based on CHimiSH and pCMV-GFP, with or without HC-PEG moieties at 72 hrs post-transfection of dissociated dorsal root ganglia cultures (DRGc; median±interquartile range), **B**) classification of DRGc transfected cells as neuron and non-neuron cells (n=4, Aver±SD; * denotes statistically difference between groups, $p<0.05$; representative data of 3 independent experiments). **C, D, E**) staining of DRGc with anti-GFP (DAB/Ni) for untreated, CHimiSH alone and CHimiSH with 1.0 HC-PEG/DNA (w/w), respectively; **F, G, H**) staining of DRGc with N200 and DAPI: untreated, CHimiSH alone and CHimiSH with 1.0 HC-PEG/DNA (w/w), respectively. Arrows indicate transfected cells. Scale bar = 50 μm.

Additionally, no toxicity was found in terms of differences in cell metabolic activity (Fig.S6, SI) for the naked and HC-PEG grafted nanoparticle formulations in relation to untreated cells.

We have previously shown that by using PEI-based HC-PEG grafted nanoparticles targeted to the PNS we could transfect 4.5% of the neurons, on a DRG dissociated culture, in a targeted dependent manner [11]. In this work we were able to transfect 2.9% of a similar neuron population.

Taken together the data from cell lines and primary cultures, we hypothesize that by grafting the HC fragment on the complex surface we are diverting the normal endocytic route from an unspecific to a receptor-mediated one. It has been shown that HC internalization in motor neurons occurs via a clathrin-dependent pathway [28]. Williamson *et al*. have reported that the passage through an acidic endosomal compartment is a pre-requisite for the tetanus toxin entry into the neuronal cytoplasm [29]. Additionally, Bohnert *et al*. reported that vacuolar (H^+) ATPases play a specific role in the early sorting events, which directs the tetanus toxin to axonal carriers, but not in the subsequent progression along the retrograde transport route [30]. These studies highlight the complexity involved in the cell internalization process and subsequent traffic of the tetanus toxin fragment. It remains to be clarified whether these mechanisms are still valid when a cargo is coupled to HC, which is the intracellular pathway followed by the internalized complexes and in what extent the HC-PEG grafting could affect the of CHimi behaviour has a DNA delivery vector. Further studies will be necessary in order to elucidate the route that the developed HC-PEG grafted nanoparticles are undertaking in order to successfully transport DNA towards the cell nucleus.

PEI is considered one of the gold standards of non-viral gene delivery systems [12], however its low biodegradability and harmful systemic effects are a major cause of concern and have been impairing its use in a regenerative application [13,14]. In this study we have used CH as a starting polymer to design a non-toxic and biodegradable gene delivery system. By using the HC fragment targeting potential and taking further advantage of the ability of this fragment to be retrogradely transported, we aim at a minimally invasive administration of the designed vectors. In addition, the HC fragment internalization is not associated with a toxicity effect [5], which enable its use in an regenerative scenario. To our knowledge this is the first report describing a targeted transfection of a specific neuron cell population by using CH as a vector. Future studies will clarify if the reported *in vitro* results are confirmed *in vivo* and if the attained levels of transfection could elicit a regenerative effect.

Conclusions

Numerous obstacles hamper the targeted delivery of genes to neurons. Therefore, innovative solutions in the development of gene delivery systems are of great importance to achieve such unmet medical need. The use of more biocompatible vehicles leads us one step closer to the clinical application. In this study, we envisaged such an approach using CH as the starting material. We developed a

multi-component nanoparticle system that successfully targeted and transfected neuronal cell lines, as well as DRG primary dissociated cultures, at no cost of cell viability. Besides targeting PNS, this versatile system can bring new viewpoints in the clinical approach of peripheral neuropathies.

Acknowledgements

This project was carried out under the Portuguese Foundation for Science and Technology (FCT) contract POCI/SAU-BMA/58170/2004. The authors would like to thank Manuela Brás (INEB) for the FTIR measurements, Patrícia Cardoso (INEB) and Susana Carrilho (IBMC) for the mycoplasma screening of the cell cultures and Raquel Gonçalves (INEB) and Simon Monard (IBMC) for the precious help with flow cytometry. Hugo Oliveira and Liliana R. Pires acknowledge FCT for their PhD scholarships, SFRH/BD/22090/2005 and SFRH/BD/46015/2008, respectively.

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Supplementary Information

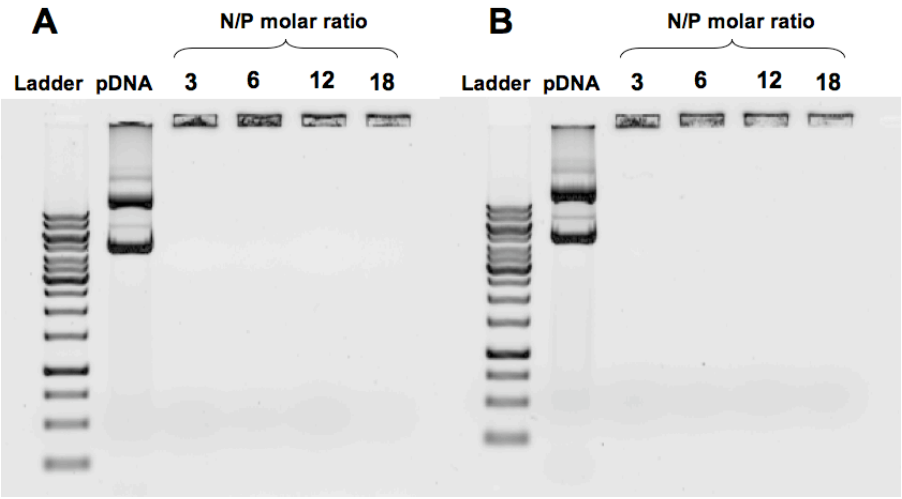

Figure S1 - Agarose gel electrophoresis of complexes of pCMV-GFP and (A) CHimi and (B) CHimiSH. Complexes based on CHimi were prepared at an N/P molar ratio of 3, 6, 12 and 18, while the CHimiSH-based formulations were prepared at the same polymer to plasmid DNA weight ratio as the correspondent CHimi-based complexes. 20 µl of each complex solution or free DNA, together with 4 µl of loading buffer (Fermentas) were loaded in a 1% (w/v) agarose (Cambrex) gel, with 0.05 $\mu g.ml^{-1}$ of ethidium bromide (Q-BioGene). The electrophoresis was run in a 90V field for 45 min, using Tris-Acetate-EDTA buffer (pH 8) as the running buffer.

