JOANA SILVA JORGE BELO MORAIS

ENHANCEMENT OF NON-VIRAL TRANSFECTION EFFICIENCY WITH NUCLEAR LOCALIZATION SIGNAL PEPTIDES

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Mestrado em Ciências Biomédicas

Trabalho efetuado sob a orientação de:

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Agradecimentos

Queria agradecer a todos aqueles que de alguma maneira contribuíram para a realização deste estudo.

À Dra. Ana Vanessa Oliveira, pela forma como orientou este estudo, por tudo o que me ensinou, pela atenção despendida, pelas suas críticas e sugestões, e principalmente por ter esticado a sua paciência até ao limite ☺.

À Prof. Gabriela Silva, pela sua acessibilidade, por ter sempre a palavra certa no momento certo, por ter disponibilizado tudo o que podia para a realização deste estudo.

À Prof. Ana Costa, queria agradecer não só pela forma espectacular como explica química orgânica mas também pela ajuda que me deu neste estudo e por todas as horas e esclarecimentos com muita paciência que me dedicou.

À equipa do laboratório, pelas respostas, conselhos e explicações que me ajudaram bastante em momentos de pânico!

Às meninas do Lab. 2.22, que fizeram o tempo no zetasizer passar muito mais rápido, à Ritinha do 3.30 e à Catarina que tornaram os tempos entre as culturas de células muito mais divertidos e à Lara do 3.31 por todas as idas para o ISEL estudar horas a fio e por me ouvires a reclamar xD

À minha família, sempre presente ao longo de toda a minha vida académica, Pais, obrigada por tudo…Maninha obrigada por me ajudares na formatação da tese se não fossem os teus conselhos ela teria ficado visualmente pouco apetitosa xD Maninho e Andreia obrigado pelos grandes momentos "libertadores de stress" ☺.

Ao meu namorado, João Fradovski, obrigada pelo apoio, pela motivação para continuar, pelo positivismo e principalmente por aturares o meu péssimo humor depois de dias longos de estudo!

Aos meus amigos, Tatxe, Moss e Alexia, as minhas cocós do coração, Kalhau obrigada por tudo desde sempre, és o meu grande pilar! Sarita obrigada pelos treinos no ginásio e os piqueniques no Continente, ajudaste-me a continuar sempre! Vanessa e Olesea as minhas babes obrigada do fundo do meu coração, Isa quando a gente se vir pode ser que já seja mestre :D, Mónica, em Lisboa ou em Cabo Verde contribuíste com muito apoio ☺, e Pipas e Vânia, obrigada pelo apoio e pelos conselhos.

The end!!!!!

Resumo

A terapia génica envolve a transferência de material genético terapêutico para células alvo, pela introdução de genes funcionais que substituam ou complementem aqueles que se encontram defeituosos, com o objectivo de tratar ou prevenir uma ampla gama de doenças, hereditárias ou adquiridas. No entanto, para o seu sucesso é necessário um sistema de entrega de material genético eficiente, capaz de proteger o DNA da degradação por nucleases e com o mínimo de toxicidade e imunogeneicidade que permita uma expressão genética estável e duradoura. Durante os últimos anos, têm sido desenvolvidos uma ampla gama de vectores, que têm sido divididos e caracterizados como vectores virais e não virais. Os vectores virais apresentam maior eficiência na transferência de material genético, tanto *in vitro* como *in vivo*, no entanto apresentam algumas limitações como a reduzida capacidade de empacotamento genético e o facto de conduzirem a respostas inflamatórias/imunológicas indesejáveis que consequentemente limitam administrações subsequentes. Por sua vez, os vectores não virais apresentam algumas vantagens sobre os vectores virais, nomeadamente, um perfil imunológico mais seguro, uma produção mais fácil, uma maior capacidade de empacotamento genético e a sua reduzida toxicidade. Contudo, o uso de vectores não virais é limitado devido às suas eficiências de transfecção relativamente baixas, marcadas pela baixa translocação nuclear, e consequentemente reduzida expressão genética. Vários esforços têm sido realizados no sentido de ultrapassar a barreira nuclear, um dos maiores passos limitantes no desenvolvimento de sistemas de entrega genética não virais eficazes. Uma das estratégias passa pela incorporação de sinais de localização nuclear em complexos poliméricos, uma vez que estes péptidos catiónicos ao serem reconhecidos pelas importinas permitem um transporte genético eficaz para o núcleo através dos complexos de poros nucleares, aumentando assim a entrega nuclear do DNA, e por consequente, a sua expressão genética.

Neste contexto, o objectivo deste trabalho foi a caracterização e optimização de vectores não virais, baseados em polímeros como o quitosano e o ácido hialurónico, que foram escolhidos devido às suas notórias propriedades de biocompatibilidade, biodegradação e ausência de toxicidade. Péptidos baseados em sinais de localização nuclear endógenos, pertencentes á família dos IGFBP, derivados nomeadamente do IGFBP-3 e IGFBP-5, foram avaliados com o intuito de melhorar a translocação nuclear sem comprometer o perfil imunológico bastante baixo dos vectores não-virais. Várias

estratégias foram testadas para avaliar a eficiência de transfecção e a expressão genética mediada por poliplexos de quitosano e ácido hialurónico em células HEK293T: nomeadamente a co-administração, a co-complexação e a ligação covalente dos péptidos IGFBP aos poliplexos de quitosano.

Os nossos resultados mostraram que as nossas formulações, com ou sem sulfato de sódio, péptidos IGFBP ou ácido hialurónico, e independentemente da estratégia usada, originaram poliplexos com um intervalo de tamanhos entre 250 nm e 750 nm e carga de superfície positiva, caracterizados através de medições no Zetasizer. Os nossos poliplexos foram ainda capazes de complexar o DNA de forma eficaz, conforme analisado através de ensaios de electroforese em gel de agarose.

Posteriormente à caracterização dos poliplexos, os péptidos IGFBP foram ainda avaliados quanto á sua citotoxicidade em dois períodos de incubação, 24 horas e 72 horas. Os ensaios de viabilidade celular não mostraram qualquer citotoxicidade para ambos os péptidos IGFBP para as várias concentrações testadas nos dois períodos de tempo testados.

Após estes resultados, e uma vez que os poliplexos apresentaram características desejáveis quanto ao seu tamanho, carga de superfície e complexação eficiente do DNA, estes foram avaliados quanto à sua eficácia através de ensaios de transfecção *in vitro*, analisados posteriormente por microscopia de fluorescência e citometria de fluxo.

A eficiência de transfecção revelou-se ser dependente da concentração dos péptidos IGFBP e variar consoante o método de entrega utilizado. Nos ensaios de transfecção, utilizando o método de co-administração dos poliplexos com os péptidos IGFBP, nenhum aumento na eficiência de transfecção foi observado. Nos métodos em que estratégias como a co-complexação e a ligação covalente dos péptidos IGFBP aos poliplexos de quitosano foram usadas, um aumento significativo da eficiência de transfecção foi conseguido, no entanto, apenas para os poliplexos associados ao IGFBP-3. Uma possível explicação para estes resultados é o facto de as acessibilidades e/ou afinidades para com as subunidades das importinas diferirem entre os péptidos IGFBP-3 e IGFBP-5, o que consequentemente, poderá levar a níveis de translocação diferentes entre os péptidos, no entanto, ainda não é claro qual o mecanismo.

Foi ainda possível verificar que combinando polímeros como o quitosano e o ácido hialurónico, que os poliplexos resultantes renderam um aumento significativo da eficiência de transfecção para ambos os péptidos, IGFBP-3 e IGFBP-5, o que poderá ser

explicado por uma possível modificação no enrolamento das cadeias do quitosano aquando da adição do ácido hialurónico.

Na sua globalidade os resultados obtidos demonstraram que, apesar de ser ainda necessário uma optimização da eficiência de transfecção, poliplexos co-complexados com péptidos IGFBP são de facto bons candidatos a sistemas de entrega genética não virais. Os poliplexos com dois polímeros combinados, quitosano e ácido hialurónico, foram os que revelaram maiores eficiências de transfecção, para ambos os péptidos, IGFBP-3 e IGFBP-5. Futuramente, seria interessante não só expandir a gama de concentrações de péptidos IGFBP testadas como também o tipo de linhas celulares, compreendendo ainda uma optimização das formulações dos poliplexos.

Palavras-Chave: terapia génica, quitosano, ácido hialurónico, sinais de localização nuclear, IGFBP-3, IGFBP-5

Abstract

Gene therapy entails the transfer of therapeutic genetic material into specific cells; however, their success requires an efficient gene delivery system, which allows a stable gene expression. Nuclear import is considered the major limiting step in the development of effective non-viral gene delivery systems; the incorporation of NLS that can mediate nuclear intake can be used as a strategy in order to enhance the internalization of DNA into the nucleus.

In this work, an endogenous NLS peptide, based on IGFBP, namely IGFBP-3 and IGFBP-5, was evaluated in order to ameliorate nuclear translocation without compromising the fairly low immunological profile of non-viral vectors. Several strategies were tested to determine their effect in chitosan and acid hyaluronic polyplex-mediated transfection efficiency in HEK293T cells: co-administration, co-complexation, and covalent ligation to chitosan polyplexes.

Our results show that our vectors are capable of an effective DNA complexation and present size and surface charge appropriated for gene delivery applications.

Transfection efficiency is concentration dependent and varies with the delivery method employed. Co-complexation and covalent ligation of IGFBP peptides to chitosan polyplexes yielded a 2-fold increase in transfection efficiency associated with the use of IGFBP-3 peptides. The incorporation of acid hyaluronic yielded a significant increase in transfection efficiency to both peptides.

Despite of the improvements in transfection efficiency it needs to be further improved, these results indicate that polyplexes co-complexed with IGFBP peptides are good candidates for non-viral gene delivery systems and would be interesting to expand the range of tested IGFBP peptides concentrations as well as the type of cell lines used.

Keywords: gene therapy**,** chitosan, acid hyaluronic, nuclear localization signals, IGFBP-3, IGFBP-5

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Abbreviations list

AIDS	acquired immunodeficiency syndrome
CAS	cellular apoptosis susceptibility protein
CD44	cluster determinant 44
CMV	cytomegalovirus
CS	chitosan
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DOPE	1, 2- dioleoyl-sn-glycero-3-phosphoethanolamine
ECM	extracellular matrix
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
eGFP	enhanced green fluorescence protein
FBS	fetal bovine serum
HA	hyaluronic acid
HARE	hyaluronic acid receptor for endocytosis
IGF	insulin-like growth factor
IGFBP	insulin-like growth factors binding proteins
LYVE-1	lymphatic vessel endothelial hyaluronan receptor-1
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MW	molecular weigh
Na_2SO_4	sodium sulfate
NLS	nuclear localization signal
NP	nanoparticles
NPC	nuclear pore complexes
PAGA	poly[alpha-(4-aminobutyl)-l- glycolic acid]
PAMAM	poly(amidoamine)
PBS	phosphate buffered saline
PdI	polydispersity index
pDMAEMA	poly[2-(dimethylamino)ethyl methacrylate]
PEG	polyethylene glycol
PEI	polyethylenimine

PLL	poly-l-lysine
RanGTP	ras-related nuclear protein guanosine triphosphate
RHAMM	receptor for hyaluronate – mediated motility
RNA	ribonucleic acid
Rpm	revolutions per minute
SV40	simian vacuolating virus 40

1. INTRODUCTION

1.1 Gene therapy

Gene therapy entails the transfer of therapeutic genetic material into specific cells (tissue or organ) of a patient where production of the encoded protein will occur, in order to treat or prevent a disease, altering an existing abnormality by replacement of a missing or defective gene, which influence the disease process (Corsi et al., 2003; Gorecki, 2006; Nayerossadat et al., 2012; Tiera et al., 2006; Yue et al., 2013). Gene therapy has been a promising strategy for the treatment of many genetic and acquired diseases, such as cancer (Bremner et al., 2004), heart failure (Doh, 2015), cystic fibrosis, emphysema, retinitis pigmentosa, vascular diseases, neurodegenerative disorders, inflammatory conditions, acquired immunodeficiency syndrome (AIDS) (Cevher et al., 2012; Nayerossadat et al., 2012), among others. Therefore, their success requires a gene delivery system of minimal toxicity, capable of protecting deoxyribonucleic acid (DNA) from nuclease enzymes until it reaches its target, allowing prolonged and stable gene expression (Machado et al., 2014; Tiera et al., 2006).

Naked DNA is often not sufficient for efficient gene transfer because it is rapidly degraded and their cellular intake is fairly low (Corsi et al., 2003; Opanasopit et al., 2009), due to their hydrophilic arrangement and large size resulting from negatively charged phosphates groups (Cevher et al., 2012). For this reason, it is necessary the help of a delivery system, referred to as vector, which tends to protect and compact the DNA (Dufes et al., 2005). Innumerous efforts have been made to develop a safe and effective gene delivery method that minimizes side effects and overcomes the major drawback of gene therapy: the low gene transfection rate (Tiera et al., 2006). Thus, the two main types of vectors used in gene therapy are based on viral and non-viral gene delivery systems (Fig. 1.1).

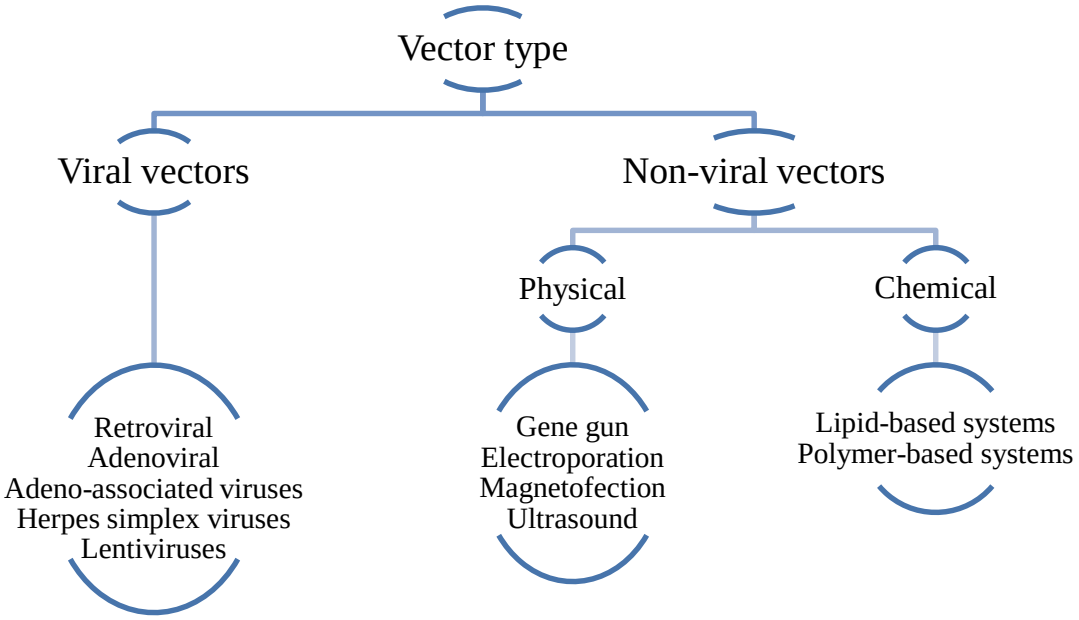

Figure 1.1 - Types of vectors: viral and non-viral vectors (adapted from (Nayerossadat et al., 2012).

1.2 Delivery vectors

1.2.1 Viral vectors

One of the most successful gene therapy vectors today are viruses-based gene delivery systems which provide high transduction effectiveness of the gene of interest to the target cells and superior levels of gene expression. Viruses, such as retrovirus, lentivirus, adenovirus, among others, can be readily transformed into viral vectors by replacing part of their genome in order to use the same space for the therapeutic gene (Yue et al., 2013). However, although viral vectors are efficient and removing part of their genome reduces their pathogenicity, their capsid can initiate a severe immune/inflammatory response. The use of these vectors could be limited due to their toxicity and oncogenicity, which limits the possibility of subsequent administrations, and moreover, have a reduced capacity to carry a large amount of genetic information and lack of optimization in large-scale production (Cevher et al., 2012; Machado et al., 2014). Viral vectors are distinguished by type of virus used, as depicted in Fig 1.2.

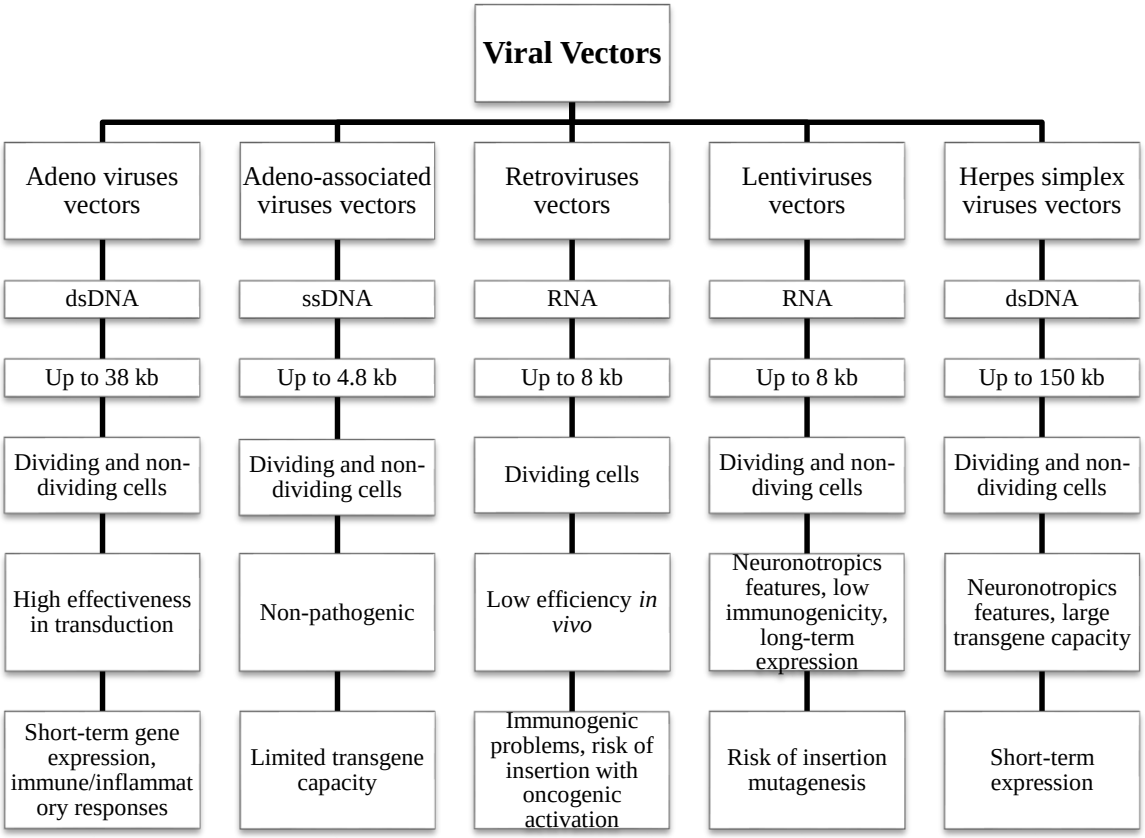

Figure 1.2 - Examples of viral vectors and their properties, such as type of viruses, viral genome, packaging capacity, type of cells transduced, vantages and disadvantages of their use (Cevher et al., 2012; Nayerossadat et al., 2012). dsDNA – double stranded DNA, ssDNA – single stranded DNA.

1.2.2 Non-viral vectors

Over the past years, the limitations of viral vectors, particularly regarding safety concerns, have led to the development of alternative gene delivery systems as non-viral vectors (Tiera et al., 2006). These vectors have many advantages, such as their safety profile, ease of production, greater structural and chemical versatility for manipulation of their properties, possibility of repeated administration, cell/tissue targeting and low immune responses; however, the biggest drawback of non-viral vectors is their low transfection efficiency (Cevher et al., 2012; De Laporte et al., 2006; Ishii et al., 2001; Tiera et al., 2006; Wong et al., 2007). The development of effective non-viral vectors has been a challenge in the field of gene therapy, and these can be divided in two categories, physical and chemical (Fig. 1.1). Physical methods, including gene gun, electroporation, magnetofection and ultrasound, are based on the application of a force to increase the permeability of the cellular membrane, allowing the gene to enter the cell and thus augment gene delivery. While chemical methods, including cationic lipids and

polymers, are based on the transport of therapeutic genes across the cell membrane up to the nuclear membrane (Cevher et al., 2012; De Laporte et al., 2006).

