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New Fully Biodegradable PEG-Dendrimers to act as siRNA Vectors to the Nervous System

Cristiana Filipa Vieira de Sousa

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Dissertação de Mestrado em Bioquímica apresentada à Faculdade de Ciências da Universidade do Porto, Instituto de Ciências Biomédicas Abel Salazar

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

“Somewhere, something incredible is waiting to be known.”

Carl Sagan

Agradecimentos

Após finalização deste meu percurso académico, cabe-me agora agradecer, aos muitos que contribuíram para que os meus objetivos fossem concretizados.

Desde já, agradecer à Faculdade de Ciências da Universidade do Porto, na pessoa do Magnifico Sr. Reitor Sebastião Feyo de Azevedo, por me ter proporcionado o ingresso neste Mestrado;

Ao Professor Doutor Pedro Fernandes e ao Professor Doutor Paulo Correia de Sá pela sua disponibilidade e apoio na qualidade de Diretor do Mestrado em Bioquímica;

À Doutora Victoria Leiro Rodríguez e à Professora Doutora Ana Paula Pêgo por me terem recebido como orientanda, por toda a ajuda, dedicação, amizade, conselhos e sugestões. Um enorme obrigada, especialmente por todos os conhecimentos que me foram transmitidos, bem como pela disponibilidade que tiveram e pelas palavras amigas sempre presentes durante a realização deste trabalho de estágio;

À Mestre Ana Spencer e à Mestre Natália Magalhães pela ajuda na realização da parte experimental deste trabalho, pelo apoio e pelos conhecimentos transmitidos;

A toda a equipa nBTT pela amizade, pelo companheirismo, pela ajuda e pelas palavras de incentivo sempre presentes;

A todos os membros do laboratório 214.S3 e técnicos do Instituto de Investigação e Inovação em Saúde que contribuíram com os seus conhecimentos para o desenvolvimento da minha dissertação;

À Ana Sousa, Rita Rodrigues e Gisela Sousa por toda a amizade, pelos momentos excecionais, pelas palavras amigas, por toda a ajuda, pelo companheirismo e pelo apoio incondicional;

À Diana Meireles, pela amizade, pelo apoio, pelos momentos incríveis, pela partilha conjunta e companheirismo;

Finalmente agradecer à minha Família, especialmente aos meus pais e irmão, por todo o carinho e amor transmitidos e por todos os sacrifícios feitos para poder obter a minha formação. Obrigada por acreditarem em mim e nunca me deixarem desistir. Obrigada pelos valores transmitidos, que fizeram de mim a pessoa que sou!

Um muito obrigada a quem de algum modo marcou de forma positiva o meu caminho e me permitiu concluir este percurso com sucesso!

Resumo

Devido ao elevado número de lesões e tumores que afetam o sistema nervoso, existe uma grande necessidade de desenvolvimento de novas formas de tratamento, como a terapia génica. Entre as abordagens que tem vindo a ser exploradas, a capacidade de inibir temporariamente a expressão génica de uma proteína através da inserção de material genético exogéneo, como pequenos RNAs de interferência (siRNA), tem tido grande potencial terapêutico em contexto clínico. Porém, este tipo de terapia requer o desenvolvimento de vetores clinicamente adequados, seguros, efetivos e biocompatíveis com capacidade de proteger e complexar os ácidos nucleicos, atravessar as barreiras biológicas, culminando na entrega do ácido nucleico terapêutico com eficiência e toxicidade mínima, às células alvo.