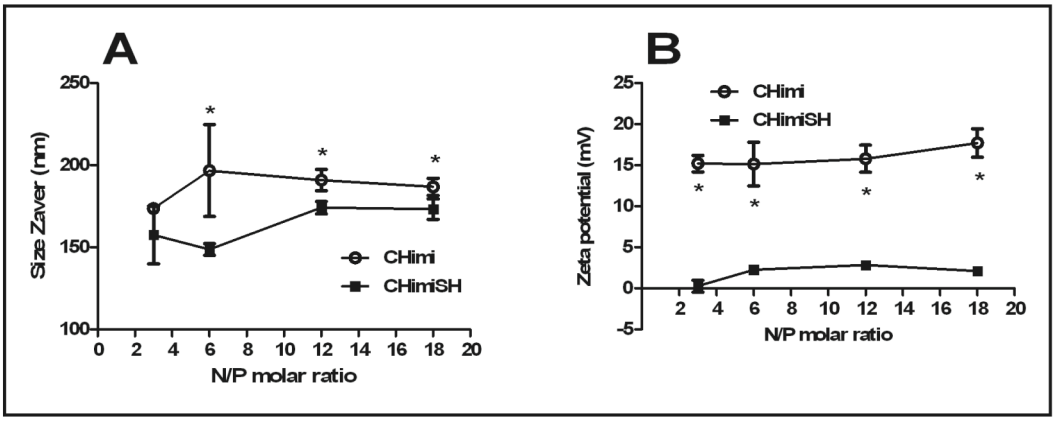

Figure S2 - Characterization of complexes based on CHimi or CHimiSH and pCMV-GFP in terms of (A) size (nm) and (B) zeta potential (mV) (n=3, Aver±SD; * denotes statistically significant difference between CHimi and CHimiSH at the same ratio, $p<0.05$).

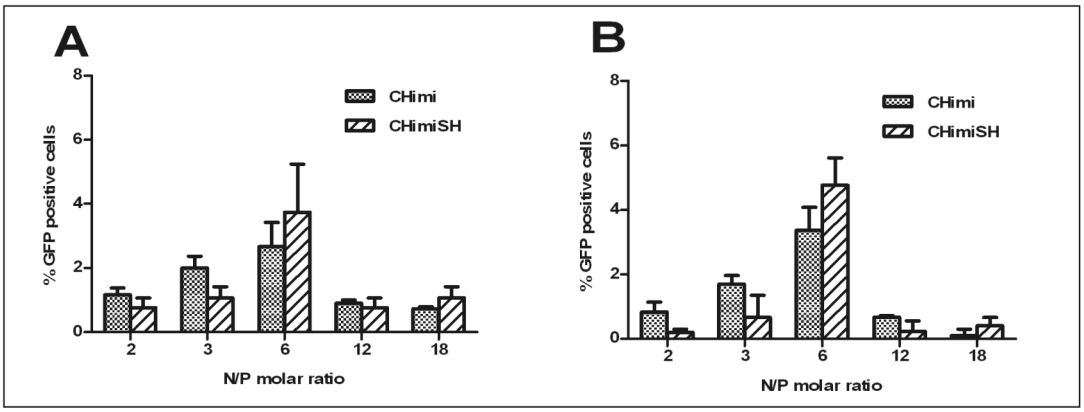

Figure S3 - Transfection efficiency of complexes based on CHimi and CHimiSH and pCMV-GFP at (A) 48 hrs and (B) 72 hrs post-transfection (n=3, Aver±SD; representative data of 3 independent experiments).

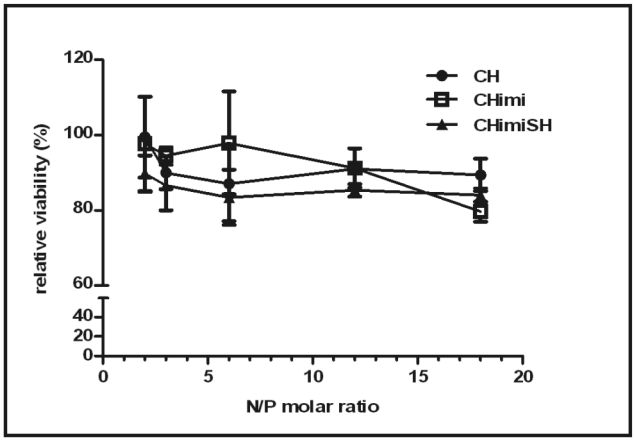

Figure S4 - Relative viability of ND7/23 cells 24 hrs post-transfection with complexes based on CH, CHimi or CHimiSH and pCMV-GFP (n=3, Aver±SD; representative data of 3 independent experiments).

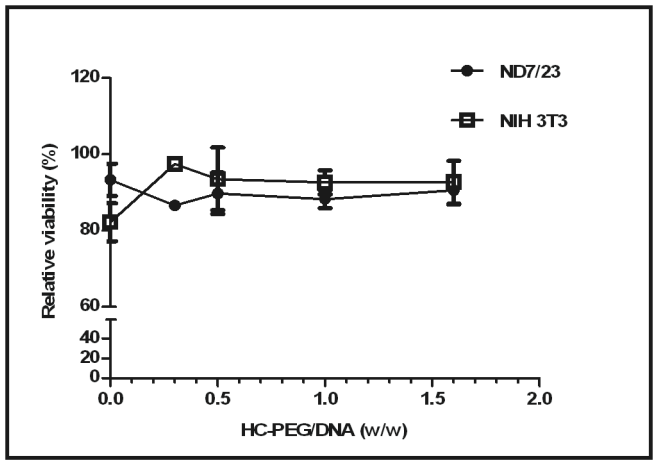

Figure S5 - Relative viability of ND7/23 and NIH 3T3 cells 24 hrs post-transfection with HC-PEG grafted nanoparticles based on CHimiSH and pCMV-GFP with increasing amounts of HC-PEG (n=3, Aver±SD; representative data of 3 independent experiments).