1.2.2.1 Physical methods

- Gene Gun

Gene gun is based on small, spherical DNA-coated heavy metal particles, including gold, tungsten and silver particles, which are accelerated to high speed by pressurized inert gas to enter into target cells/tissue, such as skin, mucosa or surgically exposed tissues. This method presents some advantages such as high reproducibility without the use of toxic chemicals or receptors, ability to carry DNA fragments of several sizes and the production of metal particles is relatively easy. However, it might cause tissue damage and their gene expression is low and short-termed (Cevher et al., 2012; Nayerossadat et al., 2012).

- Electroporation

Electroporation is based on controlled temporary destabilization of the cell membrane by insertion of a pair of electrodes into it, that produce electric pulses to increase cell permeability and to form temporary pores on the membrane surface which allows the entry of DNA into the cell. This method has been used *in vivo* for several tissues such as skin, muscle, lung, and tumor treatment, however, irreversible tissue damage might occur due to high temperature resulting from the high voltage application (Cevher et al., 2012; Nayerossadat et al., 2012).

- Magnetofection

Magnetofection is based on concentration of particles containing nucleic acid into the target cells by the use of magnetic field to increase gene transfer. This method has been tested on a wide range of cell types and presents high transfection efficiency and low toxicity (Nayerossadat et al., 2012).

- Ultrasound

Ultrasound is based on the use of acoustic cavitation, which can make some nanomeric pores in cell membranes and facilitate intracellular delivery of DNA particles

into cells of internal organs or tumors. This method is considered a relatively easy and reliable procedure, however their transfection efficiency is low (Newman et al., 2007).

1.2.2.2 Chemical methods

Non-viral gene delivery is constituted mainly by chemical methods of gene transfer, such as cationic lipids and polymers that usually form nanomeric complexes by electrostatic interactions between negatively charged nucleic acids and positively charged lipids or polymers, resulting in lipoplexes or polyplexes, respectively. These cationic gene delivery systems, when compared with other non-viral systems, have several advantages such as low toxicity and antigenicity, long-term expression with less risk of insertional oncogenesis and less tissue damage, among others. However, the major drawback is their low transfection efficiency, which can be due to 1) nonspecific interactions between cationic complexes and cell surface, 2) the process of endocytosis into endocytic vesicles, 3) compaction and release of the DNA from endosomes, which differs fundamentally for lipoplexes and polyplexes, and lastly 4) translocation of DNA to the nucleus and their expression (Cevher et al., 2012; Nayerossadat et al., 2012).

- Lipid-based systems

Lipid-based gene delivery systems can be divided according to their charges into cationic, anionic and neutral, and the most used are lipoplexes, complexes formed by a multilayered structure consisting of plasmid sandwiched between cationic lipids. Cationic lipids have tree basic constituents: the polar head group, an interconnecting linker and a hydrophobic anchor (Fig. 1.3). The hydrophilic head groups are, generally, primary, secondary, tertiary amines or quaternary amine salts, which promote interactions with DNA, through the negatively charged phosphates groups of DNA with the cationic groups present in hydrophilic head, and are largely responsible for its toxicity. Linker groups such as amides, esters and ethers, ensure a contact between the cationic head group and the negatively charged phosphates of the DNA and define the local for lipid cleavage, which affects biodegradability rate. Hydrophobic anchor represents the nonpolar hydrocarbon moiety which can be formed by a single or double chain of hydrocarbon or cholesterol, which provides self-association to form either micelles or liposomes in the presence of a helper lipid, such as

dioleylphosphatidylethanolamine (DOPE) (Cevher et al., 2012; De Laporte et al., 2006; Machado et al., 2014).

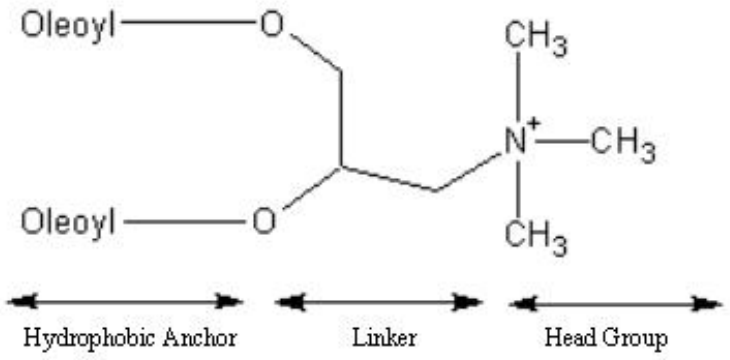

Figure 1.3 - Example of a cationic lipid (adapted from (Chesnoy et al., 2000)).

Gene delivery systems based on cationic lipids are a promising approach because they are easy to mass-produce and less immunogenic than the viral vectors, however, their transfection efficiency is not as high as viral vectors. Gene transfer efficiency of a lipoplex depends on its structural and physicochemical properties, such as stability and size, and recent findings suggest that the low efficiency of these vectors could be related to a nonspecific immunogenic response or due to instability of these vectors in the presence of serum, which creates difficulties for *in vivo* applications. The charge ratio between cationic lipids and negatively charged DNA is also an important parameter for transfection efficiency, since lipoplexes with a high charge ratio, where number of positive charges are in excess, are more efficient than neutral lipoplexes (Chesnoy et al., 2000). Positive charges on lipoplexes enhance their clearance from the circulation (Corsi et al., 2003).

Liposomes, the most used lipid-based gene delivery system, are colloidal systems with spherical form and composed of aqueous core enclosed by natural or synthetic phospholipid bilayers. Liposomes are classified based on lipid bilayers such as unilamellar or multilamellar liposomes (Fig. 1.4) and in addiction to cationic lipids may further contain a neutral/helper lipid, such as DOPE and cholesterol, which destabilizes the endosomal membrane to facilitate lipid exchange and membrane fusion between liposomes and endosomal membrane (Cevher et al., 2012; Nayerossadat et al., 2012). They are used in gene delivery to the lung, spleen, kidney, liver, skin cells, among others, due to their low toxicity and immunogenicity (Cevher et al., 2012; Mishra et al., 2011).

Although they can be produced in large scale and effective at small doses (Machado et al., 2014), when compared with viral vectors, a major problem of cationic liposomes is the low transfection, which can be influenced by factors such as size, number of layers, surface charge, namely charge ratio between DNA and cationic lipid, presence of helper lipid or by the presence of serum. The inhibitory effect of serum can be overcome either by increasing the charge ratio or modifying liposome surface with hydrophilic polymers, such as polyethylene glycol (PEG), called hydration layer, to avoid protein interaction and hence prevent their aggregation upon contact with serum, thus, to increase their circulation lifetime in the blood and transfection efficiency (Cevher et al., 2012; Chesnoy et al., 2000; Muralidharan et al., 2014).

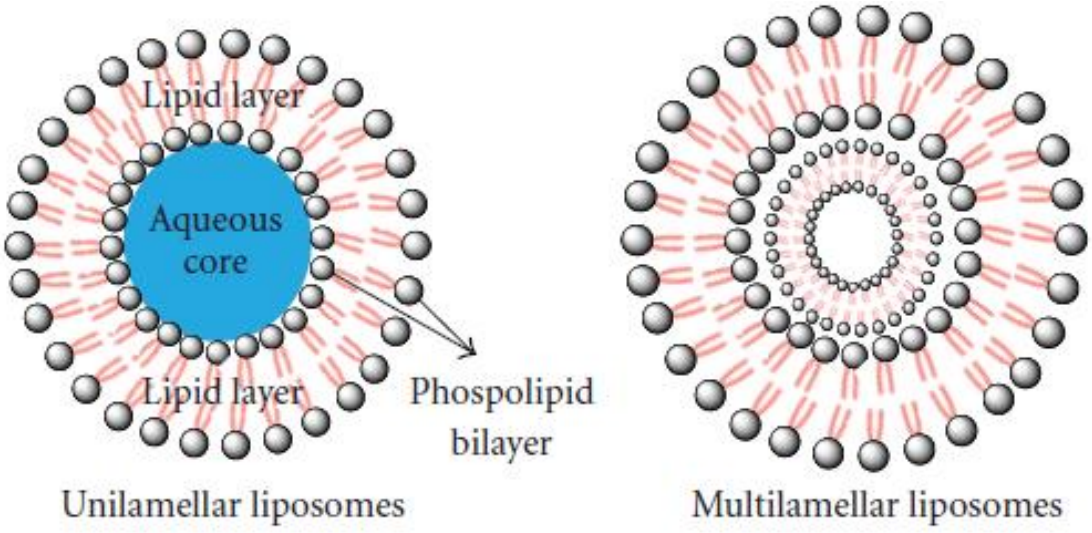

Figure 1.4 - Schematic representation of liposomal structures (adapted from (Mishra et al., 2011)).

- Polymer-based systems

Gene delivery systems based on polymers offer some specific advantages over lipid-based systems. Complexes involving cationic polymers are smaller, more stable and less cytotoxic than those involving cationic lipids. Furthermore, polyplexes condense more DNA and the efficiency with which it bind and condense DNA, allows their protection during the intracellular transport (Buschmann et al., 2013; Corsi et al., 2003; Mansouri et al., 2004).

Polymers/DNA complexes should protect negatively charged DNA from repulsion of the anionic cell surface, condense it into nano- or micro-structures for increased cellular internalization and once inside the cell protect it from nuclease degradation. In brief, three strategies have been used to produce polyplexes, either by electrostatic interactions, encapsulation or adsorption (Fig. 1.5).

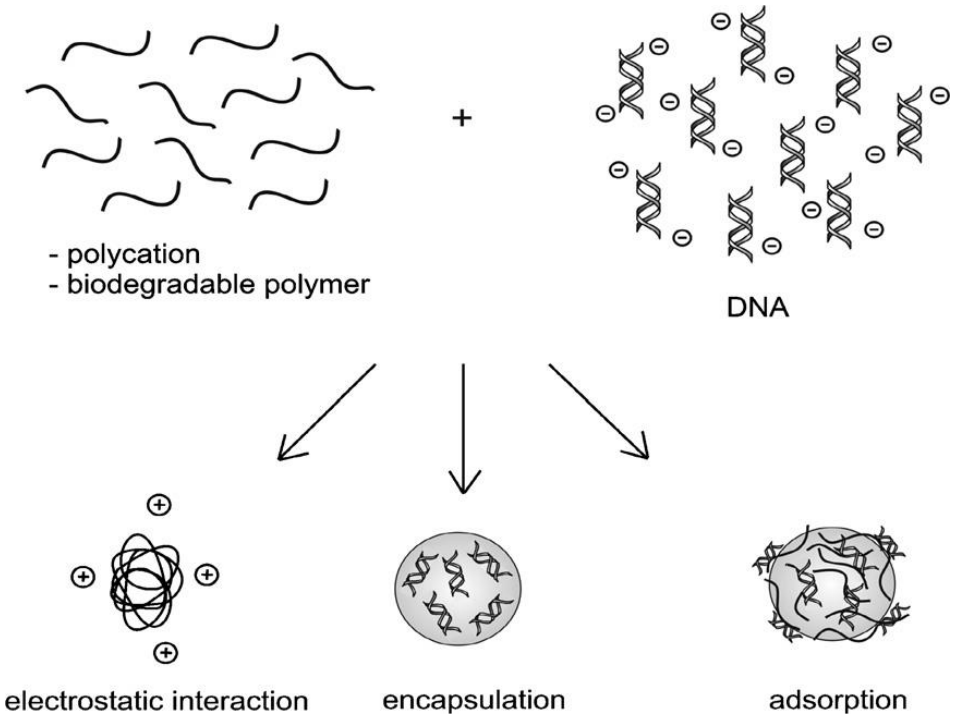

Figure 1.5 - Three main approaches to packaging DNA into polymer-based vectors: electrostatic interactions, encapsulation and adsorption (adapted from (Wong et al., 2007)).

The most common approach for polyplexes production is by electrostatic interactions, where polymer/DNA binding occurs between protonated amine groups present on polymers with negatively charged phosphate groups of DNA. This binding must occur at a sufficient nitrogen to phosphate charge ratio (N:P ratio), to promote slightly positively charged polyplexes formation and to avoid repulsions of negative cell surfaces (Wong et al., 2007). An ideal polyplex should compact anionic DNA as much as possible and be able to cross the cell membrane and protect the DNA inside the cytoplasm before it enters the nucleus, but then the polymer-DNA complexation should be weak enough to allow DNA release for transcription inside the nucleus (Yue et al., 2013).

Non-viral gene delivery systems based on polyplexes, have been considered a safe alternative to viral vectors due to their features of inducing relatively low toxicity, no significant immune responses, ability to be administered repeatedly to achieve long-

term gene expression, easy preparation, biodegradability, flexibility in use, cell-type specificity after chemical conjugation of a targeting ligand, and have capacity to package large DNA plasmids (Tang et al., 2006). Therefore, inefficient delivery at the intracellular level (Gaal et al., 2011) and hence low transfection efficiency is the major challenge of polymer-based gene therapy.

For a successfully nuclear delivery, polyplexes must efficiently enter the cells and pass through the intracellular space to the nucleus, overcome biological barriers such as plasma, endosomal and nucleus membrane (Yu et al., 2012). The properties of the complex, such as size, N:P ratio and kind of cationic polymer influence the extra and intracellular itinerary of the complex. High molecular weight polymers tend to form small and stable complexes, while complexes formed by low molecular weight polymers have lower cytotoxicity and higher ability of dissociation between DNA and cationic polymer, which increase transfection efficiency (De Laporte et al., 2006). Cationic polymers can be classified in two groups: synthetic polymers, such as polyethylenimine (PEI) and dendrimers, and natural polymers, such as chitosan (CS) and hyaluronic acid (HA).

1.3 Synthetic polymers

Synthetic polymers are the most commonly used non-viral gene delivery systems in gene therapy and PEI is one of the most effective and versatile polymer-based vectors. PEI is able to efficiently promote the DNA condensation, protecting DNA from degradation by DNases, and forming nanoparticles easily endocytosed by different cell types. PEI also has a strong buffering capacity at almost any pH because of the great number of primary, secondary and tertiary amino groups, which give PEI an opportunity to escape from the endosome (proton sponge effect). This polymer can be synthesized in its linear or branched form, with the linear form being the most efficient and with lower cytotoxicity (Nayerossadat et al., 2012). PEI, a non-biodegradable polymer, can be used with different molecular weights (MW) which is closely related to their cytotoxicity and their transfection efficiency (Tiera et al., 2006; Yue et al., 2013). PEI with MW of 25 kDa or higher, has been associated with high transfection efficiencies but increased cytotoxicity, while low MW PEI display much lower toxicity but almost no transfection efficiency (Corsi et al., 2003; Tang et al., 2006). Attaching

PEG to the surface of the particles, is a manner of decreasing the cytotoxicity and the non-specific interactions, increasing its half-life in the bloodstream (Corsi et al., 2003).

Poly-L-lysine (PLL), is a biodegradable synthetic polymer which interact electrostatically with negatively charged DNA to form polyplexes, however, PLL with high MW exhibits cytotoxicity and tendency to aggregate and precipitate depending on the ionic strength, which can be ameliorated through PEGylation (Park et al., 2006). Compared with PEI, polyplexes formed by PLL are taken up into cells as efficiently as PEI polyplexes, however, with a lower transfection efficiency (Tiera et al., 2006), while poly[alpha-(4-aminobutyl)-L-glycolic acid] (PAGA), a derivative of PLL, has showed a significantly higher transfection efficiency than PLL with no measurable cytotoxicity detected (Park et al., 2006).

Poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) is a water-soluble cationic polymer which has primary and secondary amines that can facilitate complexation (De Laporte et al., 2006; Park et al., 2006). PDMAEMA is more effective than branched PEI and PLL due to their ability to condense DNA into small and positively charged particles which are able to transfect various cell types (Tiera et al., 2006).

Another approach is the use of dendrimers that are synthetic branched polymers with tendency to adopt a globular shape and consist of symmetrical branches projecting from a central core, with functional groups on their surface that can be used to bind DNA and form complexes termed dendriplexes. Dendriplexes protect DNA from nucleases and have high transfection efficiency with low cytotoxicity, however, transfection efficiency depends on the size, shape and number of primary amino groups on the surface of the polymer (Cevher et al., 2012; Tiera et al., 2006).

Poly(amidoamine) (PAMAM) dendrimers were the first dendrimer family to be synthesized and characterized (Esfand et al., 2001), built from an ethylenediamine or ammonia core by addition, through stepwise polymerization process, of layers or generations of methacrylate and ethylenediamine. Cytotoxicity of PAMAM dendrimers increases with generation, however, is lower than high MW PEI and PLL polymers (Dufes et al., 2005).

Dendrimers have been used in gene expression, immunodiagnostics and controlled and targeted delivery due to a large number of different molecules that can be conjugated to functional groups on the surface of the dendrimer (Cevher et al., 2012).

Polymers such as polyamides, polyamines, polyesters, among others, also are used to building dendrimers (Lee et al., 2005).

1.4 Natural polymers

Natural polymers have specific advantages over the most used gene delivery systems, the synthetic polymers, such as environmental responsiveness via degradation and remodeling by cell-secreted enzymes. Among natural polymers, collagen, gelatin, alginate and, described in more detail, CS and HA, have been used for gene delivery and are, in general, non-toxic, even in large doses, biocompatible, biodegradable and mucoadhesive (Dang et al., 2006).

Collagen is the major insoluble fibrous protein in the extracellular matrix (ECM) (Zuber et al., 2015) and in connective tissue, and their structural and biochemical properties make it a promising polymer for gene delivery (Urello et al., 2014). Collagen is a non-toxic and low antigenic polymer, which can be easily combined with other materials, such as synthetic polymers, forming DNA complexes at low pH due to rapid aggregation and its low stability in serum at neutral pH (Jun Wang et al., 2004; Zuber et al., 2015). Collagen has superior biocompatibility when compared with gelatin (Zuber et al., 2015).

Gelatin, a denatured form of collagen, obtained by acid and alkaline processing of collagen (Dang et al., 2006; Malafaya et al., 2007), contains many glycine, proline and 4-hydroxyproline residues and have been used for pharmaceutical and medical applications due to its inexpensiveness and high availability, furthermore, is highly biocompatible and biodegradable in a physiological environment (Malafaya et al., 2007; Santoro et al., 2014). Gelatin nanoparticles maintain plasmid DNA structure, protecting it from nucleases degradation, and improve the transfection efficiency upon intracellular delivery (Xu et al., 2012).

Alginate is a naturally occurring anionic polymer, typically obtained from marine brown algae and bacterial species as *Azotobacter vinelandii* and several *Pseudomonas* species. This linear polymer is composed by regions of D-mannuronic acid, regions of L-gluluronic acid and regions of both, depending on the natural source. Alginate has been used in biomedical applications due to their properties as biocompatibility, low toxicity, good mucoadhesive and slight gelation by addition of divalent cations as Ca^{2+},

forming hydrogels widely used in wound healing, drug delivery and tissue engineering (Lee et al., 2012; Malafaya et al., 2007).

1.4.1 Chitosan

Chitosan is a cationic polymer obtained by deacetylation of chitin, which can be used at different degrees of deacetylation, usually between 70% and 95%, and composed of two subunits: D-glucosamine and N-acetyl-D-glucosamine, which are linked by a (1-4) glycosidic bond (Fig. 1.6) (Dang et al., 2006; Malafaya et al., 2007).

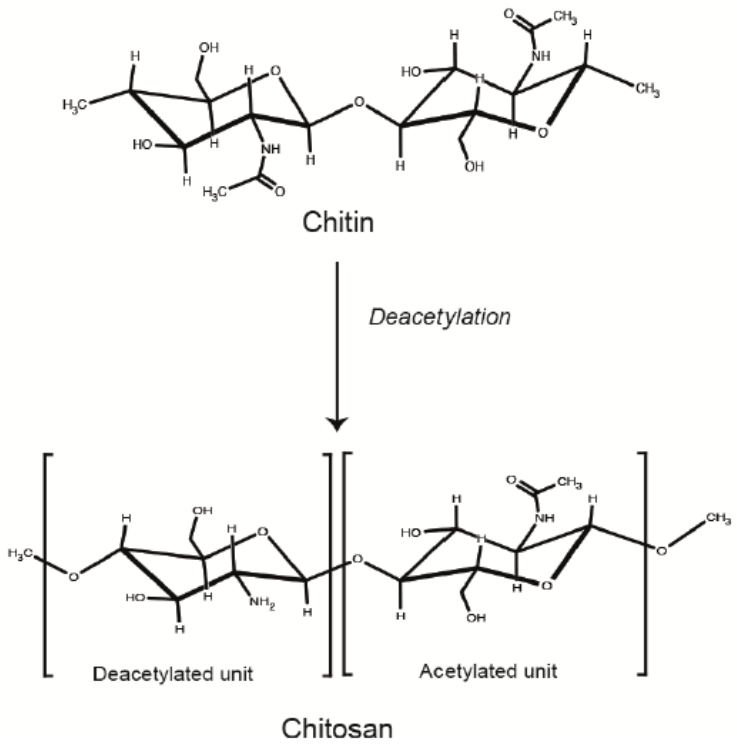

Figure 1.6 - Schematic representation of chitosan structure (adapted from (Agirre et al., 2014)).