Os dendrímeros são excelentes vetores de entrega de ácidos nucleicos devido à sua estrutura globular, bem-definida, ordenada e altamente ramificada, tamanho ajustável, presença de vários grupos terminais capazes de serem funcionalizados com diferentes ligandos e à sua capacidade de proteger e complexar o ácido nucleico em nanoestruturas, denominadas “dendriplexos”. Contudo, uma desvantagem importante dos dendrímeros mais utilizados atualmente é a sua não degradabilidade sob condições fisiológicas, o que consequentemente leva a que estes se acumulem no interior de células e tecidos, podendo desencadear efeitos citotóxicos. Devido a isto, os nossos objetivos focam-se no desenvolvimento de PEG-dendrímeros. Contudo, isto é uma tarefa altamente desafiadora devido à degradação indesejável/ prematura observada durante a sua síntese e/ ou aplicação. Portanto, não existe um grande número de estruturas dendríticas biodegradáveis na literatura, aplicadas a funções específicas em biomedicina. Devido a isto, uma nova família de PEG-dendrímeros biocompatíveis e biodegradáveis foi recentemente proposta pelo nosso grupo de investigação para atuar como vetores versáteis em diferentes aplicações biomédicas.

Neste projeto, sintetizamos PEG-dendrímeros completamente biodegradáveis de geração 3, com muito bons rendimentos. Posteriormente, eles foram funcionalizados através de “click” chemistry com dois grupos amina diferentes para avalia-los como vetores de entrega de siRNA para o sistema nervoso, testando a sua capacidade para entregar siRNA a uma linha de células neuronais. Os nossos PEG-dendrímeros mostraram boa capacidade para complexar e proteger o siRNA. Os dendriplexos resultantes mostraram tamanhos entre 70-88 nm e cargas de superfície positivas, o que são propriedades físico-químicas adequadas para internalização celular. Além disso, os dendrímeros e correspondentes siRNA dendriplexos mostraram um perfil não-tóxico e boa capacidade de internalizar o siRNA numa linha de células neuronais, levando a excelentes níveis de eficiência de transfeção.

Palavras-chave: Dendrímeros, Terapia génica, siRNA, Nanopartículas, Biodegrabilidade, Células Neuronais

Abstract

Due to the high incidence of injuries and tumors that affects the nervous system, there is a growing need of development news forms of treatment, such as gene therapy. Among the approaches being explored, the capacity to inhibit temporarily the gene expression of a protein through the insertion of exogenous genetic material, like small interference RNA (siRNA), has a good therapeutic potential in a clinical setting. Nevertheless, this therapy requires the development of clinically suitable, safe, effective and biocompatible vectors with the ability to protect and complex the nucleic acid, to cross the biologic barriers, and culminating in the delivery of the therapeutic nucleic acid with high efficiency and minimum toxicity to the target cells.

Dendrimers are excellent vectors for nucleic acid delivery due the globular, well-defined, ordered and highly branched structure, controllable nanosize, the presence of several terminals groups that can be functionalized with various ligands and their ability to protect and complex nucleic acids in nanostructures, named “dendriplexes”. However, one important drawback of the most currently used dendrimers is their non-degradability under physiological conditions, therefore they can accumulate inside the cells and tissues, triggering cytotoxic effects. So, recent interest has been focused on the development of biodegradable dendrimers. However, this is a highly challenging task because of the undesired and/or premature degradation observed during their synthesis and/or application. Therefore, there is no in the literature many examples of biodegradable dendrimers applied for specific functions in biomedicine. Because of this, a new family of biocompatible and biodegradable PEG-dendrimers has been very recently proposed by our research group that can act as versatile vectors in different biomedical applications.

In this project, we synthesize the fully biodegradable PEG-dendrimers, until generation 3 in very good yields. Moreover, we have successfully functionalized them, by means of “click” chemistry with two different amine moieties to evaluate them as siRNA delivery vectors to the nervous system, testing their ability to deliver siRNA to a neuronal cell line. Our PEG-dendrimers showed a good ability to complex and protect siRNA. The resulting dendriplexes showed sizes between 70-88 nm and positive surface charges, which are suitable physicochemical properties for cellular uptake. Moreover, these dendrimers and their corresponding siRNA dendriplexes showed a non-toxic profile and a great capacity to internalize siRNA into a neuronal cell line, leading to excellent levels of transfection efficiency.