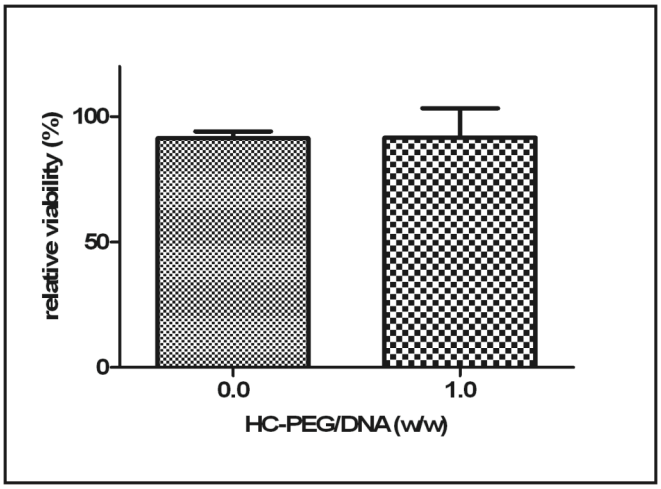

Figure S6 - Relative viability of dissociated dorsal root ganglia cultures (DRGc) 24 hrs post-transfection of complexes based on CHimiSH and pCMV-GFP, with or without HC-PEG moieties (n=4, Aver±SD; representative data of 3 independent experiments).

Chapter VII

Preliminary *in vivo* evaluation of peripheral nervous system targeted poly(ethylene imine) based non-viral vectors

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Abstract

The peripheral nervous system (PNS) may be subject to damage by a wide range of injuries. The modulation of gene expression can bring new answers to address peripheral neuropathies. Here we report the use of a poli(ethylene imine) (PEI)-based system grafted with a non-toxic fragment from the tetanus toxin (HC), which has been previously shown to interact specifically with peripheral neurons and to undergo retrograde transport. Previously we have shown that these nanoparticles could target primary sensorial neurons *in vitro*. In this study we aim to test if this system could be used in an *in vivo* scenario. Subcutaneous peripheral administration of the developed vectors in a rat model led to the transgene expression in the lumbar dorsal root ganglia (DRG) innervating the injection site, with negligible expression in other tissues, in contrast with naked DNA. Five days post-injection with the PEI-based nanoparticles, the reporter protein expression was detected and transfection efficiency estimated. The % of GFP positive DRG neuron cells ranged from 56.3 to 64.1%, with the apparent lack of harmful effects.

Introduction

A variety of neuropathies affecting the peripheral nervous system (PNS) still await efficient treatment strategies, as conventional therapies have primarily been palliative rather than curative. Growing interest has been drawn into gene therapy, which relies on the introduction of transgenes encoding proteins that can augment the natural survival and repair processes of the nervous system, such as neurotransmitters, neurotrophic factors and their receptors, cytokines, neuronal-survival (anti-apoptotic) agents, among others [1]. Such therapeutic molecules, if expressed in sufficient amounts, can arrest or delay the degeneration process and/or promote the regeneration of neuronal cells [2].

A non-systemic administration of a drug to sensory peripheral neurons can be achieved by the injection to the intrathecal space or directly to the dorsal root ganglia (DRG) [3, 4]. However, that would implicate an invasive procedure. Hence, the subcutaneous injection of a system that could target sensorial neurons and undergo retrograde transport can be seen as beneficial regarding a therapeutic application. Viral vectors have been widely used in order to achieve transgene expression in peripheral neurons (e.g. herpes simplex virus and adenovirus) due to their ability to infect neurons *in vitro* and *in vivo*, and undergo retrograde transport [5-8]. On the other hand, the original pathologic and latent infectious nature of these viruses and the inability to obtain sustained expression can limit their therapeutic applications [9], which has drawn increasing interest to non-viral vectors. A successful strategy has been the use of cationized gelatin-based DNA containing nanoparticles, which have lead to the transgene expression in the DRG neurons as early as 60 hours post-transfection upon subcutaneously injection in the rat footpad [10].

Recently, we have developed poly(ethylene imine) (PEI)-based vectors targeted to the PNS [11] by means of grafting PEI-DNA nanoparticles with the non-toxic carboxylic terminal fragment from tetanus toxin (HC). This fragment undergoes cell-specific internalization and neuronal retrograde transport [12, 13], and previous *in vivo* studies have shown that systemic administration of enzyme-HC conjugates resulted in their delivery to the brain stem, motor neurons of the spinal cord and to a lesser extent the DRG [14-16]. When functionalized with the HC-PEG moieties the developed PEI-based nanoparticles showed to be able to transfect in a targeted fashion neurons in primary cultures of dissociated DRG [11]. Thus, expectedely allowing the possibility of delivery of these nanoparticles to a peripheral neuron cell population by a minimally invasive method in a cell targeted fashion. In this study we aimed to determine if the previously optimized PEI-based system was able to target

and access PNS sensorial neurons in a rat model, after a minimal invasive and peripheral administration in the animal footpad. Here we focused primarily in the determination of the biodistribution profile of the developed nanoparticles, in relation with naked DNA, by assessing the transgene expression in a wide range of tissues as a function of time. By means of immunohistochemistry we further investigated if the transgene protein expression was achieved in a targeted fashion in the DRG neurons that innervate the zone of administration.

Materials and Methods

Polymer

Branched PEI (25 kDa, Sigma) was thiolated with 2-iminothiolane (Sigma) and purified as previously described [11]. The thiolated PEI (PEISH) was dissolved at 1 $mg.ml^{-1}$ in a 5% (w/v) glucose solution (pH 7.4) and stored at -80 ºC until further use.

Plasmid DNA

The plasmid DNA used encoded for the green fluorescent protein (GFP) and the β-Galactosidase (β-Gal; pVIVO1-GFP-LacZ, 8.4 kb, Invivogen). Plasmid was produced in a DH5α competent *E. coli* strain transformed with the respective plasmid. Subsequently, DNA purification was performed using an endotoxin-free Maxiprep kit following the manufacture's instruction (GenElute, Sigma).

Tetanus toxin production and modification

The non-toxic fragment of the tetanus toxin (HC) was produced recombinantly using the BL21 *E. coli* strain. The plasmid encoding for the HC fragment was a kind offer from Prof. Neil Fairweather (King's College, UK). The HC production in the BL21 *E. coli* strain and purification was performed as previously described [17]. The obtained HC fragment was additionally covalently linked to a bi-functional poly(ethylene glycol) (PEG) spacer. Briefly, a bifunctional 5 kDa PEG (JenkemUSA, China) bearing an N-hydroxysuccinimide (NHS) and a maleimide (MAL) end group was used as indicated by the manufacturer, at a 2.5 PEG/HC protein molar ratio [11].