Chitosan has several advantageous qualities in comparison with other non-viral vectors, such as their biocompatibility, biodegradability, non-toxicity and non-antigenicity (Chae et al., 2005; Grenha et al., 2005), making it useful and widely employed in several biomedical fields such as gene delivery, tissue engineering and drug delivery (Chae et al., 2005; Huang et al., 2005). Due to their mucoadhesive properties, CS-based gene delivery systems have been successfully applied in several mucosal routes, such as the nasal and ocular (Buschmann et al., 2013; Dang et al., 2006; Grenha et al., 2005).

Furthermore, CS is a weak base with a pKa value of the D-glucosamine residue of about 6.2-7.0, making it a pH responsive polymer, which is insoluble at neutral and alkaline pH. At pH below physiological pH, the primary amines in the CS backbone become protonated making it soluble in acidic medium (Agirre et al., 2014; Opanasopit et al., 2011). These positively charged amine groups enable CS to bind and package large molecules of DNA, condensing it into particles (Kim et al., 2003; Weecharangsan et al., 2008) and avoiding its degradation by DNases. The interaction of CS-DNA complex is driven mainly by electrostatic interactions between the positively charged amino groups of CS and the negatively charged phosphates groups of DNA (Tiera et al., 2006).

Several particle preparation methods can be used to prepare CS polyplexes, as the direct mixing of CS over DNA pipetting up and down or a more vigorous vortex agitation, which can be performed at room temperature or 50-55ºC, or even factors such as presence or absence of salt, in a very dilute or concentrated regime, with equal or different volumes of CS and DNA solutions, among other factors (Agirre et al., 2014; Buschmann et al., 2013). However, regardless of the mixing conditions used, an excess of CS conferring positive charge to the polyplex seems to be the crucial parameter in all formulations that have successfully transfected cells *in vitro* (Buschmann et al., 2013). The size of polyplexes depends of MW of CS, plasmid concentration and N:P ratio of CS/DNA, which may affect the blood circulation time and the cellular uptake and thus transfection efficiency (Tiera et al., 2006).

Transfection efficiency referred as ability of complex CS/DNA to induce gene expression (Buschmann et al., 2013) is influenced by several factors, including the degree of deacetylation and MW of CS, stoichiometry of complex, plasmid concentration, N:P ratio, serum concentration and pH of medium (Dang et al., 2006; Huang et al., 2005; Ishii et al., 2001; Opanasopit et al., 2011; Sajomsang et al., 2009; Weecharangsan et al., 2008). Furthermore, transfection efficiency of CS/DNA complexes is cell type-dependent (Dang et al., 2006; Mansouri et al., 2004; Sajomsang et al., 2009), as seen in several *in vitro* transfections that have been reported in various cell types, such as human embryonic kidney cells (HEK293), human lung adenocarcinoma epithelial cells (A549), B16 murine melanoma cells, COS-1 and HeLa cells. The cellular uptake depends on cellular membrane composition that varies among cellular types and may facilitate or hinder the binding of the complex and their internalization (Corsi et al., 2003).

The MW is one of the most important factors, because it also influences the binding affinity between CS/DNA and dissociation of DNA from the complex (Agirre et al., 2014). The binding affinity defines the ability of the polymer to complex, release and protect the DNA from degradation, as well as polyplex stability (Agirre et al., 2014; Buschmann et al., 2013), and hence the transfection efficiency. A balance between the DNA protection and intracellular DNA unpacking is necessary, because polyplexes must be stable enough to retain the DNA but also must be able to release the DNA once inside the cell (Agirre et al., 2014). Low MW CS tends to form less stable polyplexes leading to a more efficient intracellular release, while release of DNA by high MW CS is limited, due to high stability and strong affinity of polymer to DNA (Agirre et al., 2014; Dang et al., 2006).

Chitosan can be modified in order to improve its solubility and stability allowing an increased circulation lifetime (Agirre et al., 2014) or to increase proton sponge capacity improving endosomal escape (Buschmann et al., 2013), among others. These modifications can be achieved grafting certain molecules or polymers on the C2 amine, the C6 hydroxyl or both groups, through of PEGylation, quaternization or glycolyzation (Agirre et al., 2014; Buschmann et al., 2013; Chae et al., 2005). Furthermore, addition of a polyanion, as HA, is another approach to increase transfection efficiency of CS/DNA complexes, since incorporation of HA destabilizes the interactions between CS and DNA, facilitating DNA release and improving, thus, transfection efficiency of complexes (Buschmann et al., 2013; He et al., 2010).

1.4.2 Hyaluronic acid

Hyaluronic acid, also called hyaluronan, is a high MW anionic biopolymer composed of two subunits: D-glucuronic acid and N-acetyl-D-glucosamine linked by a glycosidic bond (Necas et al., 2008; Oh et al., 2010). HA can be easily produced in large scale from bacterial sources through microbial fermentation (Becker et al., 2009; Ito et al., 2006) being synthesized by hyaluronan synthases and degraded by hyaluronidases (Cho et al., 2011). HA is abundant in the ECM but can be found also in the synovial fluid, skin, lung, intestine, umbilical cord, vitreous of human eye and blood (Necas et al., 2008; Oh et al., 2010). The biological functions of HA include regulation of tissue hydration and water transport, maintenance of the elastic viscosity of joint synovial and eye vitreous fluid, among others, lubrication, control immune response,

cell proliferation and migration, wound healing, among others (Becker et al., 2009; Necas et al., 2008; Raemdonck et al., 2013).

HA has been used in drug delivery, tissue engineering, ocular and plastic surgery and gene delivery (Boeckel et al., 2014; Oh et al., 2010) due to its viscoelastic, mucoadhesive and shock-absorption properties, as well as, their non-immunogenicity, non-antigenicity, biocompatibility and biodegradability (Boeckel et al., 2014; Contreras-Ruiz et al., 2011; Raemdonck et al., 2013). As described above, HA have been used in ternary complexes as an anionic additive incorporated into existing gene delivery systems, such as CS/DNA complexes, because HA can coat complexes without disrupting their structures. Moreover, HA protects complexes against serum proteins and blood cells by decreasing nonspecific interactions (Ito et al., 2006) and improves transfection efficiency of complexes by a loosening effect of HA on the tightly compacted DNA molecule allowing the access of transcription factors to DNA (Ito et al., 2010). HA can be used also as a signaling molecule that interacts with specific cell surface receptors, as cluster determinant 44 (CD44), receptor for hyaluronate – mediated motility (RHAMM), HA receptor for endocytosis (HARE) and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (Raemdonck et al., 2013), leading to intracellular signaling, influencing cell migration, proliferation and gene expression (Becker et al., 2009; Necas et al., 2008). These receptors are present on the cell surface and extracellular matrix of some specific tissues, such as liver and kidney, and most of cancer tissues (Oh et al., 2010; Park et al., 2010). Furthermore, HA has several functional groups available for chemical conjugation that have been successfully used in conjugation reactions with drugs (Plattt et al., 2008).

1.5 Extracellular and intracellular barriers

For efficient gene transfection, non-viral vectors need to overcome many barriers as cell binding and internalization, escape from endosomes and nuclear translocation (Ito et al., 2006). To date a variety of polyplexes have been developed taking into account several factors, such as non-specific interactions either with serum components or with negatively charged cell surface (De Laporte et al., 2006). Because, at a cellular level, the first obstacle encountered by polyplexes are biological fluids, which can affect their stability and might cause aggregation or degradation of complexes. Once in proximity of a cell, to achieve gene expression, polyplexes need to bind to the cell

surface. This cell attachment can occur through non-specific electrostatic interactions between positive charge of the polyplexes and negative charge of the cell surface or by receptor ligands added to the polyplexes, to target specific cell types. Cellular uptake is achieved mainly by endocytosis and can occur, at least, through five different pathways: phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis, which depend on the particle size and cell type.

Upon cellular entry, the intracellular itinerary of endocytic vesicles depends on the pathway by which polyplexes were internalized, including recycling back to the surface cell, transformation into acidic degradative vesicles (lysosomes) or delivery to an intracellular organelle (e.g. reticulum endoplasmic or Golgi apparatus). However, polyplexes must escape efficiently of endocytic vesicles to avoid enzymatic degradation within lysosomal compartments. The proton sponge effect is the most studied escape from endosomes and depends on the buffering capacity of the polymer (Agirre et al., 2014; Wong et al., 2007). The proton sponge effect is based on acidification of endo-lysosomes by pumping of protons, leading to an influx of Cl^- that cause an increase of osmotic pressure and water absorption, resulting on endo-lysosome swelling. The combination of increased osmotic pressure and endo-lysosome swelling leads to its destabilization, rupture and release of its content into the cytoplasm before its degradation (Tiera et al., 2006; Yue et al., 2013).

Following escape of the endo-lysosomal pathway, polyplexes must be transported to the nucleus and internalize DNA across the nuclear membrane for its subsequent transcription (Agirre et al., 2014; De Laporte et al., 2006). A balance between DNA protection and intracellular DNA unpacking must exist on polyplexes, once high binding affinity between polymer and DNA can hamper the release of DNA and, hence its transfection efficiency (Agirre et al., 2014). Finally, DNA released from polyplexes can enter the nucleus by 1) passive diffusion, where DNA enters the nucleus during cell division when nuclear membrane is temporarily disintegrated or by 2) active transport, where DNA enters the nucleus via nuclear pore complexes (NPCs) present on the nuclear membrane (Fig. 1.7) (Tiera et al., 2006).

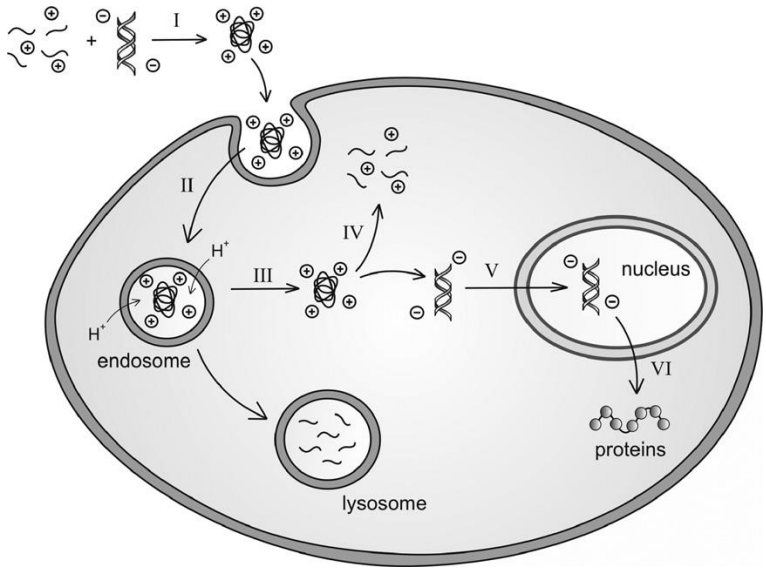

Figure 1.7 - Itinerary of gene delivery systems based on polymers. I) electrostatic interactions between positively charged polymer and negatively charged DNA to polyplex formation and subsequent entry into cells by endocytosis, II) once within the endocytic vesicles, polyplexes can be degraded on lysosomes or III) escape from endosome by proton sponge effect. IV) Latter, DNA is dissociated of polymer and V) should be able to cross nuclear membrane for its subsequent transcription and VI) transduction (adapted from (Wong et al., 2007)).

NPC allows passive diffusion of small molecules (diameter ≤ 9 nm or MW ≤ 45 kDa), while large molecules, as polyplexes used for gene delivery (≥ 100 nm), are transported actively through NPC by specific nuclear proteins, as importins, namely importin-α and importin-β (van Gaal et al., 2011; Yue et al., 2013). Importins can bind to the polyplexes by recognition of specific sequences, called nuclear localization signals (NLS), previously attached to these, which have been used to improve nuclear import and hence the efficiency of gene expression (Boulanger et al., 2005).

1.6 Nuclear localization signals

NLS are cationic peptides sequences that consist of either one or two stretches of highly basic amino acids of arginine/lysine, which are attached to DNA complexes, as polyplexes, and recognized by importins that direct their transport into the nucleus (Wong et al., 2007; Yue et al., 2013). NLS attached to polyplexes, can bind directly to importin-β or through the adapter protein, importin-α, which in turns bind to importin-β and form a complex. The resulting complex binds to the NPC, through of association with its cytoplasmic filaments, and is translocated through the pore to the nucleus. Finally, the complex dissociates and importins are recycled back to the cytoplasm and

are available for a next import cycle (Cartier et al., 2002; Görlich, 1997; Keller et al., 2003; van Gaal et al., 2011). Complex formation and dissociation is achieved by an energy-dependent mechanism involving Ras-related nuclear protein guanosine triphosphate (RanGTP) and other proteins (Fig. 1.8) (Boulanger et al., 2005; Cartier et al., 2002; Cherezova et al., 2011; Ciolina et al., 1999).

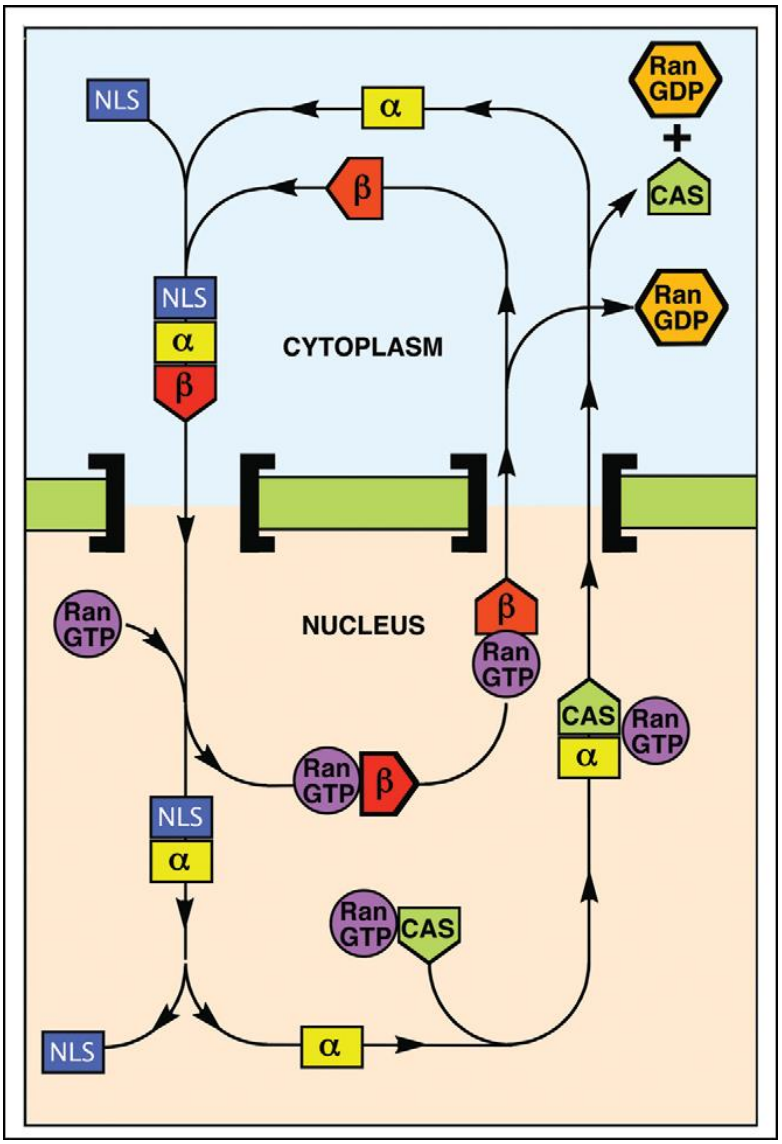

Figure 1.8 - Nuclear import mediated by importin-α and importin-β. Importin-β recognizes NLS-containing polyplex or protein and binds via importin-α adapter. Complex is translocated through the NPC into nucleus and then is dissociated by an energy-dependent mechanism mediated by RanGTP. Importin-β are recycled to the cytoplasm complexed with RanGTP and importin-α is exported with a RanGTP/CAS (cellular apoptosis susceptibility protein) complex. Finally, GTP hydrolysis dissociate the export complexes and release the importins for another nuclear import cycle (adapted from (Conti et al., 2006)).

The use of NLS for non-viral gene therapy has been widely investigated due to inefficient transfer of DNA from the cytoplasm to the nucleus that limits gene expression, mainly in post-mitotic and quiescent cells, where there is no disintegration of nuclear membrane (Boulanger et al., 2005; Bremner et al., 2004; Hu et al., 2012). This approach was first used by virus to efficiently integrate their genetic material into the host DNA (Zanta et al., 1999), and to date, NLS have been used in several studies with the goal of overcoming gene cytoplasmic degradation through an effective transport into the nucleus and, hence, improve nuclear delivery of DNA and, thus, increase gene expression (Boulanger et al., 2005; Bremner et al., 2004; Hébert, 2003). However, size and type of DNA (linear or plasmid), method used in the incorporation of NLS (covalent or non-covalent attachment of NLS to DNA or polymer), type of polymer used, among others, are some of several factors that influence the nuclear transport of DNA (Opanasopit et al., 2009; Wong et al., 2007). Furthermore, the type of NLS used in non-viral gene therapy can be monopartite or bipartite. Monopartite NLS can be characterized by a cluster of basic residues with a general sequence of K(K/R)X(K/R), as NLS derived from simian virus 40 (SV40) large T antigen, the NLS most used in gene therapy (PKKKRKV). While bipartite NLS can be characterized by two clusters of basic residues separated by 10-12 neutral residues, with a general sequence of (K/R)(K/R)X10-12(K/R)3/5, where at least 3 of 5 consecutive residues are arginine or lysine (Cherezova et al., 2011; Cokol et al., 2000; Marfori et al., 2011; van der Aa et al., 2005), as NLS derived from endogenous Insulin-like growth factor binding-3 (IGFBP-3) and -5 (IGFBP-5) (Schedlich et al., 2000).

1.6.1 IGFBP-3 and IGFBP-5

Insulin-like growth factor binding proteins (IGFBPs) are a family of six mammalian multifunctional proteins (Baxter, 2001; Hwa et al., 1999), which are involved in regulation and transport of insulin-like growth factors, IGF-I and IGF-II, in the circulation (Baxter, 2001; Ständker et al., 1998). IGFBPs can inhibit or stimulate cell growth and cell differentiation through regulation of binding of IGFs to type I IGF receptor (Schedlich et al., 2000). Furthermore, several studies have reported IGF-independent cellular activity, to IGFBP-3 (Butt et al., 2000; Cohen et al., 1997) and IGFBP-5 (Berfield et al., 2000; Miyakoshi et al., 2001), as their capacity of inducing nuclear transport (Hwa et al., 1999; Schedlich et al., 2000; Wang et al., 2014). Site-

specific mutagenesis has recognized that C-terminal region of IGFBP-3 and IGFBP-5 (Table 1.1), contain a domain with strong homology with bipartite NLS sequence (Baxter, 2001; Hwa et al., 1999; Schedlich et al., 2000). Mutations in this sequence leads to reduction or even to abolition of nuclear accumulation (Iosef et al., 2008; Schedlich et al., 2000), suggesting that this 18-amino acid region of IGFBP-3 and IGFBP-5 (Table 1.1) is essential and sufficient for nuclear uptake of the binding proteins and nuclear accumulation of IGFBP-3 and IGFBP-5 in a several cell lines (Baxter, 2001; Hwa et al., 1999; Schedlich et al., 2000).

Table 1.1 - Derived NLS sequences of C-terminal region of IGFBP-3 and IGFBP-5.

Peptide	Sequence
IGFBP-3	^{215}**KK**GFYKKKQCRPS**KGRKR**^{232}
IGFBP-5	^{201}**RK**GFYKRKQCKPS**RGRKR**^{218}

These IGFBP peptides were already used to successfully deliver heterologous proteins, such as GST (Goda et al., 2008), however, to our knowledge, they were never used for gene delivery. Taking into account their properties of nuclear uptake and accumulation of the binding proteins, these peptides (Table 1.1) may be considered good candidates for gene therapy, and the development of gene delivery systems where they can be incorporated could be potential carriers for nucleus-targeting gene delivery.