Keywords: Dendrimers, Gene therapy, siRNA, Nanoparticles, Biodegradability, Neuronal Cells

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List of Abbreviations

AGO-2	Protein argonaute-2
AONs	Antisense oligonucleotides
bis-HMPA	2,2-bis(hydroxymethyl)propionic acid
CuAAc	Cu(I)-catalyzed azide-alkyne cycloaddition
DLS	Dynamic Light Scattering
dsRNA	Double-stranded RNA
fbB	Fully Biodegradable Benzylamine-terminated PEG-dendrimer
fbD	Fully Biodegradable Diamine-terminated PEG-dendrimer
FDA	Food and Drug Administration
FTIR	Fourier-transform infrared spectroscopy
GATG	Gallic Acid-Triethylene Glycol
G	Generation
GATGE	Gallic Acid Triethylene Glycol Ester
miRNA	Micro RNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
NMR	Nuclear Magnetic Resonance
N/P	Amine-to-phosphate ratio
PAMAM	Poly(amido amine)
PB	Phosphate Buffer
PBS	Phosphate Buffer Saline
PDI	Polydispersity Index
pDNA	Plasmid DNA
PEI	Poly(ethylene imine)
PEG	Poly(ethylene glycol)

PEPE	Poly(ether) copoly(ester)
PLL	Poly-L-lysine
PPI	Poly(propylene imine)
Pri-miRNA	Primary pri-miRNA
RES	Reticuloendothelial system
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Room Temperature
siDNA	Small Interfering DNA
siRNA	Small interfering RNA

Chapter 1 – Introduction

1.1 Gene Therapy

Nervous system can be affected by many numbers of lesions, diseases and tumors, leading to the partial or total loss of motor, sensorial or autonomic functions.[1-2] Among the approaches being explored, gene therapy has gained significant attention as a promising therapeutic strategy for the treatment of a wide variety of these injuries and diseases,[2-4] such as cancer,[5] arthritis,[2] viral infections,[3] neurodegenerative diseases[4] and autoimmune diseases[6] or hereditary diseases.[7]

Gene therapy consists on the introduction of nucleic acids to modulate temporarily the gene expression of a protein through the insertion of exogenous genetic material for therapeutic benefits.[8-9] However, the effect on the cells is dependent on the type of nucleic acid that is introduced, for example, plasmid DNA (pDNA) can be used to compensate the total lack of expression of a certain protein or the expression of a non-functional protein. Otherwise, the insertion of antisense oligonucleotides (AONs) or a small interfering RNA allows mediate the down-regulation of defective genes.[10-13]

However, when administered intravenously, free nucleic acids can suffer from endogenous nucleases degradation and they are incapable of crossing the plasma membrane, due to their negative charge and hydrophilicity.[14-18] Moreover, in the case of reaching the interior of cell, they are again subjected to endonucleases degradation in the cytoplasm or in endosomes/lysosomes. Thus, therapeutic nucleic acids need to be carried to their specific sites (nucleus in the case of pDNA, and cytoplasm in the case of siRNA and AONs), protected from degradation to achieve the desired biological effects. Therefore, the inclusion of nucleic acids into a major molecule, named vector, provides them some capacity for overcome the intra and extracellular barriers.[19-20]

Therefore, the success of this therapy is dependent of drawing a suitable carrier/vector, capable of protect nucleic acid from the degradation *in vivo* environment, provide a sufficient circulation time by avoiding premature clearance and internalize efficiently into the target cells for posterior delivery the therapeutic agent.[11-13] Initially, researches were focused on using viral carriers genetically altered, as retrovirus and adenovirus, in clinical trials and laboratory studies due to their high transfection levels. However, viral vectors demonstrated various inconvenient, namely, insertional mutagenesis, inflammatory effects, immune response and safety issues for cells.[14] These limitations in viral vector systems lead to the development of non-viral vectors, safe, efficient, specific and non-pathogenic for gene delivery. However, non-viral vectors present lower transfection efficiencies than viral ones, because of this, over the last few years, researchers have been focused on improving these transfer efficiencies.[15] Since

the primary human gene therapy test performed by Rosenberg in 1989, many clinical trials have been followed. In August of 2017, the Food and Drug Administration (FDA) approved the first gene therapy in the USA, which will have as target kids' population with acute lymphoblastic leukemia. The implementation of these type of techniques constituted a mark in treatment of patients in a country that is one of the world's greatest powers.[16-17]