Ternary complex formation

DNA-polymer complexes were prepared as described elsewhere [18] by mixing, while vortexing, equal volumes of plasmid DNA and PEISH solution (in 5% (w/v) glucose aqueous solution, pH 7.4). The core complexes were formed using PEISH at an N/P molar ratio of 3 and let to stabilize for 15 min. Subsequently, at a final

concentration of 7.5 µg per 2 µg of plasmid DNA, HC-PEG was added to the complex mixture and ternary complexes were let to form for 24 hrs at room temperature. The described formulation was chosen as it has been previously shown to be optimal regarding neuronal cell targeting potential *in vitro* [11]. Prior use, the dispersion of ternary complexes was concentrated to a final plasmid DNA concentration of 500 $\mu g.ml^{-1}$ in 5% (w/v) glucose aqueous solution (pH 7.4) using a 30 kDa cut-off filter (Amicon Ultra, Millipore).

Animal procedure

All the animal procedures were conducted following the European animal care guideline 86/609/CEE according to an approved protocol by the ethics committee of the Portuguese official authority on animal welfare and experimentation (*Direção Geral de Veterinária*). Twenty male 4-month rats, with an average weight of 350-400 g were used. Animals were housed in cages with free access to food and water. Prior nanoparticle/naked DNA administration, animals were anesthetized by intraperitoneal injection of ketamine (75 mg/kg body weight). Nanoparticles were prepared as previously described and 150 µl, corresponding to 75 µg of plasmid DNA, was subcutaneously injected in the left posterior footpad (FP) of the animal using a 25-gauge needle (Hamilton). The following test groups were formed:

A1 (n=14): FP / HC-PEG nanoparticles

A2 (n=6): FP / naked DNA

At 3 and 5 days post-injection animals were sacrificed by a lethal intraperitoneal injection of 20% (v/v) sodium pentobarbital. In the **A1** group and per time point the tissue samples of 5 animals were processed for gene expression analysis (RT-PCR and Real time-PCR) and of 2 animals animals processed for immunohistochemistry (IHC) using an anti-GFP antibody. In the **A2** group 2 and 1 animals were used, respectively. Tissues were collected and snap frozen or fixed in 4% (v/v) buffered formaline, for ribonucleic acid (RNA) extraction or histology, respectively. The following tissues were collected: posterior footpads (ipsilateral and contralateral to the site of injection), left and right DRGs (L4, L5), ventral roots (L1 to L4), left and right sciatic nerves, brain, kidney, heart, lungs, spleen, liver, lymphatic nodes that drain the back limb region (i.e. popliteal and inguinal) and blood.

RNA isolation and real-time Reverse Trancriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from designated tissues using the PureLink RNA Mini kit (Invitrogen). Reverse transcription was obtained from 500 ng of total RNA employing

the Superscript First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions. For real-time quantification of mRNA levels, the synthesized cDNAs were amplified in duplicate in an iCycler iQ5 (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). At the end of the PCR cycling, correspondent melting curves were performed in order to ascertain the amplification of a single product and the absence of primer dimmer formation. The primers used are listed in table 1.

Table 1- Sequences of primers used for quantitative real-time RT-PCR analysis, of green fluorescent protein (GFP) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GFP	CTGTCCAACTGGCAGACCATTACC	TTCCTGCTGCTGTCACAAACTCC
GAPDH	TTCACACCCATCACAAACAT	GCCATCAACGACCCCTTCAT

Results were normalized to GAPDH as endogenous control, using the primers indicated in table 1. Relative expression levels (RE) were determined as follows:

$$RE = 2^{-((C_{T,sample} - C_{T,GAPDH})_x - (C_{T,sample} - C_{T,GAPDH})_{control})} \quad \text{equation 1,}$$

where C_T stands for cycle threshold and in this case sample is GFP expression.

Histology

Following fixation, the tissues were processed and embedded in paraffin. Four µm thick sections were sequentially recovered, de-waxed in xylene and rehydrated in a decreasing gradient series of ethanol, followed by water. Sections were prepared for standard haematoxylin and eosin staining.

Immunohistochemistry

Deparaffinized and rehydrated sections were incubated with 3% (v/v) hydrogen peroxide in methanol for 20 min. After washing with phosphate buffered saline (PBS), these were incubated overnight at 4ºC with rabbit anti-GFP (1:500, A11122, Invitrogen). After washing with PBS the slides were incubated for 30 min at room temperature with anti-rabbit peroxidase polymer-labelled (Dako) as indicated by the manufacturer. After washing with PBS the GFP detection was revealed with diaminobenzidine (DAB, Vectorlabs. After extensive washing in water, the tissues

were counterstained with haematoxylin, dehydrated and mounted in Histofluid (Sofdan).

Results and Discussion

In order to assess the ability of the developed ternary complexes to efficiently access the PNS in an *in vivo* scenario, their minimally invasive peripheral administration was tested. As the HC fragment has been shown to target peripheral neurons and to undergo retrograde transport after peripheral administration [14-16], we expect that following the same administration the developed nanoparticles will be able to access neuronal cells. As seen in Figure 1, we hypothesize that after footpad injection, the nanoparticles would contact the sensorial terminals and be internalized in a cell specific fashion, be transported to the DRGs where reside the nucleus of the neurons that innervate the injection site, and transfection would result in the local expression of the introduced transgene.

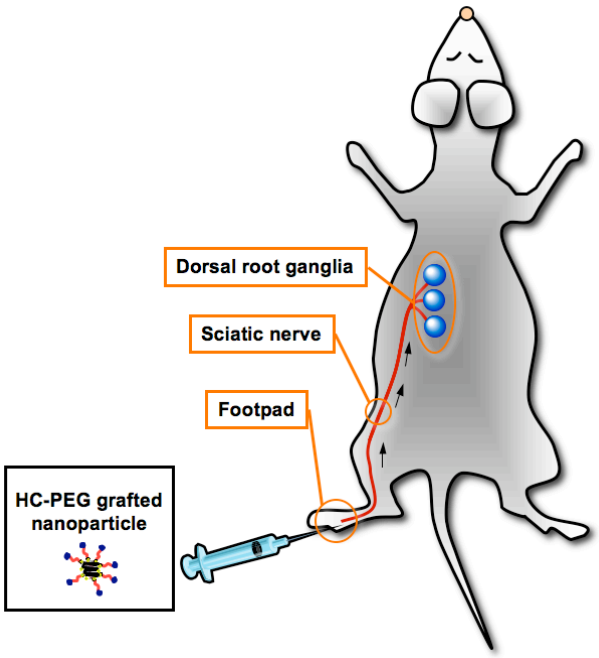

Figure 1- Schematic representation of the proposed administration of the developed targeted gene delivery nanoparticles by a minimally invasive peripheral subcutaneous injection in the posterior footpad.