1.7 Aims

For an efficient gene delivery, several barriers need to be overcome and the nuclear import is considered the major limiting step in the development of effective non-viral gene delivery systems.

In this context, this work has as main aim to characterize and optimize several non-viral gene delivery systems based on natural polymers, as CS and HA. To overcome the nuclear barrier, incorporation of NLS derived of IGFBP peptides, an

endogenous NLS, to the polyplexes formulation is one of the strategies that can be used in order to enhance the internalization of the DNA in the nucleus.

Taking this into account, three strategies to incorporate IGFBP peptides were tested, namely, co-administration, covalent ligation to CS polymer and co-complexation of IGFBP peptides into polyplexes. Moreover, a second polymer, HA, was incorporated into polyplexes and co-complexed with IGFBP peptides.

The polyplexes were extensively characterized regarding their size, polydispersity, zeta potential and efficiency of DNA complexation. The effectiveness of polyplexes was evaluated through *in vitro* transfection assays using HEK293T cell line.

Furthermore, analysis of the cytotoxicity of IGFBP peptides was carried out in HEK293T cells.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasmids

During this study, three plasmids (Fig. 2.1) were used:

- pAAV2,1CMVeGFP3, encoding the enhanced green fluorescence protein (eGFP), driven by the cytomegalovirus (CMV) promoter and conferring ampicillin resistance, which was kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA) and used in all polyplexes.
- pCMVIGFBP-3 and pCMVIGFBP-5, driven by the CMV promoter and conferring kanamycin resistance, were used to encode the NLS derived of IGFBP peptides, respectively (Table 1.1), which contain a histidine tag at the N-terminal. These plasmids were previously constructed by S. Calado (unpublished data).

The plasmids were amplified in Top10 *E.coli* bacteria and extracted using a Plasmid Maxi kit (Qiagen, USA), according to the manufacturer's instructions. Later, plasmids were dissolved in TE buffer and their concentration was determined at 260nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

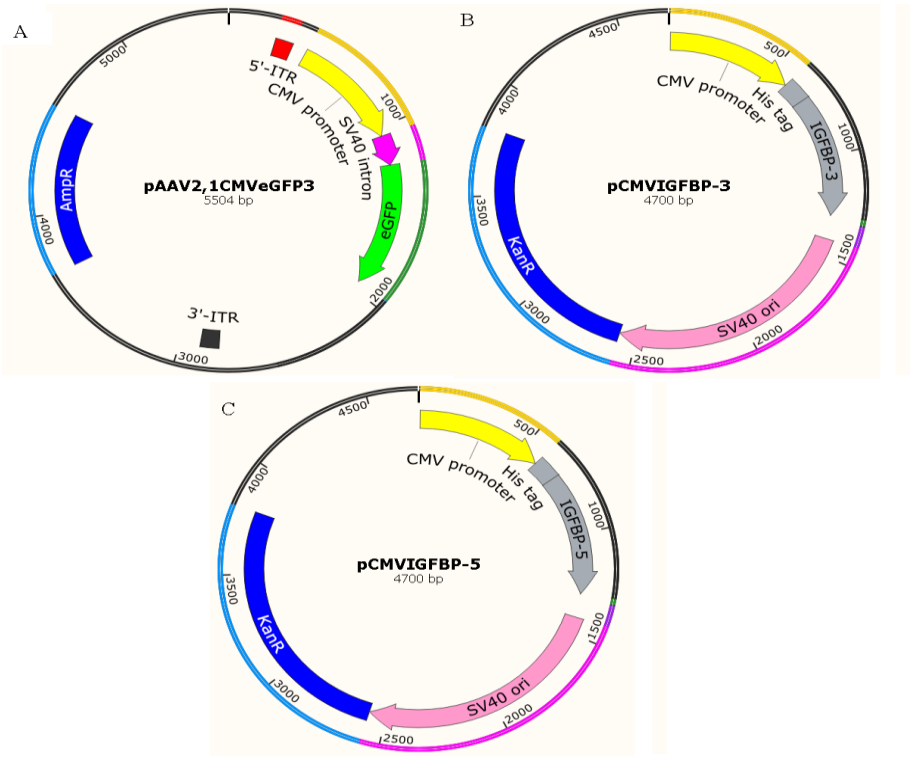

Figure 2.1 - Schematic representation of the structure of plasmids. (A) pAAV2.1CMVeGFP3 used for expression of GFP (B) pCMVIGFBP-3 used to encode IGFBP-3 peptide and (C) pCMVIGFBP-5 used to encode IGFBP-5 peptide. AmpR and KanR are genes for resistance to ampicilin and kanamycin, respectively.

2.1.2 Cell line and cell culture

For the *in vitro* assays, the HEK293T cell line was used, which is a cell line widely used in transfection assays because of their ease of transfection (kindly provided by Dr. Guilherme Ferreira, University of Algarve, Portugal). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin solution, and maintained at 37°C under a 5% CO_2 atmosphere. When 70-80% of confluence was reached, the cells were trypsinized with trypsin-EDTA, and transferred to new culture flasks. All cell culture reagents were acquired from Sigma-Aldrich (St. Louis, MO/USA).

2.1.3 Polymers

Ultrapure chitosan CL 113 (CS), with MW of 80 kDa and degree of deacetylation of 83%, was purchased from Novamatrix (FMC BioPolymer AS, Norway). Hyaluronic acid (HA), with MW of 132 kDa, was purchase from Lifecore Biomedical Inc. (USA). Polymer solutions of 1 mg/ml were prepared dissolving the polymer in milliQ water, and the pH of the solutions was adjusted to 5.5 with sodium hydroxide. The solutions were sterile filtered through a 0,2 μm filter.

All other reagents were acquired from Sigma-Aldrich (St. Louis, MO/USA).

2.2 Methods

2.2.1 Bacterial transformation

For bacterial transformation, aliquots of competent bacteria (E.coli TOP 10) were thawed and kept on ice. Then, 30 ng of plasmid (pCMVIGFBP-3 and pCMVIGFBP-5, respectively) were added to 100 μl of competent bacteria suspension and keep on ice for 15 minutes (min), followed by a heat shock at 42ºC for 90 seconds (sec). Posteriorly, 300 μl of S.O.C medium were added (containing 98% of S.O.B medium (triptone, yeast extract and NaCl), 1% of Mg^{2+} and 1% of glucose) and incubated the bacterial suspension at 37ºC, 180 revolutions per minute (rpm) for 30 min. Then, 100 μl of

transformed bacteria were spread in a pre-warmed LB agar plates containing kanamycin (30 µg/ml), and incubated overnight at 37°C.

2.2.2 IGFBP peptides extraction

The IGFBP peptides were extracted from the bacteria using the B-Per 6xHis Fusion Protein Purification Kit (Thermo Scientific, USA), according to manufacturer's instructions, and quantified by Bradford method (Bradford, 1976).

Thereafter, the peptides were dialyzed against 10 mM HCl solution for 6 hours and then against milliQ water, for 12 hours, on dialysis tubing with MW cut-off 2 kDa (Sigma-Aldrich, St. Louis, MO/USA). After dialysis, the peptide solutions were frozen at -80ºC and concentrated by sublimation in a vacuum pump. Their concentrations were determined by measurements at 280 nm (Kumar et al., 2013).

2.2.3 Polyplexes preparation

2.2.3.1 CS polyplexes

Several research groups have worked in the optimization of CS-based vectors, suggesting that the mixing technique of CS with DNA and the incubation conditions influence the final gene expression (Lavertu et al., 2006). Different methods, mainly driven by electrostatic interactions, adapted from Mao et al., 2001, were used to prepare the polyplexes. All polyplexes were prepared with an excess amount of CS to DNA, 250 µg of CS to 26.5 µg of DNA (N:P ratio of 15:1). All of nanoparticles were used for transfection assays without purification.

- $CSNa_2SO_4$

DNA was diluted in a sodium sulfate solution (25 mM) and an equal volume of CS solution was preheated to 55°C for 5 min, and quickly mixed together, placed on ice for 30 min and stored at 4°C (Fig. 2.2). Different concentrations of IGFBP peptides; 10, 25, 50, 100 and 150 µg for IGFBP-3 and 10, 25 and 50 µg for IGFBP-5; were co-administrated later at the time of transfection in the cell culture well.

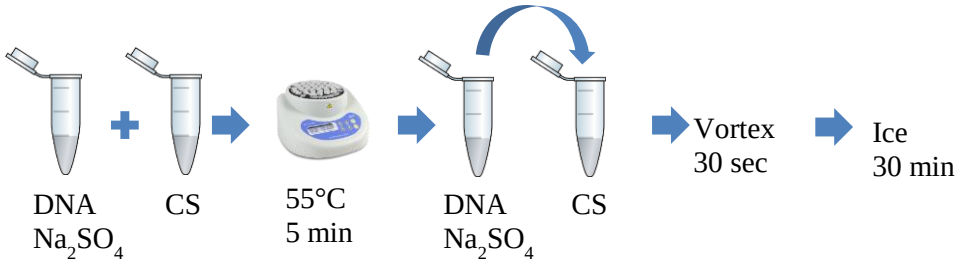

Figure 2.2 - Schematic representation of CSNa_2SO_4 polyplexes preparation.

- CS

In order to evaluate if polyplexes were affected by the presence of salts, sodium sulfate solution was removed, and polyplexes were prepared by adding the DNA directly to the CS solution (Fig.2.3).

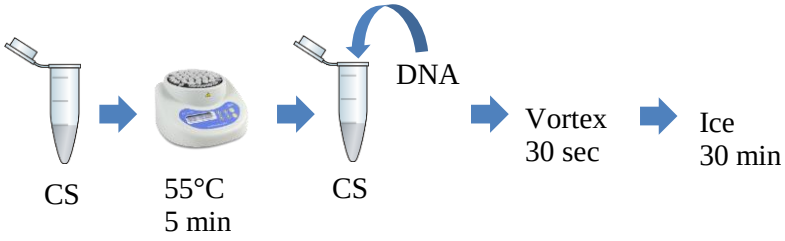

Figure 2.3 - Schematic representation of CS polyplexes preparation.

- CS3 and CS5

The total amount of IGFBP peptides was either added to the CS solution (T) or divided into equal parts and added to both the CS solution and DNA solution (S) and quickly mixed together as depicted in Fig. 2.4 and 2.5, respectively.

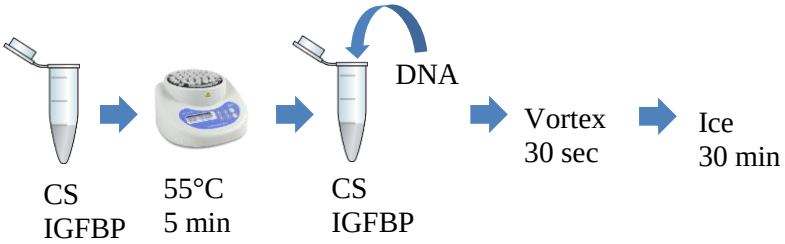

Figure 2.4 - Schematic representation of CS3 and CS5 polyplexes preparation, where the total amount of IGFBP peptides was added to the CS solution (T).

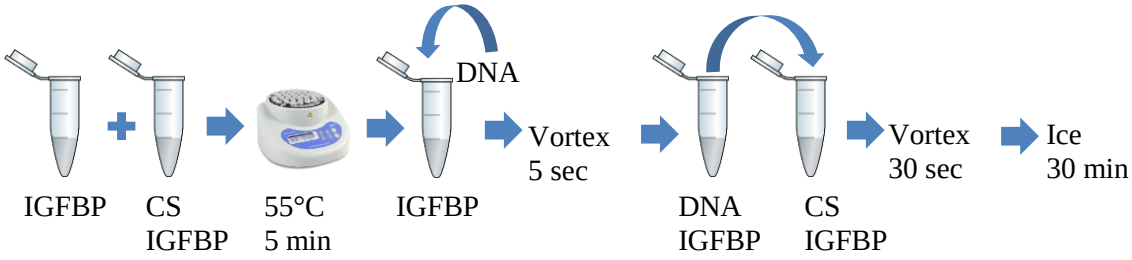

Figure 2.5 - Schematic representation of CS3 and CS5 polyplexes preparation, where the total amount was divided into equal parts and added to both the CS solution and DNA solution (S).

- 15:1CS3 and 15:1CS5

For formulations 15:1CS3 and 15:1CS5, we chose a fixed peptide concentration of 100µg. In order to evaluate differences in complexation, polyplexes were prepared considering the total amount of amine groups, including peptides and polymer, at a IGFBP-3 or -5/CS:DNA (N:P ratio) of 15:1, and the amount of CS solution was adjusted. This formulation was prepared the same way as previously described (Fig. 2.4 and Fig. 2.5).

- CSedac3 and CSedac5

In this formulation, a one and a half molar excess (relative to the carboxylic acid groups in peptides) of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC) were added to 100µg of IGFBP peptides, respectively, and stirred at 4˚C for 24h (Fig. 2.6). Then, the mixture was added to polyplexes previously prepared (described above, Fig. 2.3) and stored at 4°C.

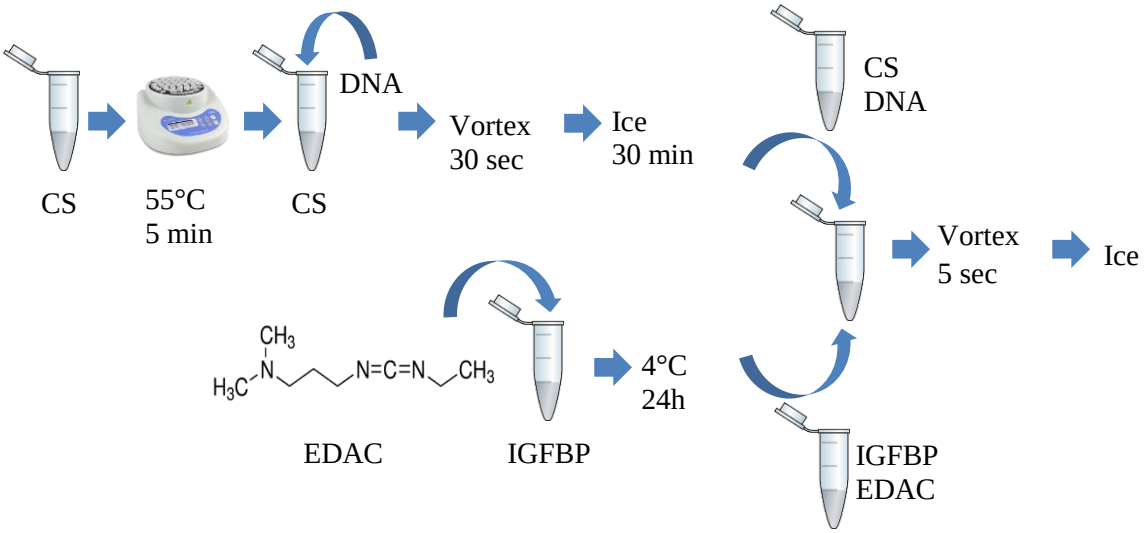

Figure 2.6 - Schematic representation of CSedac3 and CSedac5 polyplexes preparation.

2.2.3.2 HA polyplexes

- CSHA3 and CSHA5

Polyplexes with HA polymer were prepared using a CS:HA weight ratio of 5:1, and a CSHA:DNA N:P ratio of 15:1, these ratios were chosen based on previous work (Oliveira et al., 2014). The total amount of IGFBP peptides, at different concentrations, respectively, was divided into equal parts and was added to CS solution and HA, milliQ

H_2O and DNA solution, preheated to 55˚C for 5 min, quickly mixed together and stored at 4˚C (Fig. 2.7).

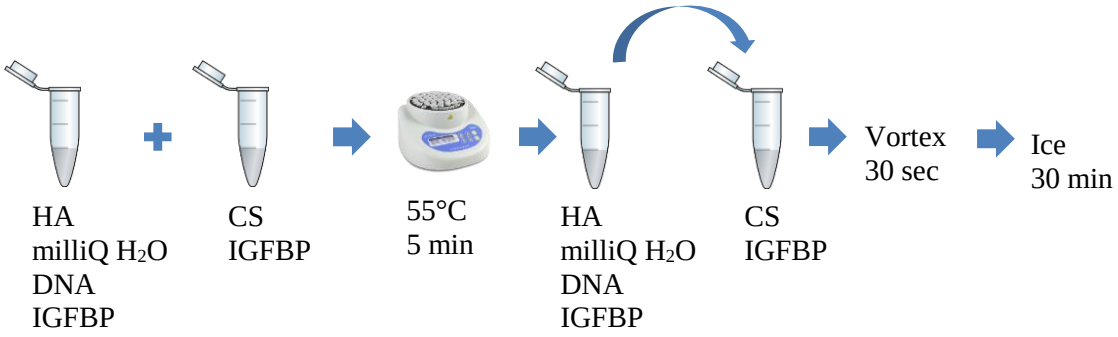

Figure 2.7 - Schematic representation of CSHA3 and CSHA5 polyplexes preparation.

2.2.4 Polyplex characterization

Freshly prepared samples were diluted in milliQ H_2O and polyplex size measurements were performed by dynamic light scattering (DLS), at 25˚C with a detection angle of 173º, using a Zetasizer Nano ZS (Malvern Instruments, UK). The zeta potential (surface charge) was measured with laser Doppler velocimetry at 25˚C on the same instrument. After characterization of polyplexes, preparations were stored at 4ºC, to avoid DNA denaturation and complex dissociation.

2.2.5 Polyplex complexation

The DNA complexation efficiency in the polyplexes was assessed by a retardation assay using agarose gel electrophoresis. Agarose gels with 1% (W/V) agarose in TAE buffer with GreenSafe® Premium (NZYtech, Portugal) were prepared. The polyplexes were loaded in each well and the electrophoresis was carried out for approximately 60 min at 90mV. The samples were visualized under UV light.

2.2.6 Cell viability evaluation

To evaluate the cytotoxicity of IGFBP-3 or IGFBP-5 peptides on HEK293T cells, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in a 48-well plate at a density of 15 000 cells per well in 500µl of DMEM and allowed to grow for 24h, at 37°C under 5% CO_2 atmosphere. After this

time, medium was removed and five different concentrations of IGFBP-3 and IGFBP-5 peptides (10, 25, 50, 100 and 150 µg) were tested by adding peptides to 500 µl of free DMEM. As in the transfection assay, after 5 hours, the medium was changed to DMEM with FBS, and cells were incubated up to 72h, at 37°C under 5% CO_2 atmosphere.

At the end of the incubation time, the medium was removed and 25 µl of MTT solution was added (5 mg/ml in phosphate buffered saline (PBS)) and the cells incubated for 4 hours. For formazan crystals solubilization, formed in living cells, 250 µl of HCl/Isopropanol (0,04 N HCl in isopropanol) was added to each well. After one hour, the absorbance was measured on a microplate reader (Tecan Infinite M200, USA) at 570 nm, for formazan solution, and 630 nm, for cellular debris. Non-treated cells were used as positive control, and cells treated with latex extract were used as negative control, and cell viability was calculated using the following equation:

Absorbance samples = absorbance at 570 nm - absorbance at 630 nm

Cell viability (%) = (absorbance samples ÷ absorbance positive control) × 100

2.2.7 *In vitro* transfection assays

Cells were seeded at a density of 200 000 cells per well in a 6 well culture plates with DMEM supplemented with FBS, 24h prior to transfection, at 37°C under a 5% CO_2 atmosphere. We performed the transfection using 1 µg of DNA per well and FuGENE® HD (Promega, USA) as positive transfection control, according to the manufacturer's instructions. Non-transfected cells were used as negative transfection control.

Polyplexes, prepared as describe above, were added to the cells and incubated in FBS free medium for 5h. After 5 h, the medium was changed to complete medium and cells were analyzed for transfection efficiency after 72h. All polyplexes were added to the cells on a single step, except the $CSNa_2SO_4$, where the polyplexes were added to the cells and then different concentrations of IGFBP-3 or IGFBP-5, respectively, were added (Fig. 2.8).

All transfection experiments were performed in triplicate. Transfected cells were visualized, 48h and 72h after transfection, using a fluorescence microscope (Axiovert 40 CFL, Zeiss) in order to evaluate GFP expression.