1.1.1 Gene Therapy Strategies

Gene therapy can follow different pathways, consisting the most classic approach on the introduction of pDNA in nucleus, which uses later the cellular machinery to express the therapeutic gene, resulting in the expression of therapeutic proteins.[11] To control the gene expression, the pDNA sequence can be further modified to carry promoters, enhancers, splicing and polyadenylation sites. So, if correctly applied, gene therapy could be used to treat patients with lack of a specific protein or set of proteins. For instance, many studies have already been done towards the delivery of factor VIII, involved in blood clotting process.[13] Another approach consists of the down-regulation of specific deregulated genes, which actively produce excessive amounts of deleterious proteins. This approach is achieved using AONs and siRNA. Both nucleic acids are synthesized knowing the sequence of the disease-causing gene, which will bind to the messenger RNA (mRNA) through the bases pairing stipulated by Watson-Crick, resulting in the inhibition of translation and consequently in the silencing of protein expression.[21-22]

Some AONs were shown to effectively inhibit the ribosomal access and movement along the mRNA by steric hindrances. One alternative mechanism for AONs is the RNAse-H mediated cleavage of the mRNA sequence in the RNA/DNA duplex. So, while the mRNA sequence is degraded, the oligonucleotide fragment remains intact and therefore available to bind another complementary mRNA.[14-17] Regardless of the mechanism chosen by the AONs, silencing efficiency is dependent on the AONs sequence as well as the affinity to the mRNA. So, it is crucial the drawing of the AONs sequences well-thought and good knowledge of the target mRNA, to achieve maximum silencing activity.[23-26]

The unmodified oligonucleotides have low stability and low cellular internalization, which led to the development of new chemically modified chains to increase their stability.[18-20] The first modification was done in 1980 and consisted of the

introduction of phosphorothioate linkage between the nucleotides and modification of the 3' - and 5' -terminal with 2'-O- methoxyethyl or 2'-O- methyl. Modifications in 2' -OH ribose with the CH_3 group both in the sense and antisense chain protects the molecule from degradation by nucleases, increasing the time of circulation inside the organism. [23] Moreover, the change at ends, also enhance the bind to the target mRNA and reduce the associated collateral effects.[24] Otherwise, scientists can use vehicles to protect and carry the therapeutic nucleic acid without need to make changes in its structure. Among the vast set of strategies to modulate gene expression,[27-28] the potential of therapies based in the interference RNA (RNAi) mediates by a small interfering RNA to regulate the abnormal expression of a protein or mutated proteins involved in human diseases,[29] is a strategy quite promising in clinic context for treat genetic problems in humans.[30-31] RNAi process was initially observed in plants (petunias) by Napoli and Jorgensen, in 1990,[32] and in 1998 in nematodes (*Caenorhabditis elegans)* by Andrew Fire and Crag C. Mello.[33-36] In 2001, was demonstrated similar processes in mammalian cells, which led to the emergence of new tools for study genetic function.[37]