In the first hours post-injection mild inflammation in the site of injection was observed, characterized by a moderate swelling of the footpad. However, 24 hrs post-injection the rat footpad regained the normal appearance and the animal demonstrated a normal use of the limb. Moreover, haematoxylin and eosin staining of footpad tissue

did not show significant infiltration of inflammatory cells, for both groups tested, in relation to control footpads (see Figure 2 for an example at day 5).

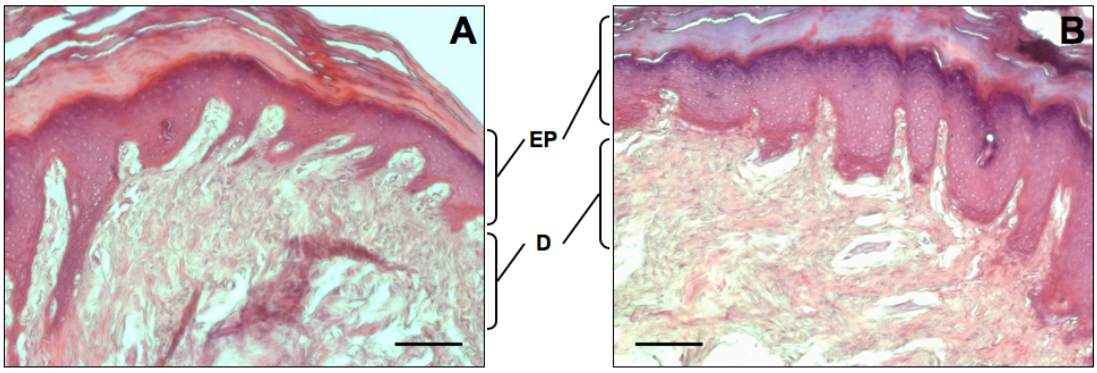

Figure 2- Haematoxylin/ eosin staining of rat footpad at day 5, injected with DNA alone (A) or complexes based on HC-PEG grafted PEISH (B) (EP - epidermis and D-dermis). No cell infiltration or apparent inflammatory response is observed (scale=100 µm).

Additionally, rat weight variation was followed during the study with no significant weight reduction being detected. Altogether the presented results indicate that the developed system does not induce a relevant harmful effect at the site of injection under the tested conditions.

In order to evaluate if the nanoparticles were indeed able to target and transfect the aimed neuronal cell population - the two main DRGs innervating the correspondent injected footpad (i.e. DRG lumbar 4 and 5) - the biodistribution of the injected material was assessed by evaluating the transgene expression in a number of tissues by means of Q-PCR. The relative quantification of the transgene expression (GFP) was performed in the following tissues (from the ispilateral side of injection, when applied): DRGs L4 and L5, sciatic nerve, ventral root (VR), brain, spleen, liver, kidney, heart, lung and the lymph nodes draining the footpad (i.e. the popliteal and the inguinal lymph nodes). Following previous studies regarding the peripheral administration (i.e. posterior rat footpad) of cationized gelatine-based nanoparticles [10], which reported transgene expression from 60 hrs to 6 days, we chose as evaluation time-points, 3 and 5 days post-injection. As control, animals were injected with the same amount of naked DNA. The GFP gene expression levels were normalized to GAPDH gene expression levels, at the respective sample, and expressed as relative to GFP gene expression in the DRG tissues, at day 5 (indicated by an arrow), as the DRGs were the target tissue in this study.

In the case of naked DNA test group, 3 days post injection GFP gene expression was only detected in the footpad (Figure 3). At day 5 the transgene expression was detected again in the posterior footpad and additionally in the left sciatic nerve, liver, heart, lung and blood. In the HC-PEG grafted nanoparticles test group at day 3, GFP relative expression was found in the footpad for all animals, and only in 1 animal was detected expression of GFP, in the lung and in the lymph nodes (Figure 3). Noteworthy, the positive tissues were from different animals. For this test group at day 5 the expression of the reporter gene was observed in the DRG (in 3 out of 5 animals) and in the lymph nodes and blood (1 out of 5; in non-related animals, Figure 3).