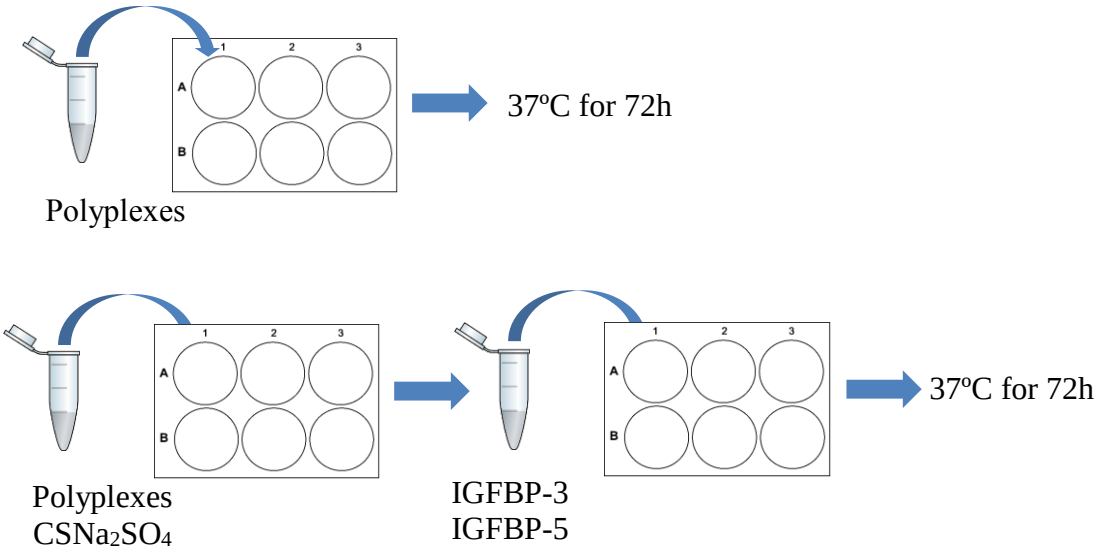

Figure 2.8 - Schematic representation of transfection assays. All polyplexes were added directly to the cells, except $CSNa2SO_4$, which were co-administrated with IGFBP peptides, respectively.

2.2.8 Transfection efficiency evaluation by flow cytometry

Transfection efficiency was evaluated quantitatively by flow cytometry. Cells were washed three times with PBS, the supernatants were discarded, and the cells were re-suspended in PBS and placed in specific tubes to be analyzed in the flow cytometer (FACScalibur, BD Biosciences, USA), for analyze of GFP expression. A total 50000 events were counted for each sample.

2.2.9 Statistical analysis

Statistical analysis was performed using a GraphPad Prism 6 software, and data were analyzed using ANOVA test (one-way ANOVA) and multiple comparisons tests using a confidence interval of 95% and considering $P<0.05$ value as significant.

3. RESULTS AND DISCUSSION

3.1 Polyplexes characterization

A positive surface charge is required on polyplexes for efficient cellular uptake, since cell entry occurs by non-specific electrostatic interactions between the positively charged polyplexes and the negatively charged cell surface (Tros de Ilarduya et al., 2010). In order to avoid repulsion by the negative cell surface, a N:P ratio of 15:1 was chosen to promote the formation of positively charged polyplexes. Parameters as N:P ratio, molecular weight, mixing technique, among others, have been widely investigated on CS/DNA complexes (Buschmann et al., 2013; Chae et al., 2005; Ishii et al., 2001; Mao et al., 2001; Opanasopit et al., 2009), and described as factors that influence the binding affinity between CS/DNA, size and zeta potential of polyplexes, cellular uptake and dissociation of DNA, and thus, transfection efficiency (Agirre et al., 2014; Buschmann et al., 2013; De Laporte et al., 2006).

The mixing technique of the CS solution and DNA solution used was adapted from Mao et al., 2001, where polyplexes were prepared by solubilization of DNA in a Na_2SO_4 solution, used as desolvating reagent, and mixed with CS solution. Both solutions were preheated at 55ºC to allow polyplexes formation with less aggregation (Mao et al., 2001). Polyplexes were formed as result of electrostatic interactions between positively charged amine groups of CS and negatively charged phosphate groups of DNA, and posteriorly, Zetasizer measurements were made to evaluate their size, polydispersity index (PdI) and zeta potential.

This preparation method of polyplexes yielded homogeneous preparations, with PdI below 0.3, and mean size of 285.85 ± 56.50 nm (Table 3.1). Regarding zeta potential, positively charged polyplexes were prepared, with mean values of zeta potential of 15.45 ± 0.21 mV. These polyplexes were prepared without IGFBP peptides, and used, later, on transfection assays, where several concentrations of IGFBP-3 and IGFBP-5 peptides were co-administrated at the time of transfection, respectively, as described above.

Table 3.1 - Size, PdI and zeta potential of $CSNa_2SO_4$ polyplexes.

Polyplex	Z-average size (nm)	PdI	Zeta potential (mV)
$CSNa_2SO_4$	285.85 ± 56.50	0.12 ± 0.05	15.45 ± 0.21

Values are presented as mean ± S.D.

To evaluate if sodium sulfate salts affected polyplexes, this was removed from the polyplexes preparation. Moreover, several concentrations of IGFBP peptides were co-complexed with polyplexes, where the total amount of IGFBP peptides was divided into equal parts and added to both the CS solution and DNA solution, as described above. Polyplexes were characterized regarding their size, PdI and zeta potential, as depicted in Fig. 3.1.

According to Mao et al., 2001, concentrations up to 25 mM of sodium sulfate do not significantly affect the mean size of polyplexes, however, CS polyplexes, without sodium sulfate and IGFBP peptides, yielded particles with an increased mean size of 729.27 ± 116.77 nm and an increased positive charge with a mean zeta potential of 27.17 ± 10.43 mV. These results suggest that sodium sulfate has a role on the entanglement of CS with DNA, which results in polyplexes with smaller size and lower positive surface charge.

Regarding polyplexes prepared with several concentrations of IGFBP peptides, statistical differences were found for the mean size of polyplexes co-complexed with concentrations of IGFBP-5 peptides between 50 μg and 250 μg, when compared with polyplexes without IGFBP peptides (Fig. 3.1). However, despite not having found statistical differences between polyplexes prepared with different concentrations of IGFBP-3 peptides and polyplexes without IGFBP peptides, size similarities were found when comparing them to polyplexes with IGFBP-5 peptides. This preparation method of polyplexes produced less homogeneous solutions, with PdI above 0,3, which can be result of some aggregation. Regarding to zeta potential, mean values of surface charge increased, as expected, since the NLS sequences used (Table 1.1) are rich in basic amino acids resulting on an increase of the overall positive charges and, hence, an increase of surface charge of polyplexes. These results indicate that addition of IGFBP peptides to polyplexes influenced their physical properties, namely their size and zeta potential, as previously reported (Opanasopit et al., 2009).

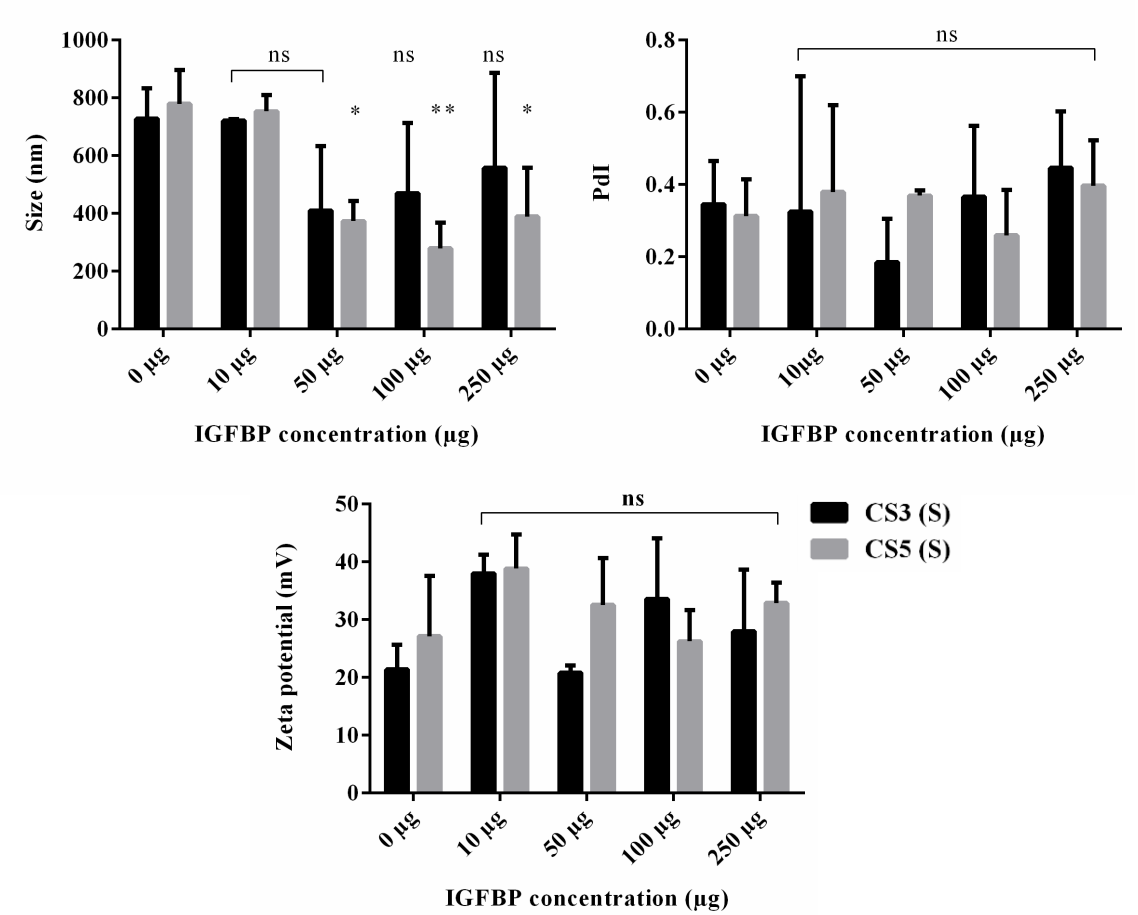

Figure 3.1 - Physical characterization of CS3 (S) and CS5 (S) polyplexes. Statistical differences, compared to polyplexes without IGFBP peptides, were calculated using Dunnett´s multiple comparisons test (**p<0.01; * p<0.05 and ns - not significant).

Since the mixing method can influence parameters such as polyplex size, (Buschmann et al., 2013), to evaluate if dividing (S) or adding the total amount of IGFBP peptides directly to CS solution (T), had a significant impact on polyplexes formation, we decided to select the IGFBP concentration of 100 µg, which presented a better transfection efficiency (described in the next section, Fig. 3.11). We characterized the produced polyplexes (Table 3.2) and compared them with CS (S) formulation with 100 µg of IGFBP peptides (Fig. 3.2).

Table 3.2 - Composition, size, PdI and zeta potential of CS3 and CS5 (T) polyplexes.

Polyplex	IGFBP (µg)	Z-average size (nm)	PdI	Zeta potential (mV)
CS3 (T)	100 µg	713.83 ± 270.28 ^{ns}	0.58 ± 0.09 ^{ns}	32.57 ± 8.46 ^{ns}
CS5 (T)		325.73 ± 14.04 ^{ns}	0.45 ± 0.13 ^{ns}	26.40 ± 3.38 ^{ns}

Values are presented as mean ± S.D. Statistical differences between formulations were calculated using Sidak´s multiple comparisons test (ns - not significant).

No statistical differences were found between formulations, (S) and (T), with IGFBP-3 and IGFBP-5 peptides, respectively (Fig. 3.2). However, a slight increase on polyplex mean size was observed in (T) formulation when compared with (S) formulation. These results might indicate that when the total amount of IGFBP peptides is added to CS solution, polyplexes formation can be hampered due to a slight increase of polyplex aggregation accompanied by the increase of their mean size, leading to more heterogeneous preparations. Regarding zeta potential, similar results were observed between formulations (Fig. 3.2).

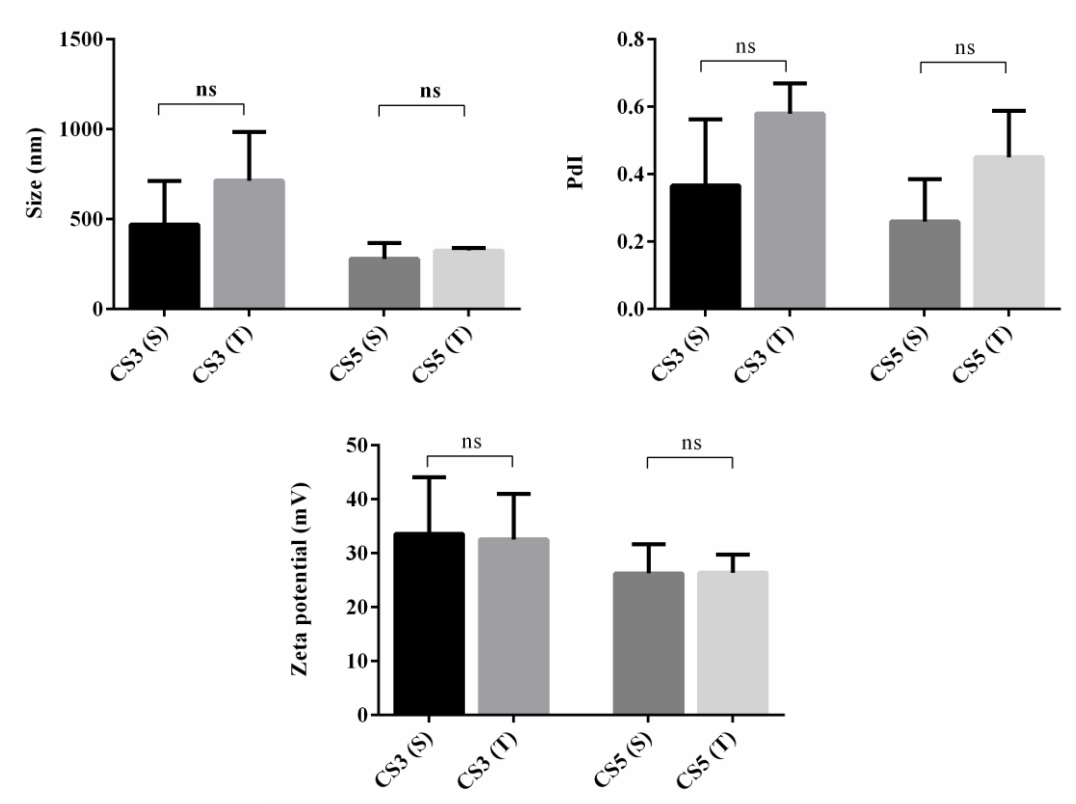

Figure 3.2 - Comparison of physical characterization between CS3 (S) and CS3 (T), and CS5 (S) and CS5 (T) polyplexes, with 100µg of IGFBP peptides, respectively. Statistical differences between polyplexes, with same kind of IGFBP peptides, were calculated using Sidak´s multiple comparisons test (ns - not significant).

For formulations 15:1CS3 and 15:1CS5, we also choose a fixed peptide concentration of 100 µg and in order to evaluate differences of polyplex formation, these were prepared considering the total amount of amine groups, including peptides and polymer, at a IGFBP-3 or -5/CS:DNA (N:P ratio) of 15:1 and were characterized regarding their size, PdI and zeta potential, as depicted in Fig.3.3.

This formulation yielded polyplexes with similar results in terms of mean size and zeta potential, when comparing both 15:1CS (T) and 15:1CS (S), with 100 μg of IGFBP-3 and IGFBP-5 peptides, respectively (Fig. 3.3). Regarding zeta potential, although there has been a reduction in amine groups available to interact with phosphate groups when IGFBP peptides were included in the ratio 15:1 (CS:DNA) and CS solution was adjusted, the surface charge of polyplexes was not affected yielding positively charged polyplexes (Fig. 3.3).

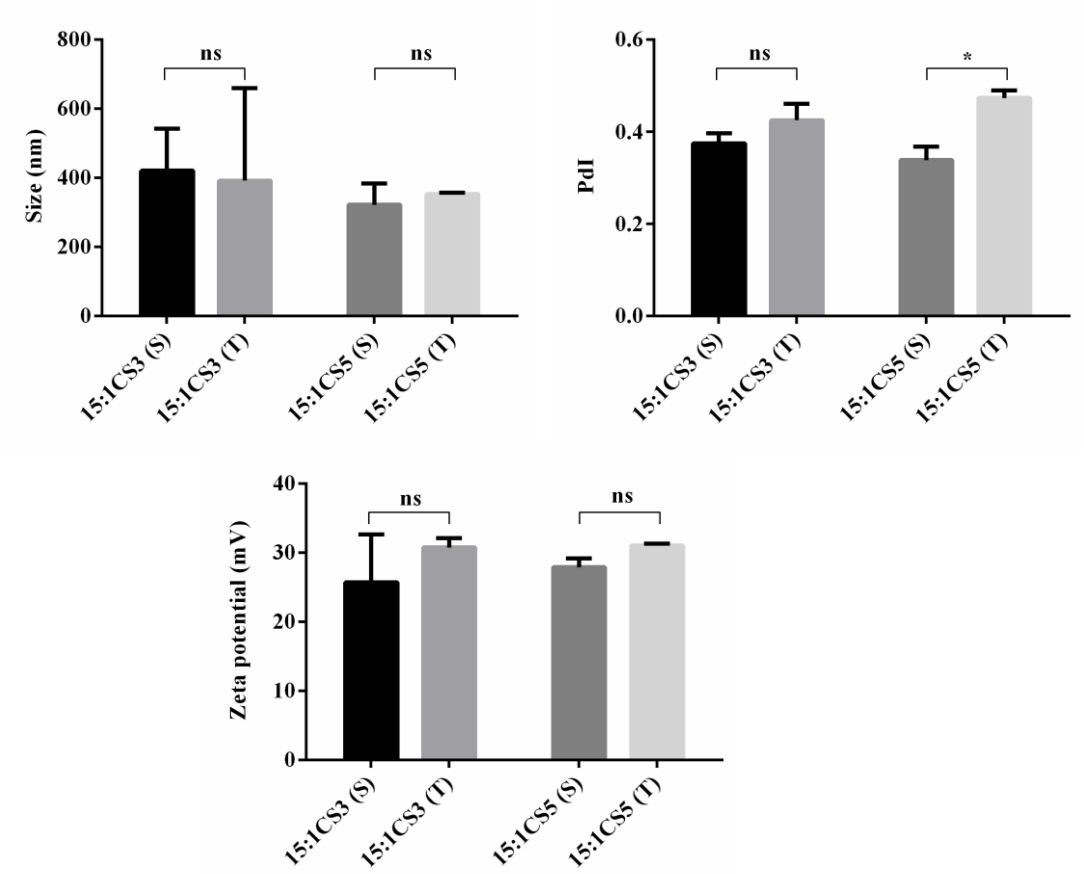

Figure 3.3 - Comparison of physical characterization of polyplexes 15:1CS, (S) and (T), with IGFBP-3 or IGFBP-5, respectively. Statistical differences between polyplexes, with same kind of IGFBP peptides, were calculated using Sidak´s multiple comparisons test (* $p<0.05$ and ns - not significant).

These results suggest that the reduction of amine groups did not significantly affect size and surface charge of polyplexes. According to the literature, N:P ratio is one of the factors which influence polyplexes size (Buschmann et al., 2013), in our study, a decrease in N:P ratio yielded polyplexes with similar sizes and surface charge to CS formulations.

Several methods have been used to covalently bind NLS peptides to DNA, as described by Ciolina et al., 1999, which associated covalently NLS peptides to DNA by photoactivation, although plasmid-NLS conjugates have not been detected in the nucleus. Nagasaki et al., 2003, observed an increase on gene expression only when 5 NLS peptides were covalently coupled to DNA by diazo coupling through a PEG chain and not when the NLS peptides were directly coupled to DNA. Zanta et al., 1999, obtained success on ligation of one NLS-oligonucleotide conjugated covalently to one or both ends of a linear DNA. However, extensive chemical modification of DNA causes reduction or inhibition of transfection process (Cartier et al., 2002; Nagasaki et al., 2003; Opanasopit et al., 2009; Yoo et al., 2007).

In our study, to avoid chemical modification of DNA, NLS peptides were linked covalently to CS polymer (Fig. 2.6), and polyplexes were characterized regarding size, polydispersity and zeta potential, as shown in Table 3.3.

Table 3.3 - Physical characterization of CSedac3 and CSedac5 polyplexes.

Polyplex	IGFBP (µg)	Z-average size (nm)	PdI	Zeta potential (mV)
CSedac3	100 µg	758.05 ± 159.88 ^{ns}	0.54 ± 0.16 ^{ns}	32.70 ± 12.02 ^{ns}
CSedac5		689.20 ± 22.91 ^{ns}	0.39 ± 0.06 ^{ns}	41.20 ± 1.98 ^{ns}

Values are presented as mean ± S.D. Statistical differences were calculated using Sidak´s multiple comparisons test (ns - not significant, compared to control CS).