1.1.1.1 Gene Therapy with siRNA: mechanism, advantages and barriers

RNAi works like a regulatory mechanism post-transcriptional[24, 27, 29, 38] of genes, proteins and RNA[37] and its synthesized in two steps.[39] In the first phase, initiation phase, two effectors molecules (micro RNA (miRNA) (21-25 nucleotides) and smalls interference RNAs (21-23 nucleotides) are produced through the degradation of double-strand RNA (dsRNA).[40] This phase starts with the transcription of primaries precursor of mRNA (pri-miRNA) (80 nucleotides) in the nucleus by RNA polymerase II. Then, the pri-miRNAs are processed in the nucleus by DGCR8 and Drosha, producing a structure with the form of harpin with 60-70 nucleotides, structure named "pre-miRNA". Once finishing the nuclear process by Drosha, the pre-miRNA is exported to the cytoplasm with the aid of caryopherina-5 and their co-factor Ran (vie exportin 5).[37] Then, in the second phase, effector phase, Dicer cleaves the pre-miRNAs into small and imperfect miRNA duplexes that contain the miRNA passenger strand and guide strand.[5, 28] The passenger strand is degraded and the guide strand interacts with the RNA-induced silencing complex (RISC)[33] in conjunct with multifunctional protein argonaute-2 (AGO2).[35] Guide strand remains in complex[39] for guiding complex AGO2-RISC to bind to the target complementary mRNA. If the homology in base pairing is high, this results in the cleavage of the RNA in the cell in complementary sequences at guide strand for

degradation.[29] In the opposite, when the homology between the guide strand and the target complementary mRNAs is poor, the translation of the mRNA will be blocked (Figure 1.1).[37, 40-42]

siRNAs has capacity to regulate untargeted genes and the recognition of long exogenous dsRNAs reflects an innate cellular defense mechanism against double-strand RNA viruses. Another way to trigger this mechanism, is by the introduction of chemical modifications of the siRNA that will reduce the immunostimulatory effects and the insertion of siRNA fragments that mimic Dicer-cleaved, endogenous micro RNAs and interact directly with RISC. Unlike pDNA, siRNA do not have to cross the nuclear membrane for carrying out their activity and therefore there is no need the action of exportin 5 to aid the nucleic acid to leave the nucleus since acts on the cytoplasm. Thus, is expected a faster development and higher transfection efficiencies with siRNA strategies.[43-45]