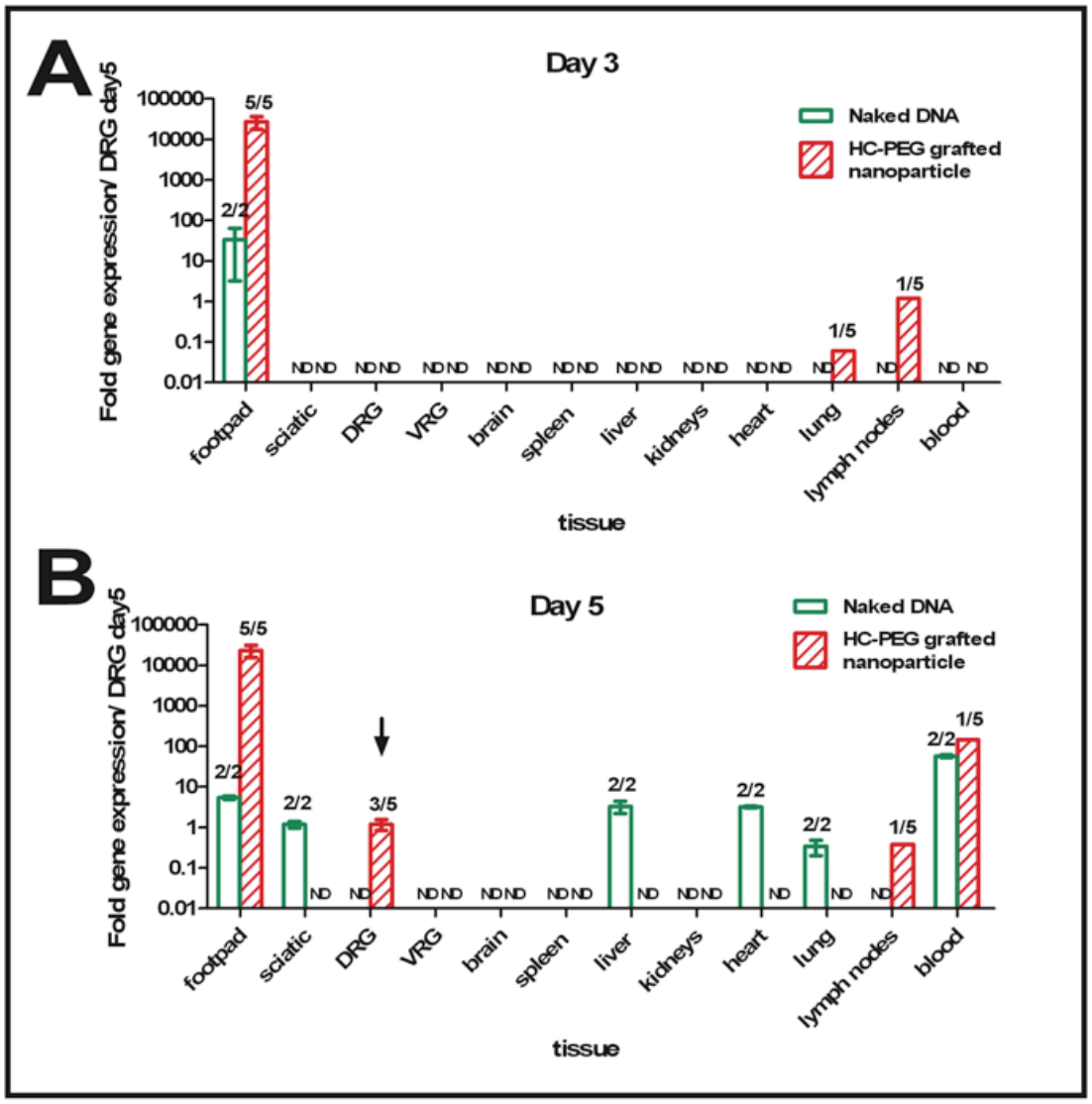

Figure 3- Green fluorescent protein (GFP) gene expression normalized by GAPDH gene expression levels, relative to GFP expression in the dorsal root ganglia (DRG) at day 5 (arrow). The following tissues were analysed: footpad, sciatic nerve (from the ipsilatera side of injection), L4 and L5 DRGs (from the ipsilateral side of injection), ventral root (VR), spleen, liver, kidney, heart, lymph nodes (popliteal and inguinal) and whole blood. ND denotes “non detected”; the number of tissues where GFP gene expression was positive in relation to the total of animals tested is indicated over the columns (i.e. 1/5 indicates that 1 analized tissue was found positive for GFP expression out of the total number of animals analyzed at the correspondent time point; Aver±SD).

The overall GFP expression distribution shows that the PEI-based system grafted with the HC fragment was able to spatially limit the transgene expression, in relation to naked DNA. The transgene expression in DRG was only observed at 5 days post-

injection, suggesting that nanoparticle access to the target cell nucleus and the subsequent efficient expression of the transgene occurred between 3 and 5 days post-administration. It is described that for large particles (>30 nm) passive diffusion cannot account for the distance that nanoparticles have to travel inside cells [19]. The HC-PEG grafted nanoparticles are in the range of 60 nm [11], one can then assume that active transport has to occur in order to efficient transfection to take place. Indeed, several viruses have developed features that enable them to take advantage of the retrograde neuronal transport in their journey towards cell nucleus [20, 21]. This faster active transport has been shown to operate at the rate of 100 to 250 mm per 24 hrs for endosomal vesicles, lysosomes and herpes simplex viruses, along spinal and sciatic nerves [22, 23]. Our data fits the rate of fast retrograde transport. Neverthless, further studies are necessary in order to clarify this hypothesis.

To determine if the transgene expression was resulting in protein expression in the target cells an immunohistochemistry for GFP in the DRGs was performed. Indeed, and by observing Figure 4 (A and C), one could see DRG neurons where immunoreactivity for GFP was detected (brown staining). When a similar treatment was applied to the contralateral DRG no specific staining was observed (Figure 4, B and D).