This formulation yielded polyplexes with an increased size, which might be due to the fact that IGFBP peptides with EDAC had been added to polyplexes previously prepared by method described on Fig. 2.3, corresponding to CS polyplexes (Fig. 3.1, 100 µg IGFBP peptides). These results indicate that the addition of IGFBP peptides to CS formulation previously prepared result in similar sizes. Regarding the zeta potential, an increase of its mean values was expected, since IGFBP peptides were added to polyplexes previously prepared, resulting in the covalently bind of IGFBP peptides to the available amine groups of CS in the polyplex surface, giving it positive charge.

Several studies have reported the potential use of the polymer HA in ternary complexes for gene delivery applications (De La Fuente et al., 2008 (a); de la Fuente et al., 2008 (b)), which can be incorporated into complexes without disrupting their

structures (Ito et al., 2006, 2010). In our study, this anionic polymer, HA, was incorporated into polyplexes preparations, and several concentrations of IGFBP peptides were added, namely, 10, 50 and 100 µg, as described above (Fig. 2.7). Polyplexes were characterized regarding their size, PdI and zeta potential, as shown in Table 3.4.

Table 3.4 - Composition, size, PdI and zeta potential of CSHA3 and CSHA5 polyplexes.

Polyplex	IGFBP (µg)	Z-average size (nm)	PdI	Zeta potential (mV)
CSHA	0 µg	375.20	0.62	37.80
CSHA3	10 µg	353.60 ± 167.87 ^{ns}	0.64 ± 0.51 ^{ns}	37.35 ± 1.06 ^{ns}
CSHA5		264.35 ± 55.65 ^{ns}	0.63 ± 0.53 ^{ns}	34.10 ± 3.96 ^{ns}
CSHA3	50 µg	241.15 ± 18.03 ^{ns}	0.25 ± 0.04 ^{ns}	30.25 ± 2.62 *
CSHA5		400.20 ± 75.80 ^{ns}	0.45 ± 0.02 ^{ns}	25.25 ± 2.47 *
CSHA3	100 µg	281.85 ± 44.19 ^{ns}	0.26 ± 0.06 ^{ns}	29.95 ± 1.06 *
CSHA5		556.80 ± 218.07 ^{ns}	0.55 ± 0.14 ^{ns}	18.85 ± 4.03 **

Values are presented as mean ± S.D. Statistical differences were calculated using Dunnett's multiple comparisons test (**p<0.01; * p<0.05 and ns - not significant, compared to control CSHA (nanoparticles without IGFBP peptides)).

According to the literature, addition of HA causes variations in size and zeta potential of polyplexes (De La Fuente et al., 2008), namely an increase in size and a decrease in surface charge (Ito et al., 2006, 2010). This was not observed in our study, since polyplexes without IGFBP peptides, CSHA, yielded polyplexes with a decrease in size and an increase in potential zeta when compared with CS polyplexes, without IGFBP peptides and HA. These results might suggest that when HA was added to CS solution, which might have affected the entanglement of CS chains, it was mostly entrapped inside the polyplexes, and thus not contributing to a decrease of the zeta potential.

Regarding polyplexes prepared with several concentrations of IGFBP peptides, no statistical differences were found on mean size of polyplexes when compared with CSHA polyplexes, yielding polyplexes with mean size between 250-550 nm. However, statistical differences were found in the zeta potential, which was dependent of

concentration of IGFBP peptides, when compared with CSHA polyplexes. These results indicate that addition of IGFBP peptides on polyplexes preparation influenced their size and zeta potential, and mainly the decrease of zeta potential was concentration-dependent of IGFBP peptides.

The efficiency of cellular uptake, as well as endocytic pathway of particle entry and intracellular tracking are factors size-dependents of polyplexes. According to literature, large particles present less internalization than small particles but, however, have a higher rate of gene release into the cytosol due to the prolonged residence time in cytosol of large particles. This prolonged residence time indicates that large particles may avoid rapid lysosomal degradation. Particles with size >200 nm up to >1 μm are internalized mainly by caveolae-mediated endocytosis (Rejman et al., 2004), wherein the motility of caveolae is relatively low but depends on the actin filaments and microtubules network (Le Roy & Wrana, 2005). Taking into account that all formulations in our study yielded polyplexes with a distribution of very heterogeneous size, with ranges between 250 nm up to 750 nm, these results suggest that the predominant polyplexes internalization pathway may be caveolae-mediated endocytosis. The surface charge also affects the cellular uptake level of the polyplexes, and according to the literature positively charged polyplexes interact efficiently with the cell membrane (Agirre et al., 2014). All formulations in our study yielded polyplexes positively charged favoring the internalization.

These results suggest appropriated characteristics of polyplexes, either size as surface charge, which indicate that may be applied as gene delivery systems.

3.2 Evaluation of DNA complexation

The DNA complexation by all polyplexes formulations described above was evaluated by agarose gel electrophoresis. DNA complexation was achieved for all formulations which was observed by the absence of free DNA migration into the gel (Fig. 3.4). These results indicate that all polyplexes complexed DNA effectively regardless of the IGFBP concentration tested.

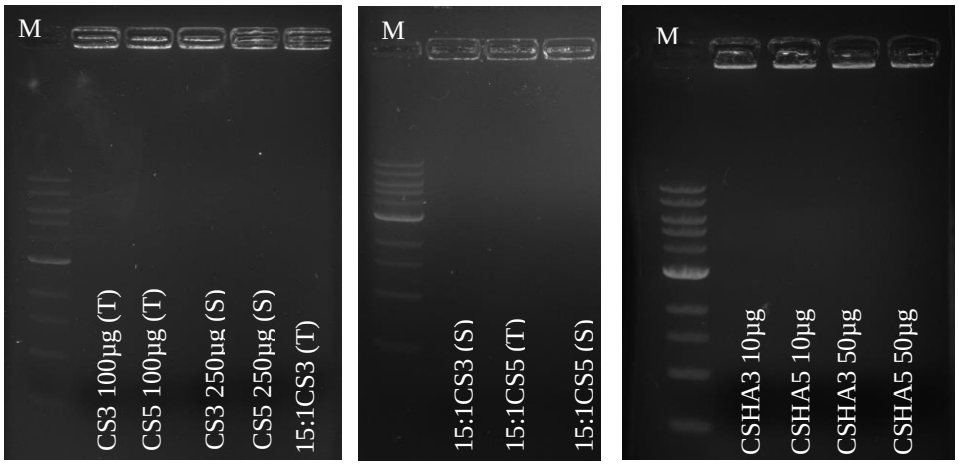

Figure 3.4 – Representative images of evaluation of DNA complexation by polyplexes by agarose gel electrophoresis. Polyplexes showed an efficient DNA complexation, visualized by GreenSafe Premium (data shown for some formulations). M - DNA marker

3.3 Evaluation of cell viability

The cytotoxicity of IGFBP peptides on HEK293T cells was measured through a MTT assay, at 24h and 72h, with increasing amounts of IGFBP-3 or IGFBP-5 peptides, as depicted in Fig. 3.5. No cytotoxicity was observed for either IGFBP peptides regardless of the tested concentration, since cell viability (%) was above 70% for all tested concentrations. These results suggest that the IGFBP peptides are not cytotoxic to cells and may even show some proliferative effect shown by the high values of cell viability at 24h, followed of a decrease at 72h. According to the literature, IGFBP-3 has been described as having the potential to modulate apoptosis (Baxter, 2001), while IGFBP-5 has an important role in controlling cell survival, differentiation and apoptosis (Baxter, 2001; Beattie et al., 2006), which may explain these results. However, it would be interesting to test other peptides concentrations but due to time constrictions and lack of available biological material it was not possible to do so.

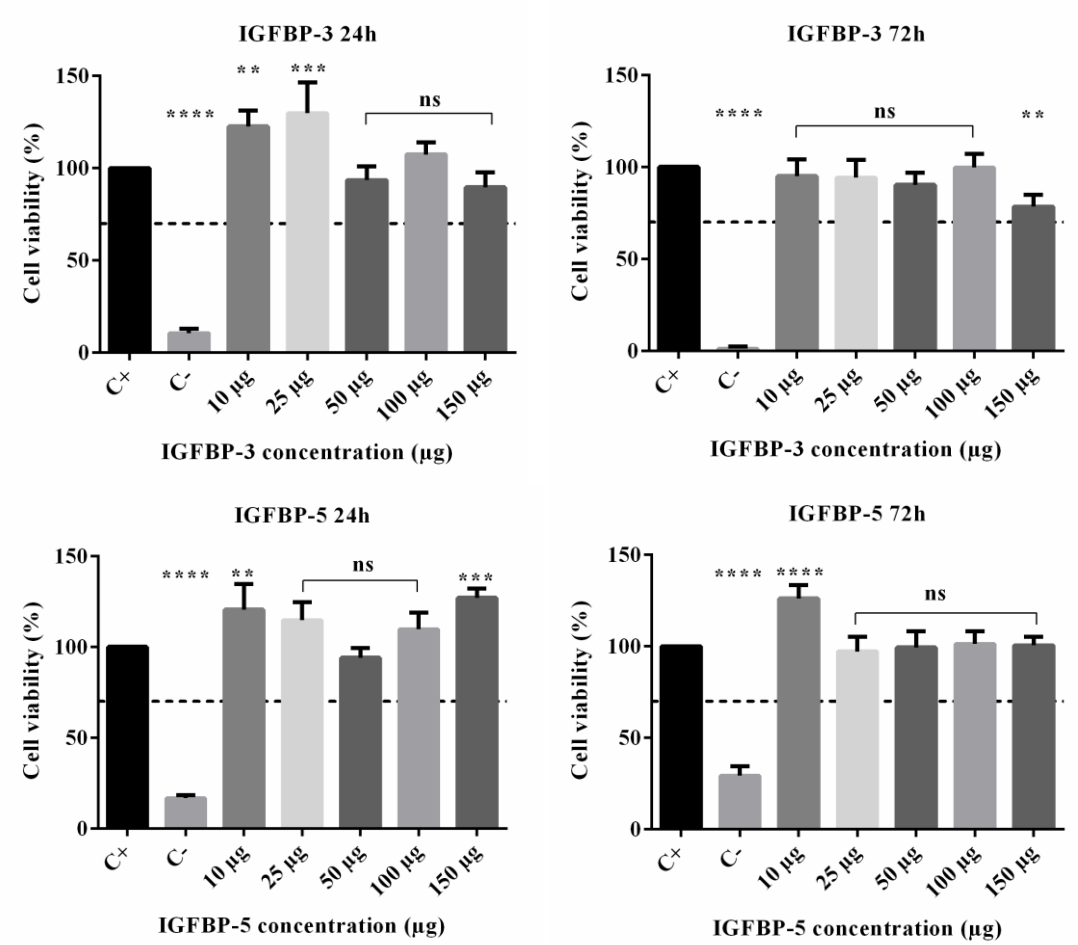

Figure 3.5 - Cell viability (%) after 24h and 72h of incubation with several IGFBP peptides concentrations, respectively. Cells untreated were used as positive control and cells incubated with latex extracts as negative control. Statistical differences, compared to positive control, were calculated using Dunnett's multiple comparisons test (**** $p<0.0001$;*** $p<0.001$, ** $p<0.01$ and ns - not significant).

3.4 Transfection efficiency evaluation

To evaluate transfection efficiency of formulations characterized above a series of transfection assays were performed in HEK293T cells. Gene transfection was evaluated through GFP expression, which was visualized by fluorescence microscopy after 48h and 72h, and lastly, transfection efficiency was analyzed by flow cytometry at 72h.

To evaluate transfection efficiency of $CSNa_2SO_4$ polyplexes, a range of concentrations between 10 µg and 150 µg of IGFBP-3 peptides, corresponding to $CSNa_2SO_43$, were co-administrated at the time of transfection as described above. Polyplexes associated with IGFBP-5, corresponding to $CSNa_2SO_45$, were co-administered with only the three lower concentrations, since no improvement was observed with higher concentrations of peptides (Fig. 3.8) and due to limited amount of available biological material. Polyplexes were visualized by fluorescence microscopy at 48h and 72h, as shown in Fig. 3.6 and Fig. 3.7.

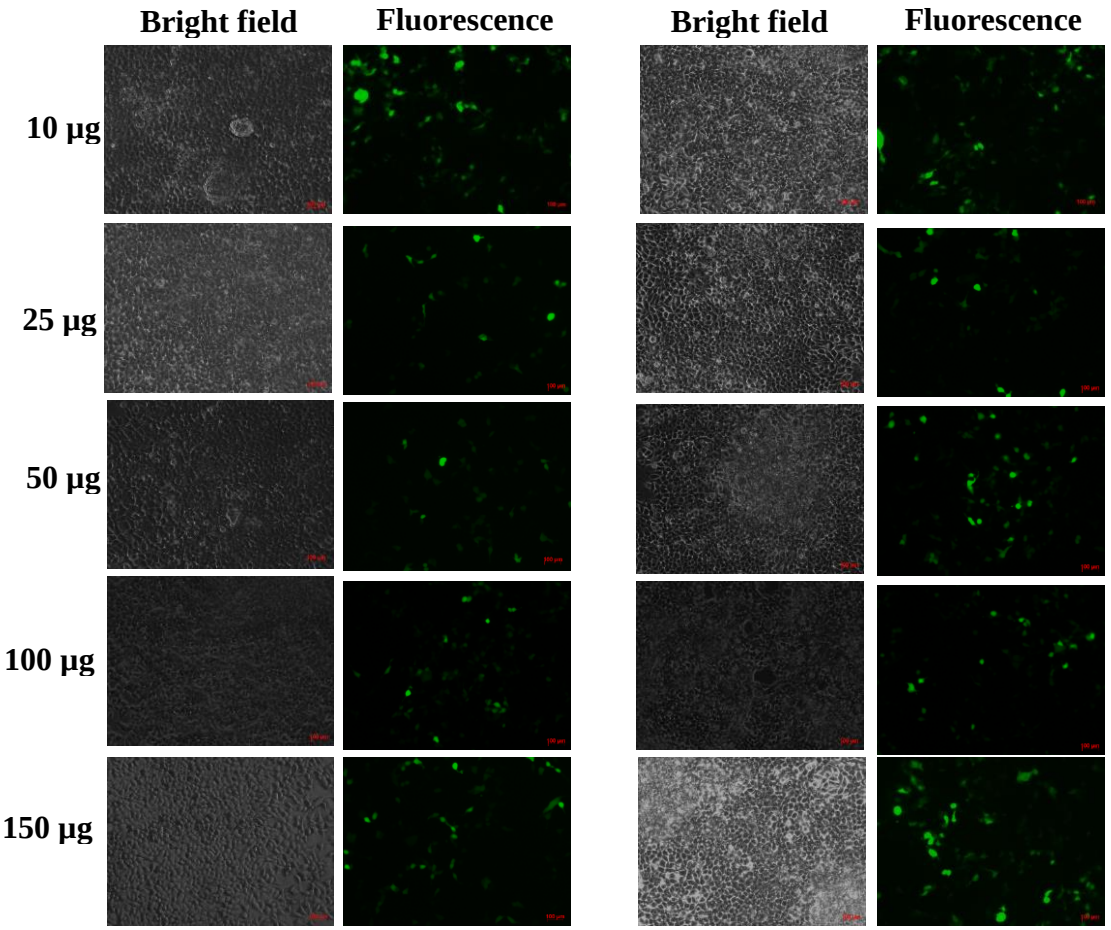

Figure 3.6 - Representative images of fluorescence microscopy of transfected cells by $CSNa_2SO_43$ polyplexes with several concentrations of IGFBP-3, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1µm.

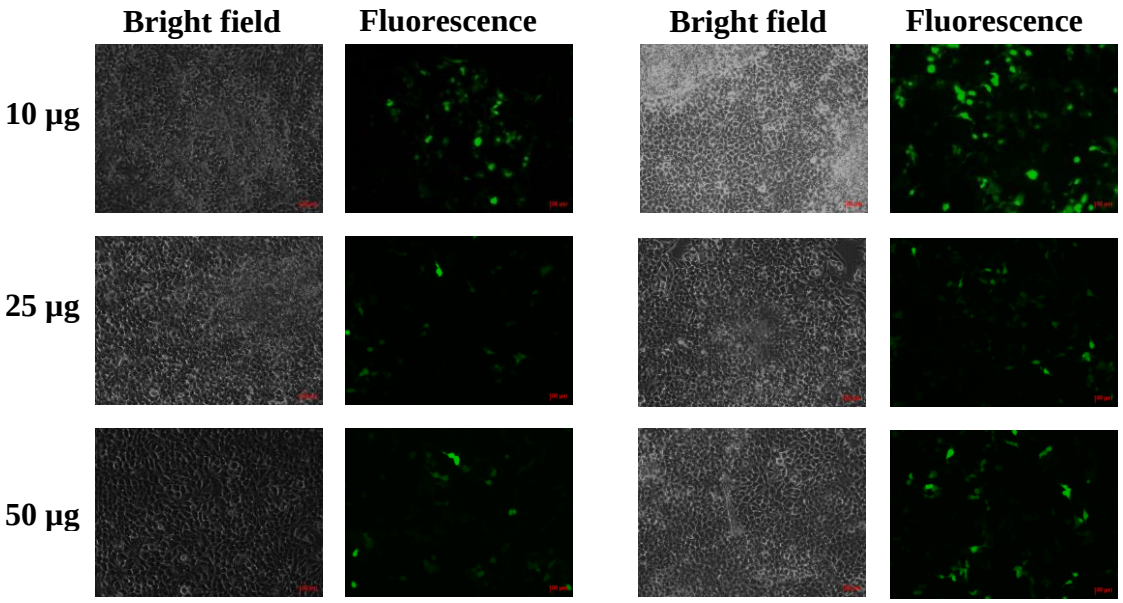

Figure 3.7 - Representative images of fluorescence microscopy of transfected cells by $CSNa_2SO_45$ polyplexes with several concentrations of IGFBP-5 peptides, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm..

The *in vitro* transfection ability of CS/DNA complexes, without IGFBP peptides, was evaluated in HEK293T cells and displayed in Fig. 3.8. The results of flow cytometry analysis, that detect GFP expression, indicated a relatively low transfection efficiency of polyplexes without IGFBP peptides when compared with cells transfected with FuGENE®, a commercial transfection reagent, which was used as positive control. Similar results were achieved in a study, with the same preparation method of polyplexes in HEK293 cells, where a low transfection efficiency of CS/DNA complexes was obtained when compared with Lipofectamine™, a transfection reagent (Mao et al., 2001).

Regarding to polyplexes co-administrated with several concentrations of IGFBP peptides, contrary to our expectations, no transfection efficiency increase was observed for either peptide regardless of the tested concentrations, when compared with polyplexes without IGFBP peptides (Fig. 3.8). Moreover, polyplexes with IGFBP-5 peptides registered a significant decrease in transfection when compared with polyplexes without IGFBP peptides. These results indicate that despite polyplexes presenting an appropriate size for gene delivery, lower than 500 nm, and positive surface charge, the co-administration of increasing amounts of IGFBP peptides did not improve transfection efficiency of polyplexes. Although NLS cationic peptides,

containing lysine and arginine, are capable of binding nonspecifically to DNA by electrostatic interactions (Bremner et al., 2004), in our study, when IGFBP peptides are co-administrated with polyplexes, the interaction between IGFBP peptides and DNA might have been hampered, and thus nuclear entry of DNA appears to be similar with and without IGFBP peptides.

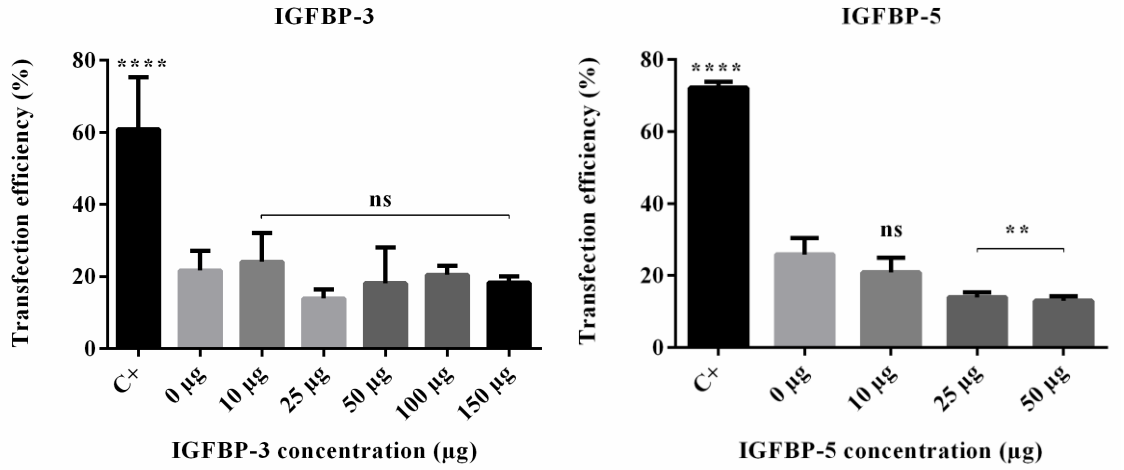

Figure 3.8 - Transfection efficiency represented by percentage of GFP positive cells. Statistical differences were calculated using Dunnett´s multiple comparisons test compared with polyplexes without IGFBP peptides (**** $p<0.0001$; ** $p<0.01$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

As described above, to evaluate if sodium sulfate influences polyplexes formation, this was omitted, and CS3 and CS5 polyplexes were prepared with increasing concentrations of IGFBP-3 and IGFBP-5 peptides, respectively, which were co-complexed at the time of polyplexes preparation. GFP expression was evaluated by fluorescence microscopy, as shown in Fig. 3.9 and Fig. 3.10.