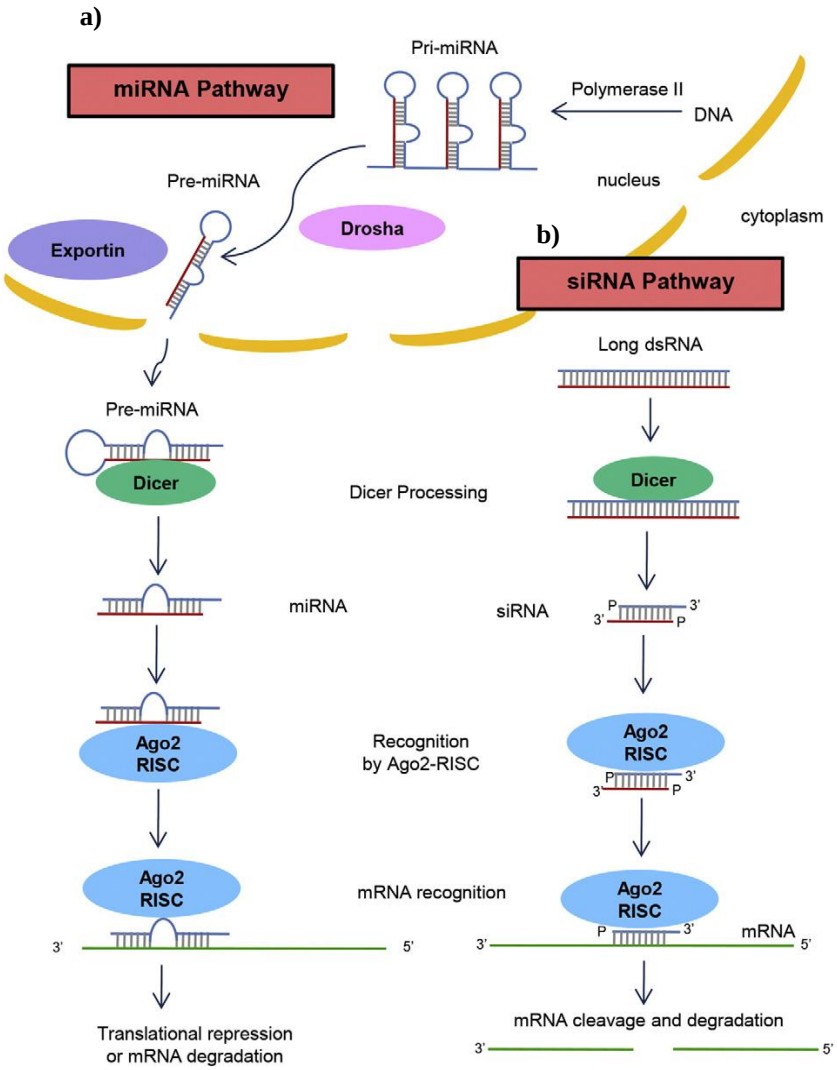


Figure 1. 1 - The current model for the biogenesis and post-transcriptional suppression of micro RNAs and small interfering RNA. a) The long precursor miRNA (pri-miRNA) are transcribed from a gene by RNA polymerase and they are processed by the Drosha protein into precursors of ~70 nucleotides (pre-miRNA) within the nucleus. Pre-miRNA are exported to the cytoplasm by Exportin 5 and then it is cleaved by Dicer (RNAse II) into a small miRNA duplex with imperfect complementarity. miRNAs are recognized by Argonaute 2 (AGO2) and RNA-induced silencing complex (RISC), leading to the separation of the strands and removal of the passenger strand. The strand guide miRNA guides the AGO2- RISC complex to bind a complementary region on an mRNA and inhibits translation of the message. **b)** Cytoplasmic long double-stranded RNA is cleaved by the endonuclease Dicer to form a small *siRNA* (21-23 nucleotides). siRNA is recognized by the AGO2-RISC enzyme complex and one of the strands is degraded (passenger strand) and the other strand guides the complex to recognize mRNA sequences, resulting in cleave and degradation of target.[37]

Among the various mechanisms to silence the protein expression,[29] the siRNA-based therapies are a really good choice[44] because the ease to discover unmodified siRNAs that work with high potency and specificity.[33] There are several studies showing that the therapy with siRNA was effective to silence genes in very varied cells and diseases,[45] acting like antiviral, anticancer and therapeutic agents of the central nervous system, inflammation and cardiovascular therapy, among others.[39] siRNA has two strands, making them more resistant to degradation by endonucleases. Moreover, its antisense strand is fully complementary to the target mRNA whereas, for instance,

dsRNA is only partially complementary to the target RNA.[29, 36-38] Because of both characteristics, the siRNA therapeutic effect is more potent and prolonged. It was shown that the large silencing capacity can last for 5-7 days in rapidly dividing cells and for several weeks in non-diving cells.[23] Moreover, the siRNA synthesis is easy and does not require any system of cellular expression neither purification complex of proteins. Therefore, siRNA is an attractive nucleic acid for therapy,[5] allowing, this way, the development of a siRNA synthesis methodology able off selectively a target gene.[23] There are already many commercially available ones with the ability to silence almost any gene in several different animals.[35]

Nevertheless, despite the great advances made in recent years in the siRNA gene therapy field, there are still obstacles to overcome to reach its clinical application. These include: 1) instability *in vivo* because of the degradation in extracellular medium that could be resulting from siRNA short half-life in serum (only a few minutes),[28] possibility of aggregation with serum proteins, elimination by reticuloendothelial system (RES) and renal filtration,[46] which consequently conduces at 2) low cellular internalization[6] and 3) off-target silencing gene, leading at biological results that are caused by different interactions than those that occurs with the intended target, which can lead to toxicity[7, 27, 39] and 4) initiate an innate immune response which gives rise to side effects such as introduction of pro-inflammatory cytokines or interferons.[42]