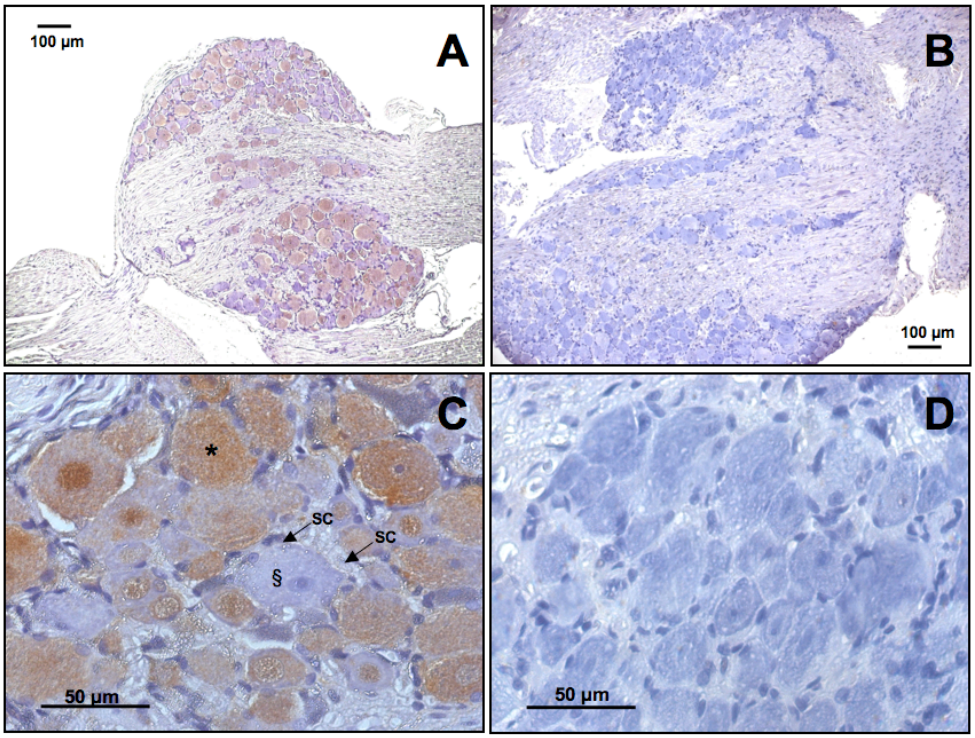

Figure 4- Immunohistochemistry of dorsal root ganglia (DRG) for the identification of green fluorescent protein (GFP) positive cells. Diaminobenzidine (DAB) positive neuron cells (brown staining, see * as example; also see § for negative cell) indicates positivity for GFP, as observed for DRG injected with complexes based on HC-PEG grafted PEISH at 5 days post-injection (A and C, corresponding the latter to a higher magnification of an area of A). Arrows indicates satellite cells (SC). Co-lateral DRG of the same animal was used as control (B and D, corresponding the latter to a higher magnification of an area of C).

The DRG innervating the site of injection (anterior footpad) are from the lumbar segment 3 to 6 (L3-6), although most of all sciatic DRG perikarya reside in the L4 and L5 DRGs [24]. Therefore, in order to determine the efficiency of the developed nanoparticles to transduce the target cells, the percentage of GFP positive neurons from the L4 and 5 DRGs was performed (Figure 5).

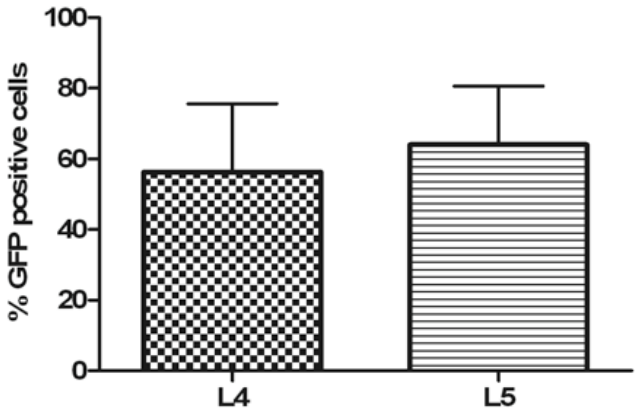

Figure 5- Transfection efficiency of lumbar dorsal root ganglia (DRG) neurons mediated by complexes based on HC-PEG grafted PEISH at N/P=3, at 5 days post-injection. The immunohistochemistry was performed in slides from every 50 µm throughout the sample of both lumbar DRG 4 and 5 (L4 and L5, respectively; Aver±SD).

The % of GFP positive DRG neuron cells was 56.3 and 64.1%, for L4 and 5, respectively, as observed in figure 5. In a previous study regarding the use of cationized gelatin-based nanoparticle system, where authors followed the same administration mode, levels of DRG neuron transfection were of 46.9 and 47.5%, for L4 and 5 DRGs respectively, at 6 days post-injection [10]. Moreover, in the described study transgene expression was detected along the sciatic nerve at 60 hrs post-injection what may indicate that the cationized gelatin-based vectors are transfecting other cells troughout the route towards the sensorial neuron nucleus. The results obtained with the present developed system show improved transfection efficiency for DRG neurons, in relation with the previous study, with no expression of the transgene in the sciatic nerve and with the apparent lack of local inflammatory reaction or toxic effects.

Retrograde transport of naked PEI- or cationized gelatine-based vectors, following uptake into neuronal cells, has been previously described [10, 25, 26]. Following that premisse, we are currently assessing the distribution profile of the transgene mediated by PEI-based complexes, lacking the HC-PEG moieties, as well as their ability to access and efficiently transfect DRG neurons. As the nanoparticle system described in this study was designed for targeted transfection of peripheral neuron cells, these studies will clarify the ability of the present vectors to deliver genes specifically to the aimed cells.

Future studies should address extended evaluation periods of transfection, giving

new hints on the optimal transfection profile of the developed system.

The features presented by the developed nanoparticles can render this type of delivery systems as a promising tool for *in vivo* gene therapy applications in the treatment of peripheral neuropathies.

Acknowledgements

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Chapter VIII

Concluding remarks and future perspectives

The main objective of this thesis was the development of a safe and effective biomaterial-based delivery system of therapeutic genes to promote neuroregeneration in the peripheral nervous system (PNS).

The PNS may be subjected to a variety of injuries and, depending on its extent, nerve damage can have serious and unrecoverable consequences to the patient with total loss of functionality occurring in the more extreme cases. Conventional treatments for peripheral neuropathies have primarily been palliative rather than curative and, often, ineffective [1]. Indeed, one of the challenges currently facing neuroscientists is the development of effective therapies based on the advances achieved in basic research. The use of genes as pro-drugs can be faced as strategy in order to improve neuronal treatments. The principle of gene therapy, which relies on the use of genetic material as a pro-drug that can lead to the production of therapeutic proteins or to modulate gene expression within specific cells, recently got much attention.

Viruses have proved to be the most efficient vectors to mediate gene delivery and expression. However, due to the drawbacks presented by the generality of viral vectors, which include pathogenicity, mutagenesis potential, toxicity and ethical concerns [2], nowadays a crescent interest is focused on the development of non-viral vectors.

Poly(ethylene imine) (PEI) has became one of the gold standards of polymer-based non-viral delivery systems, mainly due to its high transfection efficiency, even in neurononal cells [3]. This has been attributed to the “proton sponge” effect in which unprotonated amino moieties of the polymer buffer the pH inside the endocytic vesicles, leading to endosome disruption and increased complex escape [4]. In **Chapter III**, a simple and efficient system, using PEI as starting material, that could specifically transfect peripheral sensorial neurons and bring new answers to address peripheral neuropathies is described. A binary DNA/polymer complex based on thiolated PEI (PEISH) was optimized, considering complex size and zeta potential and the ability to transfect a sensorial neuron cell line (ND7/23). The 50-kDa non-toxic fragment from tetanus toxin (HC), which has been shown to interact specifically with peripheral neurons and to undergo retrograde transport [5], was grafted to the complex core via a bi-functional PEG (HC-PEG) reactive for the thiol moieties present in the complex surface. Several formulations of HC-PEG grafted nanoparticles were tested for targeting, by assessing the extent of cellular

internalization and levels of transfection, in both the ND7/23 and NIH 3T3 (fibroblast) cell lines. The vector-targeting efficacy was found to be dependent on the HC fragment amount available in the surface of the vector, in a tightly tuned fashion for optimal targeted transfection. Moreover, our results demonstrate that the developed ternary vectors were able to preferentially transfect neurons in primary cultures of dissociated dorsal root ganglia and elicit the expression of the brain derived neurotrophic factor (BDNF) - a therapeutic relevant neurotrophic factor [6].