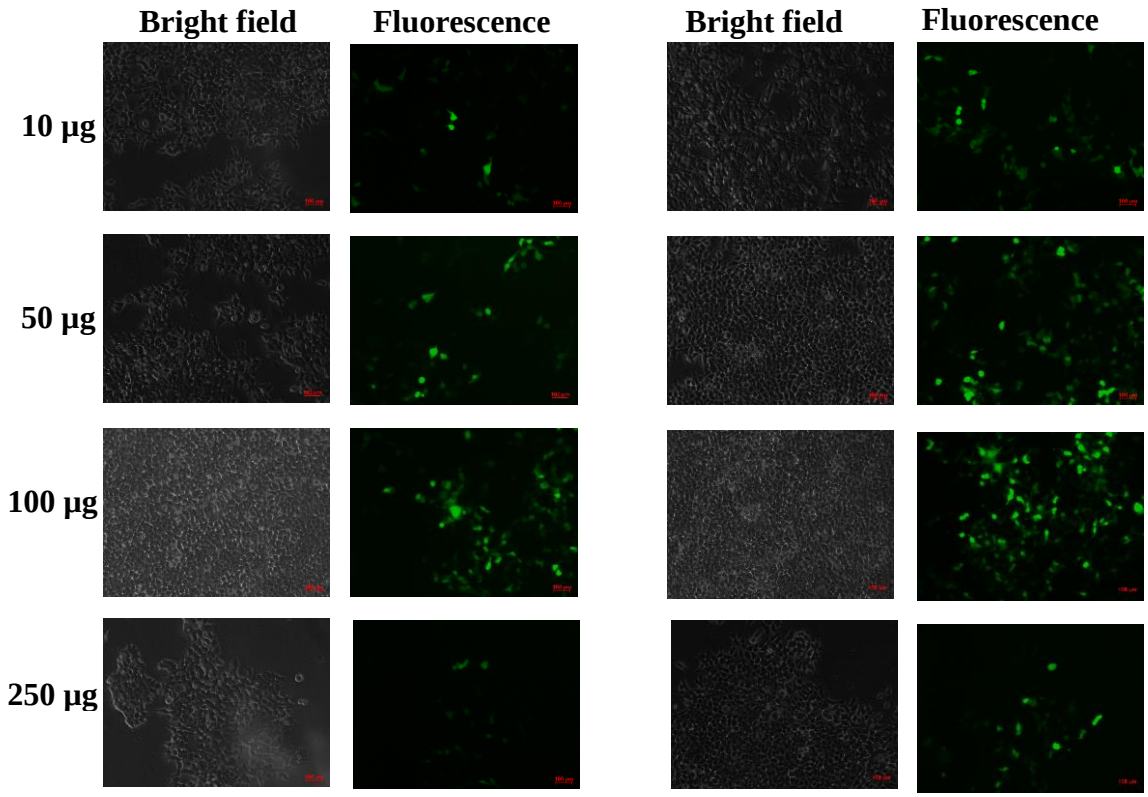

Figure 3.9 - Representative images of fluorescence microscopy of transfected cells by CS3 (S) polyplexes with several concentrations of IGFBP-3, after 48h and 72h, left and right panel respectively. Amplification of 100X and scale bar represents 1µm.

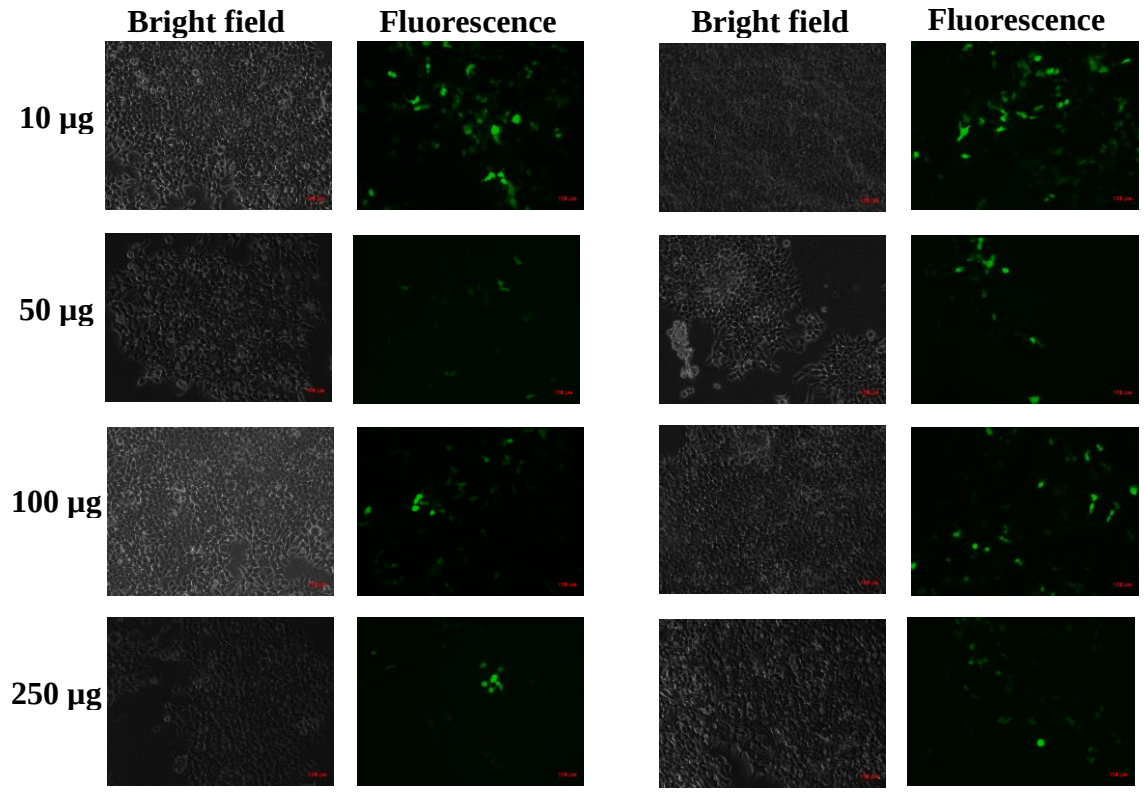

Figure 3.10 - Representative images of fluorescence microscopy of transfected cells by CS5 (S) polyplexes with several concentrations of IGFBP-5, after 48h and 72h, left and right panel respectively. Amplification of 100X and scale bar represents 1µm.

NLSs can be coupled to DNA or to vectors, to improve gene delivery, however, it is not yet clear what is the best form to incorporate the NLS peptides to gene delivery systems (Hébert, 2003). Several studies have widely investigated ways to incorporate NLS peptides, either covalently or non-covalently, to gene delivery systems. Yoo et al., 2007, attached psoralen-NLS conjugates non-covalently to DNA/PEI complexes, and an increase in transfection efficiency in COS-1 cells was observed, when compared with a mutant NLS or DNA/PEI complexes without NLS peptides. Opanasopit et al., 2009, incorporated directly, without covalent conjugation, NLS peptides to DNA or CS polymer. The CS/DNA complexes, with increasing amounts of NLS peptides co-complexed, increased transfection efficiency into Hela cells, in a NLS-dose dependent manner, in comparison to CS/DNA complexes without NLS peptides.

In our study, we prepared CS3 and CS5 polyplexes with increasing concentrations of IGFBP-3 and IGFBP-5 peptides co-complexed, respectively, and analyze GFP expression by flow cytometry at 72h. As shown in Fig. 3.11, a significant increase in transfection efficiency was observed with polyplexes co-complexed with IGFBP-3 peptides, when compared to polyplexes without IGFBP peptides. However, regarding to polyplexes co-complexed with IGFBP-5 peptides, contrary to our expectations, no significant transfection efficiency increase was observed when compared with polyplexes without IGFBP peptides.

These results indicate that IGFBP-3 peptides improve transfection efficiency of polyplexes, however, similar results were not observed with CS5 polyplexes. Previous studies reported that the affinities or accessibility to importin subunits differ between IGFBP-3 and IGFBP-5, although they are not clear (Schedlich et al., 2000), which could explain the difference of results observed in Fig. 3.11. When IGFBP-5 peptides are co-complexed with CS polyplexes, the accessibility of peptides to their nuclear receptors, importins, might be hampered and hence the DNA nuclear delivery is lower, not contributing to an improvement of transfection efficiency.

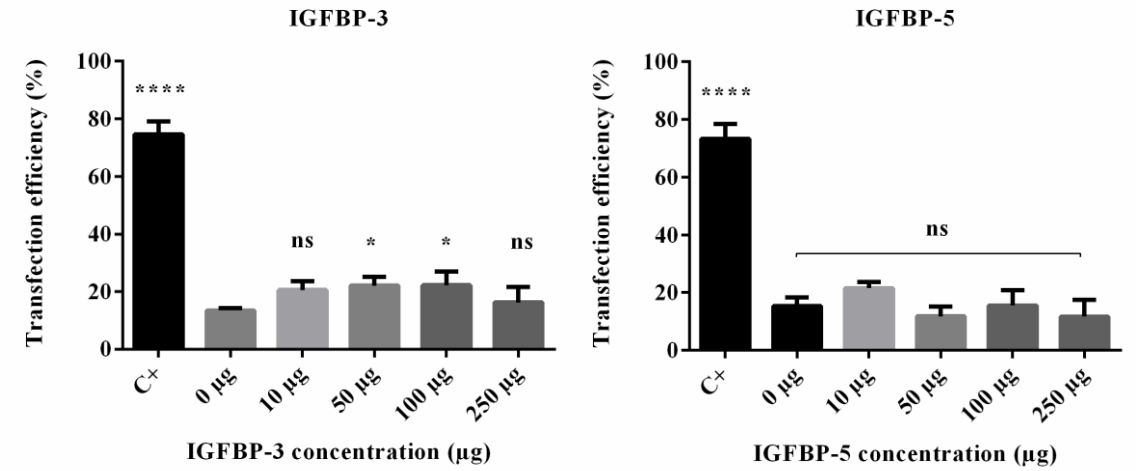

Figure 3.11 - Transfection efficiency represented by percentage of GFP positive cells, of CS3 (S) and CS5(S) polyplexes. Statistical differences were calculated using Dunnett´s multiple comparisons test compared with polyplexes without IGFBP peptides (**** $p<0.0001$; * $p<0.05$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

To evaluate if the mixing technique influences the performance of polyplexes, a fixed concentration of 100 µg was chosen based on results in Fig. 3.11, showing an increase in transfection efficiency of polyplexes, and polyplexes were prepared as described above. Transfection assays were performed in HEK293T cells and visualized by fluorescence of microscopy at 48h and 72h, as displayed in Fig. 3.12.

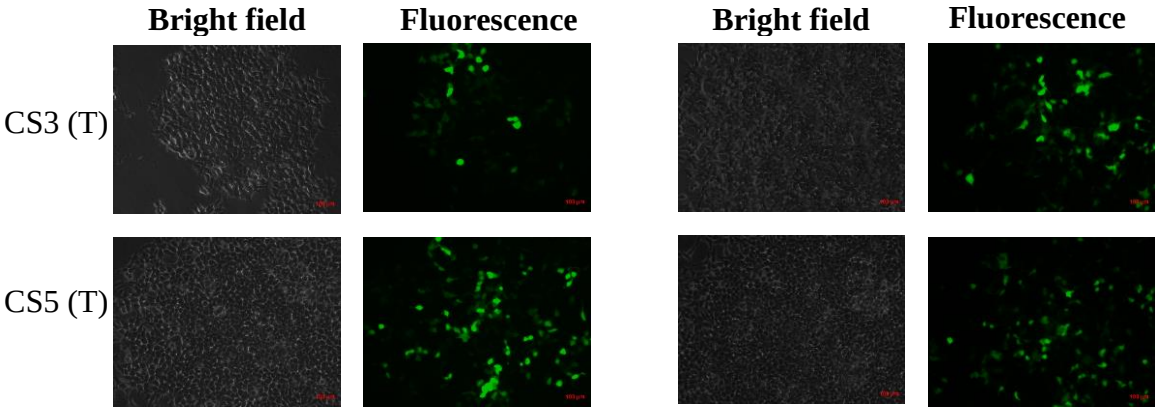

Figure 3.12 - Representative images of fluorescence microscopy of transfected cells by CS3 and CS5, (T), polyplexes with 100 µg of IGFBP-3 or IGFBP-5, respectively. Cells were visualized after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1µm.

Transfection assays of CS3 (T) and CS5 (T) polyplexes were analyzed after 72h by flow cytometry and compared with transfection assays performed with CS3 (S) and CS5 (S) with 100 µg of IGFBP peptides, respectively, and displayed in Fig. 3.13. According to the obtained results in Fig. 3.11, a significant increase in transfection efficiency of polyplexes with IGFBP-3 peptides was observed, while polyplexes with IGFBP-5 peptides obtained similar results to polyplexes without IGFBP peptides (Fig. 3.13). These results indicate that according to previous results (Fig. 3.11), IGFBP-3 peptides improve, in fact, transfection efficiency of polyplexes when compared with polyplexes without IGFBP peptides, and although, the mixing technique used affect the size of polyplexes (Fig. 3.2), it does not appear to affect their effectiveness, as previously reported (Buschmann et al., 2013). Regarding CS5 (T) polyplexes, these similar results to previous results with CS5 (S) polyplexes suggest once again that, when IGFBP-5 is co-complexed in polyplexes, the recognition of peptides by import proteins might be hampered and hence not contributing to an improve of transfection efficiency.

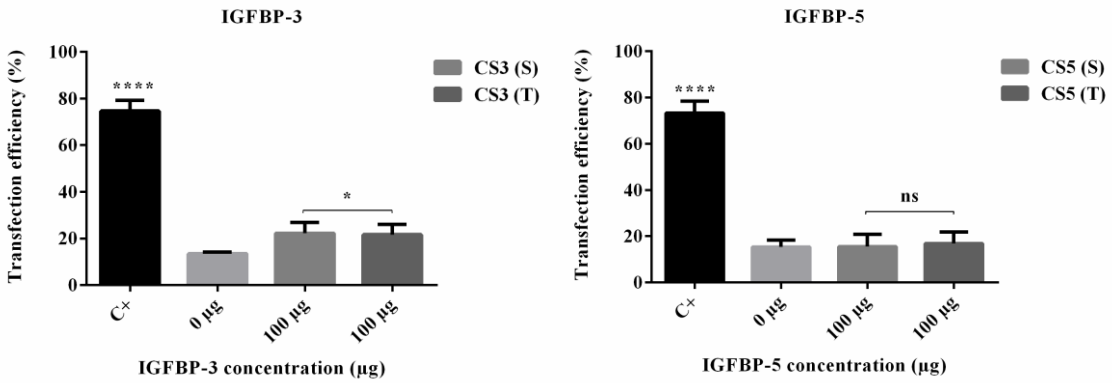

Figure 3.13 - Transfection efficiency represented by percentage of GFP positive cells of CS3 (S) and (T) and CS5 (S) and (T) polyplexes, both with 100µg of IGFBP peptides, respectively. Statistical differences compared with polyplexes without IGFBP peptides were calculated using Dunnett´s multiple comparisons test (**** $p<0.0001$; * $p<0.05$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

For formulations 15:1CS3 and 15:1CS5, (S) and (T), we also choose a concentration of 100 µg in order to evaluate the differences between formulations, and polyplexes were prepared as described above. Fluorescence microscopy was used to visualize the transfection assays after 48h and 72h, as shown in Fig. 3.14 and Fig. 3.15.

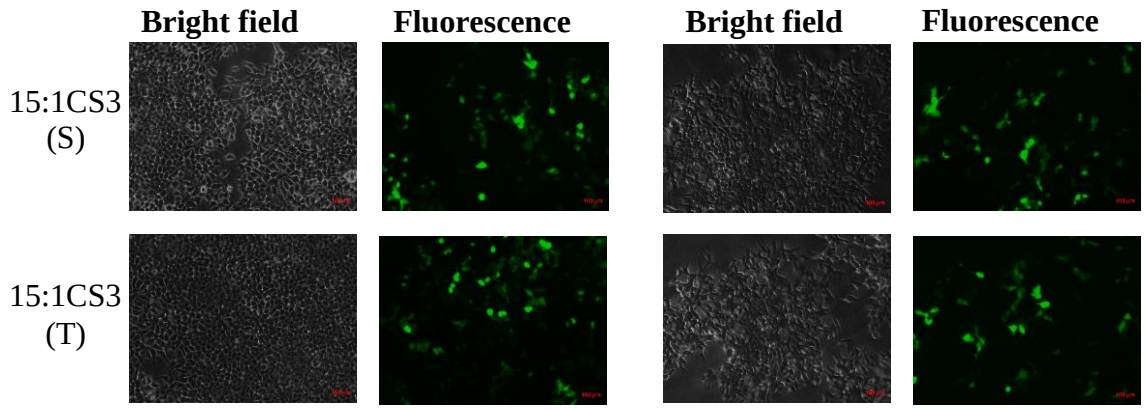

Figure 3.14 - Representative images of fluorescence microscopy of transfected cells by 15:1CS3 (S) and 15:1CS3 (T) polyplexes with 100 µg of IGFBP-3, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm.

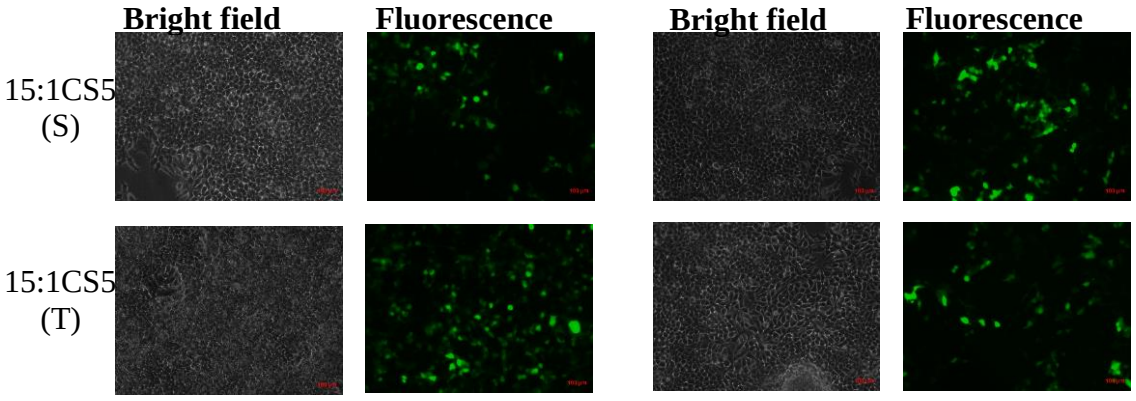

Figure 3.15 - Representative images of fluorescence microscopy of transfected cells by 15:1CS5 (S) and 15:1CS5 (T) polyplexes with 100 µg of IGFBP-5, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm.

Transfection efficiencies were analyzed through GFP expression by flow cytometry, after 72h, and shown in Fig. 3.16. For these formulations, 15:1CS3 and 15:1CS5, we also choose a fixed peptide concentration of 100 µg in order to evaluate differences between polyplexes formulations, and these were prepared considering the total amount of amine groups, including peptides and polymer, at a IGFBP-3 or -5/CS:DNA (N:P ratio) of 15:1. Although amine groups have been reduced, since the amount of CS was adjusted and peptides were included in the N:P ratio, a significant 2-fold increase in transfection efficiency was observed to 15:1CS3 polyplexes, (S) and (T), when compared with polyplexes without IGFBP peptides, corresponding to NP complexes (Fig. 3.16). According to previous results, shown in Fig. 3.8 and Fig. 3.11,

no transfection efficiency increase was observed in polyplexes with IGFBP-5 peptides when compared to NP complexes (Fig. 3.16).