As a result of the work described in **Chapter III**, it was established that the density of the targeting moieties in the developed PEI-based nanoparticle gene delivery system plays an important role in its targeted vectoring performance. So taking as a model the system developed in **Chapter III**, in **Chapter IV** we proposed molecular recognition force spectroscopy, using atomic force microscopy (AFM), as a tool to optimize the targeting moieties density of a nanoparticle towards achieving cell-specific interaction. By tailoring the nanoparticle formulation, one could show that the transfection outcome could be directly correlated with the unbinding event probability of HC functionalized nanoparticles to ND7/23 cells. The optimal ligand density allowing maximal cell-specific interaction is a critical issue in the design of targeted systems, therefore new tools are welcome in order to assist in their efficient design.

In view of a clinical application the use of more biocompatible vehicles will lead us one step closer to real application. Such an example is chitosan (CH). CH is a natural cationic polymer that, due to its low cytotoxicity, has been emerging as an alternative non-viral gene delivery system, despite showing less efficiency than PEI. In order to mimic the hypothesized mechanism of action of PEI and improve CH based vectors transfection efficiency, the grafting of imidazole grafting into CH backbone (CHimi) was performed (**Chapter VI**). We showed that CHimi promoted higher transfection efficiency than unmodified CH in two different cell line models - 293T and HEPG2 cell lines, human embryonic kidney and human liver carcinoma cells, respectively - via the improvement of its buffering capacity, which is hypothesized to promote the endosomal escaping ability of the CHimi-based complexes. Indeed, the incubation of a cell culture with bafilomycin A1 (a proton pump specific inhibitor) shown to inhibit transfection with CHimi:DNA complexes. Demonstrating that by increasing the buffering capacity of CH we could favour the escape of the complexes from the endosomes, increasing the amount of transgene that can reach the cell nucleus.

Using CHimi as a starting material and following the lessons learned in **Chapter III**, we went further and developed a CHimi-based neuron targeted gene delivery system (**Chapter VII**). The introduction of imidazole moieties in the CH backbone showed also to enhance reporter protein expression in neuronal cells. Again, the formation of a ternary complex was achieved by the linkage of the tetanus toxin fragment, via a bifunctional 5 kDa PEG, taking advantage of a thiol modification introduced in the CH backbone. Both internalization and transfection efficiency of the developed ternary vectors was assessed in view of achieving neuronal targeting. Ultimately we succeed in developing a biodegradable neuron targeted system, using CH as base material, able to transfect neuronal cell lines, as well as neurons in primary cultures of dissociated dorsal root ganglia in a specific manner at no cost of cell viability.

The ultimate evaluation of a targeted delivery system efficiency is its application in an *in vivo* scenario. Following the promising results obtained in **Chapter III** we assessed the optimal PEI based formulation (i.e. 7.5 µg of HC-PEG), following a minimally invasive peripheral administration *in vivo*, in a rat model (**Chapter VII**). The subcutaneous peripheral administration, in the rat posterior footpad, of the developed vectors led to the expression in the lumbar dorsal root ganglia, with minimal expression in other tissues. High levels of DRG neuron transfection efficiency were attained, which together with the apparent lack of harmful effect render this system as promissory for an *in vivo* scenario. Nonetheless, further studies are currently underway, in order to shed more light regarding the targeting potential of the developed nanoparticles system. The evaluation regarding biodistribution of naked nanoparticles, lacking the HC moieties, will further clarify the obtained targeted profile of HC grafted nanoparticles.

A non-toxic, targeted, simple and efficient system that can specifically transfect peripheral sensorial neurons can pave the way towards the development of new therapeutics for the treatment of peripheral neuropathies. In this thesis we propose the use of CH as starting material in order to design a safe and targeted gene delivery vector towards the PNS. However, some limitations of CH, regarding solubility and *in vivo* stability have to be addressed in order to potentiate its application in a clinical scenario. One approach could be to explore the chemical modification of CH in order to improve its solubility in physiological conditions and plasmid DNA complexing ability.

While the use of the HC fragment has been used as a targeting moiety in the nanoparticles, as well as a strategy to design vectors that could be retrogradly transported in a neuron cell, other targeting moieties could be explored, and regarding the modular nature of the developed system new vectors targeted for neuronal cells can be easily produced. Other candidates could be found in neurotoxins, exploring their natural ability to bypass the cellular barriers and access neuron cells, which can bring diversity to neuronal-targeted transfection systems.

In order to access the transcriptional machinery of the nucleus, plasmid DNA must cross the nuclear membrane. Considering the post-mitotic nature of neurons, the nuclear import of plasmid DNA can be considered as one of the main barriers for efficient gene expression in this cell population. Although some conflict still exists regarding the effectiveness of the use of nuclear localizing sequences (NLS) [7, 8]. The use of these sequences may lead to an improvement on the vector and/or DNA import to the target cell nucleus, and be a leap forward to the improvement of non-viral vectors, specifically towards neuronal cells. Thus the evaluation regarding the inclusion of NLS moieties in the developed vectors could be beneficial regarding transfection efficiency improvement.

The *in vivo* assessment of CH-based neuronal-targeted system is underway, following a minimal invasive administration, in a parallel study to the one described in **Chapter VII**. The obtained results will lead the way to new understanding regarding the application of degradable non-viral gene delivery vectors towards regeneration of the PNS. Foremost, the application of these targeted nanoparticle systems in a regeneration in vivo model can lead us closer to a real regenerative application.

The fact that the developed CH based vectors are biodegradable, non-toxic, mediate efficient gene transfer into mammalian neurons, and exhibit adequate properties for *in vivo* administration render these type of complexes as very promising tools for gene therapy based strategies to promote neuroregeneration. However, the proposed system, based on the design of modular structures and therefore more versatile ones, can be easily adapted to be targeted to different cell types and demands, broadening the scope of their possible application in regenerative medicine.

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