The N:P ratio has been widely investigated (Boulanger et al., 2005; Bremner et al., 2004; Buschmann et al., 2013; Mao et al., 2001; Opanasopit et al., 2009) and described as one of the factors which influence polyplexes formation (size and surface charge), although in our study this influence have not been observed, as well as transfection efficiency (Agirre et al., 2014).

However, taking into account our results (Fig. 3.16), although N:P ratio has been slightly changed in this formulation, it did not influence transfection efficiency of polyplexes. Polyplexes co-complexed with IGFBP-3 peptides yielded a significant increase in transfection efficiency and polyplexes co-complexed with IGFBP-5 peptides not improved of transfection efficiency, when compared to NP complexes, as expected by previous results.

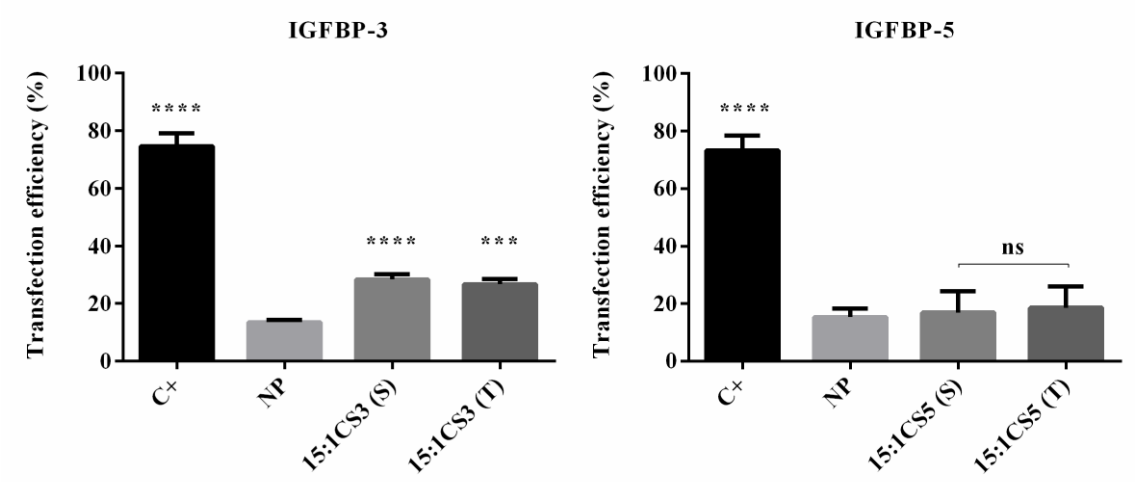

Figure 3.16 - Transfection efficiency represented by percentage of GFP positive cells of 15:1CS3 (S) and (T) and 15:1CS5 (S) and (T) polyplexes, both with 100 µg of IGFBP peptides, respectively. Statistical differences compared with NP were calculated using Dunnett´s multiple comparisons test (**** $p<0.0001$; *** $p<0.001$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

Several methods for covalent attachment of molecules to DNA have been developed and reported (Ciolina et al., 1999; Nagasaki et al., 2003; Neves, Byk, Scherman, & Wils, 1999; Zanta et al., 1999). However, the chemical modification of DNA might cause a decrease in their gene expression (Cartier et al., 2002; Yoo et al., 2007). In our study, we linked IGFBP peptides covalently to CS polymer by amide

bond formation between the carboxylic acid moieties of the IGFBP peptides and the amine groups of CS, which was mediated by a carbodiimidine (EDAC). Transfection assays were visualized at 48h and 72h by fluorescence microscopy, as depicted in Fig. 3.17.

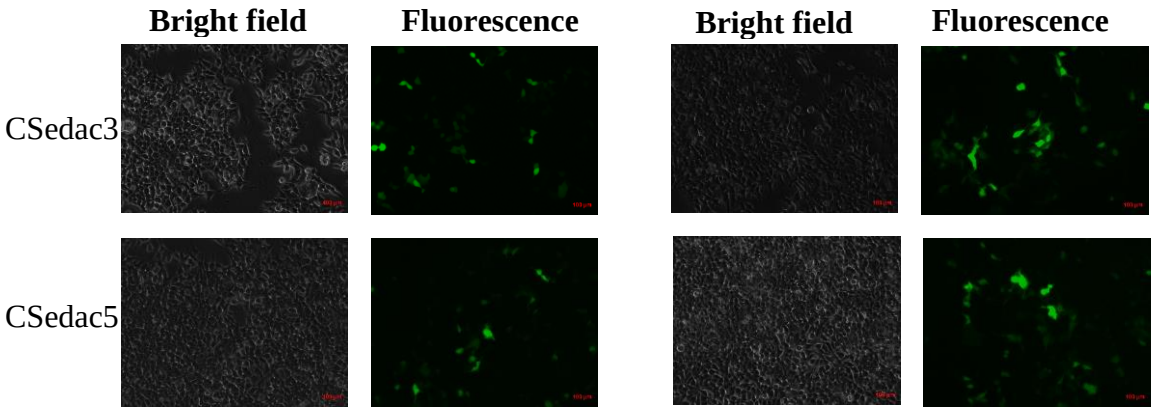

Figure 3.17 - Representative images of fluorescence microscopy of transfected cells by CSedac3 and CSedac5 polyplexes with 100 µg of IGFBP-3 or -5, respectively. Cells were visualized after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm.

As shown in Fig. 3.18, analysis of transfection efficiency at 72h by flow cytometry revealed a 2-fold enhancement in transfection efficiency with CSedac3 polyplexes, when compared to NP complexes, without IGFBP peptides. While no transfection efficiency increase was observed for CSedac5 polyplexes comparatively to NP complexes. Contrary to our expectations, CSedac3 and CSedac5 polyplexes yielded similar transfection efficiencies to previously described formulations. These results indicate that transfection efficiency is improved by polyplexes with IGFBP-3 peptides but not with polyplexes with IGFBP-5 peptides, suggesting, as previously described, that recognition of IGFBP-5 peptides by importins may be being hampered by their entanglement in polyplexes preparation.

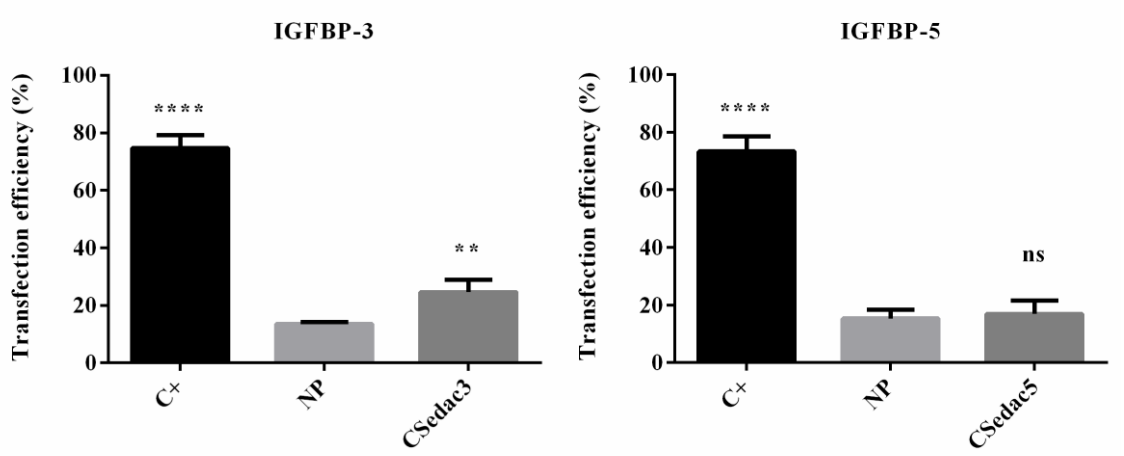

Figure 3.18 - Transfection efficiency represented by percentage of GFP positive cells of CSedac3 and CSedac5 polyplexes, both with 100µg of IGFBP peptides, respectively. Statistical differences compared with NP were calculated using Dunnett´s multiple comparisons test (**** $p<0.0001$; ** $p<0.01$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

The final goal of a gene delivery system is to produce a significant level of transfection efficiency, however, we should take into account, factors as N:P ratio, MW, pH of transfection medium, plasmid concentration, among others, since these factors can influence transfection efficiency of gene delivery systems (Contreras-Ruiz et al., 2011; Lu et al., 2011). The transfection efficiency of CS complexes has been widely investigated and can be improved by combining CS with anionic biopolymers, as HA (Lu et al., 2011). Taking this approach into account, in our study, polyplexes with HA were prepared and a ratio 5:1 (CS:HA) was chosen according to Lu et al., 2011, since transfection efficiencies of CS/HA polyplexes were investigated using ratios from 1 to 5, being the highest level reached at an N:P ratio of 5:1 (CS:HA). Polyplexes were prepared with increasing amounts of IGFBP-3 and IGFBP-5 peptides, respectively, and transfection efficiency was visualized at 48h and 72h by fluorescence microscopy, as shown in Fig. 3.19 and 3.20.

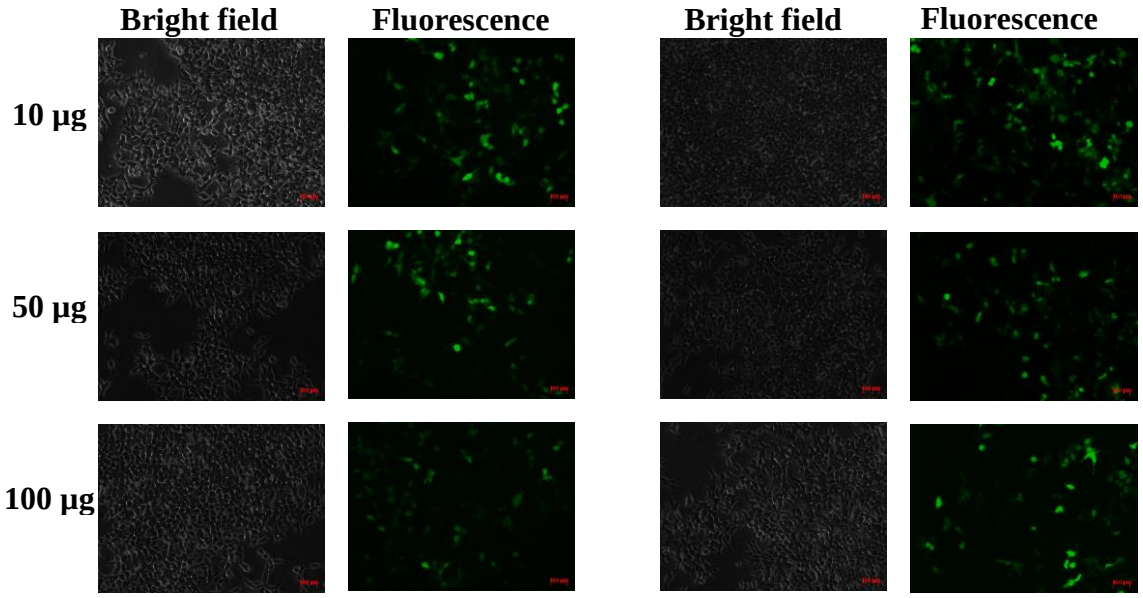

Figure 3.19 - Representative images of fluorescence microscopy of transfected cells by CSHA3 polyplexes with several concentrations of IGFBP-3 peptides, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm.

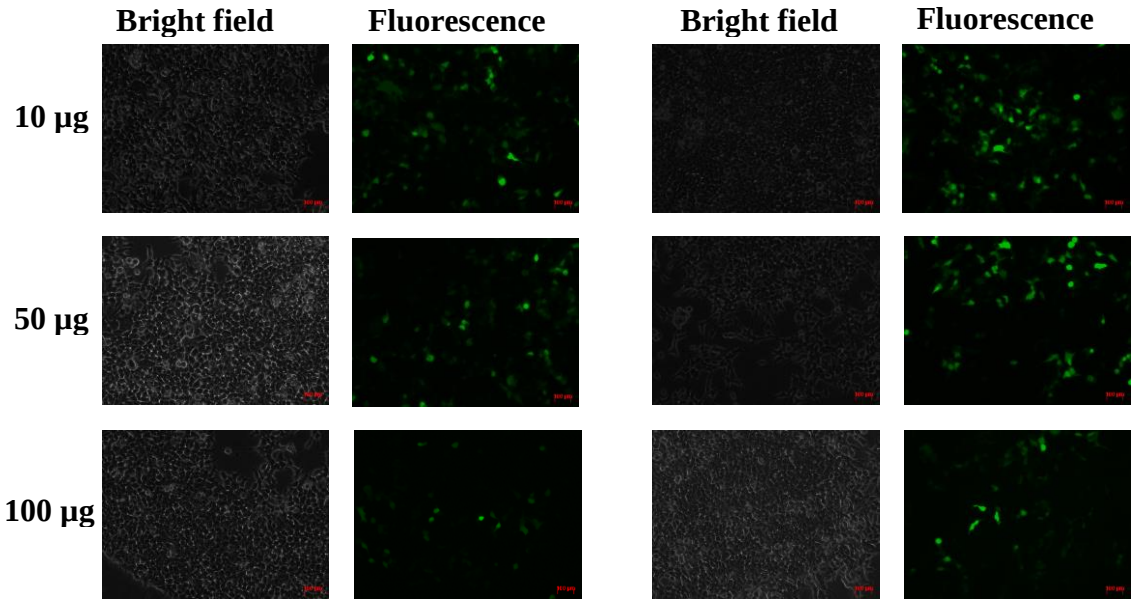

Figure 3.20 - Representative images of fluorescence microscopy of transfected cells by CSHA5 polyplexes with several concentrations of IGFBP-5, after 48h and 72h, left and right panel, respectively. Amplification of 100X and scale bar represents 1 µm.

Transfection efficiencies were analyzed at 72h by flow cytometry, as shown in Fig. 3.21. For these formulations, HA was incorporated into polyplexes preparation, and according to literature (Ito et al., 2010; Lu et al., 2011), an increase in transfection efficiency of polyplexes CS/HA, without IGFBP peptides, was expected, when compared with previously obtained results with CS complexes.

Regarding to polyplexes co-complexed with IGFBP-3 peptides, a significant 2-fold increase in transfection efficiency was observed when compared to polyplexes without IGFBP peptides. Moreover, a significant increase in transfection efficiency was also observed in polyplexes co-complexed with IGFBP-5 peptides, when compared to polyplexes without IGFBP peptides. The transfection efficiency increased when the amount of IGFBP-5 peptides increased and reached the maximum at 50 µg, followed by a decrease by further increments of IGFBP-5 peptides, suggesting that addition of HA may modify the entanglement of CS chains favoring the IGFBP-5 peptides recognition by importins, and thus, improve DNA nuclear delivery in a concentration dependent manner.

The results indicate that addition of HA to polyplexes improves transfection efficiency of polyplexes, as previously reported (Lu et al., 2011), when compared to CS polyplexes. Furthermore, these results indicate that polyplexes co-complexed with increasing amounts of IGFBP peptides, respectively, are significantly more efficient in mediating transfection, depending of concentration of IGFBP peptides, than the CS/HA polyplexes.

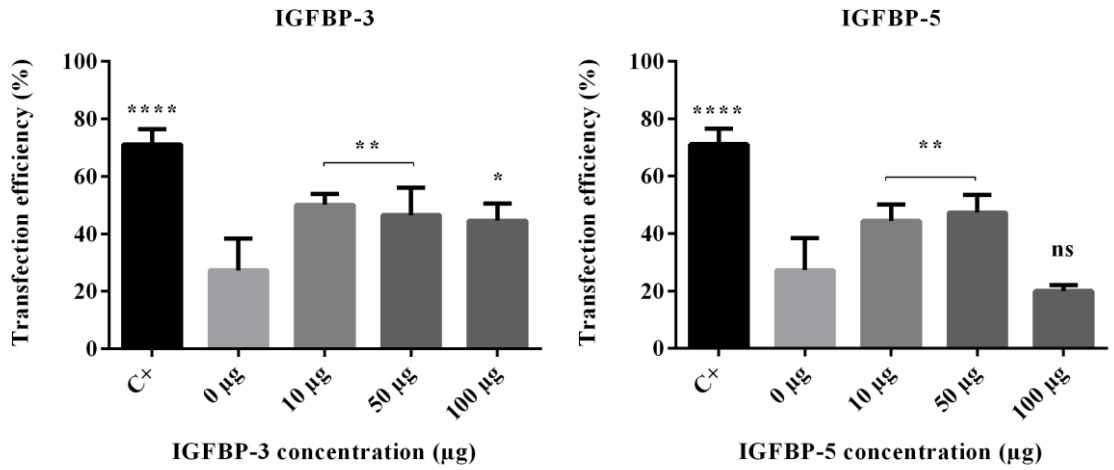

Figure 3.21 - Transfection efficiency represented by percentage of GFP positive cells of CSHA3 and CSHA5 polyplexes. Statistical differences, compared with polyplexes without IGFBP peptides, were calculated using Dunnett´s multiple comparisons test (**** $p<0.0001$; ** $p<0.01$, * $p<0.05$ and ns - not significant). Transfection was performed at a dose of 1µg of DNA for all groups and analyzed 72h after transfection.

4. CONCLUSIONS

This study had as goal to characterize and optimize CS-based non-viral gene delivery systems, improving the transfection efficiency of polyplexes by incorporation of IGFBP peptides, using HEK293T cell line to evaluate their effectiveness.

In this work, we approached three strategies of incorporation of IGFBP peptides in polyplexes, co-administration at the time of transfection, co-complexation and covalent ligation to CS polymer. Firstly, we started by characterizing the physical properties of polyplexes, as their size, PdI and zeta potential, since these are factors that can influence the cellular uptake efficiency, and hence, condition all the transfection process.

Taking our results into account, we observed, by the addition and removal of sodium sulfate from polyplexes, that this has a role in polymer entanglement, since its addition yielded polyplexes with lower size and surface charge. We also observed that addition of increased amounts of IGFBP peptides to polyplexes, influenced physical properties of polyplexes, as their size and surface charge, which were concentration-dependent of IGFBP peptides. However, regardless of the mixing technique, presence of sodium sulfate, N:P ratio, addition of IGFBP peptides, or even, incorporation of a second polymer, HA, all polyplexes formulations yielded polyplexes positively charged, capable of an effective DNA complexation, with an appropriate size (> 1 μm) to their use in gene delivery applications. The IGFBP peptides were also submitted to cellular viability assays, which revealed that peptides are not cytotoxic to HEK293T cells, however, it would be interesting to test other peptide concentrations.

Lastly, *in vitro* transfection assays were performed to evaluate transfection efficiency of polyplexes, which showed that transfection efficiency of polyplexes was concentration-dependent of IGFBP peptides as well as of gene delivery systems employed.

In transfection studies with polyplexes co-administrated with increased amounts of IGFBP peptides, no transfection efficiency increase was observed. When IGFBP peptides are co-administrated with polyplexes, these can be rapidly degraded in the extracellular environment and cellular intake may not be achieved. On the other hand, formulations of polyplexes without HA, co-complexed with increasing amounts of IGFBP peptides, revealed a 2-fold increase in transfection efficiency to IGFBP-3 peptides. A possible explanation for these results may be related to differing affinities or accessibilities to importin subunits between IGFBP-3 and IGFBP-5 peptides, although

not clear. The approach of IGFBP peptides incorporation covalently bind to CS, revealed similar results to polyplexes without HA co-complexed with IGFBP peptides.

Lastly, a second polymer was incorporated into polyplexes formulation, HA polymer. These results showed an increase in transfection efficiency to both IGFBP peptides co-complexed with polyplexes, which may be explained by the addition of HA into polyplexes, suggesting a modification in CS chains entanglement favoring increased IGFBP-5 peptides recognition by importins, and thus, improved of DNA nuclear delivery.

To the best of our knowledge, this is the first study where IGFBP peptides are associated to CS-based non-viral gene carriers used for gene delivery. On the whole, the results shown that, although optimization of transfection efficiency is still needed, polyplexes co-complexed with IGFBP peptides are indeed good candidates for non-viral gene delivery systems. The polyplexes with two combined polymers, chitosan and hyaluronic acid, were those that showed greater transfection efficiencies for both peptides, IGFBP-3 and IGFBP-5, being considered the best formulation in our study.

In the future, it would be interesting not only expanding the range of concentrations tested IGFBP peptides as well as the type of cell lines, further comprising the optimization of the formulations of polyplexes.

5. REFERENCES

5. References

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