1.2 Vectors

The intentional introduction of nucleic acids into the correct cells represents a critical step in gene delivery. Therefore, depending the type of tissues, when intravenously administered, nucleic acids has more or less facility to overcome barriers before they reach the target cells.[11] Because of this, sometimes is necessary resort to noninvasive methods or minimally invasive, which denotes the necessity to development safe, suitable and efficient vehicles for gene delivery.[23] As previously said, the unprotected genetic material can be degraded by nucleases in the extracellular medium or aggregate with serum proteins. Furthermore, mammalian cells were not designed to naturally uptake foreign genetic material and therefore, after the internalization,[31-32] the biological material is also subjected to degradation in endosomal/lysosomal compartments.[47-48] The small amount of nucleic acid that can escape from these compartments is subsequently exposed to further cytoplasmic degradation.[28-29, 39] Moreover, as reported above, in some therapeutic cases (pDNA use), the genetic

material nuclear entry is required which represents an additional barrier: to cross the nuclear membrane. All these factors contribute for a low internalization and as result a low transfection efficiency.[31, 37]

Therefore, one major focus of research is the design of a carrier with ability to compact and protect the genetic material from the extra-cellular barriers, to cross the cell membrane giving an optimal cellular absorption,[50-52] overcome the intra-cellular barriers,[28] and deliver the therapeutic substance with a minimum toxicity.[28-29, 39]

Nucleic acid carriers are divided in two groups: viral and non-viral vectors.[53]

1.2.1 Viral Vectors

Virus can be considered as biologic machines highly specialized in cellular internalization and delivery of genes.[54] Virus has natural capacity to penetrate the host cells, deliver and replicate through the cellular machinery to express their pathogenic genes, which causes harmful effects and at last cellular death.[55] The ideal vector should be maintaining this internalization, delivery and replication capacity, expressing the intended genetic material, but avoiding the toxicity. Therefore, the ideal viral vector for nucleic acids delivery, should not contemplating the viral components responsible for pathogenicity while leaving intact all the necessary components for the assembly of the nucleic acids within the capsid or the virus or the integration in host genome.[54, 56] So, a viral vector is equivalent a non-pathogenic virus that transports the therapeutic agent.[57] Several type of viruses, such as adenovirus, retrovirus, adeno-associated virus and herpes simplex virus, have been target of huge success in gene therapy applied to the cancer.[5] Despite the good transfection efficiencies observed,[6] the significant adverse effects like the presence of residual viral components that can cause insertional mutagenesis, recombination, inflammatory response, cytotoxicity and immunogenicity,[8, 39] oncogenic effects, among others limit its use *in vivo*.[5, 12] In 1999, the death of a patient with 18 years old was attributed to an inflammatory reaction due the treatment based in adenovirus to combat a deficiency in ornithine transcarbamylase.[54] Despite the adverse effects observed using viral vectors, in 2000 the insertion of a gene in defective cells as effective to the cure of a children with a rare and fatal immunodeficiency,[58] this was the first positive results in gene therapy using these type of vectors.[59] However, in 2002, almost three years after treatment, patients which received the same treatment exhibits new cases of leukemia in T cells, which has been shown to be associated with insertional mutagenesis of the gene near an oncogene.[54, 60] Consequently, new cases of leukemia

appeared in treated patients and for safety reasons, this treatment was suspended until improvements appeared. Nevertheless, there are other some successful treatment reports with the use of viral vectors in combat of diseases like chronic granulomatous, metastatic melanoma and Parkinson.[61]

These undesired effects together with the production in a low scale with high costs, storage difficulties and the limited packing capacity of nucleic acids have hampered the use of this type of vectors for clinical applications.[45, 58]

1.2.2 Non-viral Vectors

The drawback of viral vectors, principally safety issues as previously mentioned, led researches to find new alternatives, like the development of non-viral vectors.[53] These type of vectors highlight by advantages like flexibility[39], lower cytotoxicity, non-immunogenicity, stability, easier large scale production through innovative synthesis schemes, ability to load higher amounts of genetic material[51, 58] and less expensive.[33-62] However, they present low transfection efficiency in comparison with viral vectors, due their inability to overcome numerous barriers found between the site of administration and the target cell.[8] Three major classes of non-viral vectors can be distinguished, namely those based on lipids, polymers and dendrimers.[63-64] Non-viral vectors are cationic to complex the nucleic acid through electrostatic interactions with the negative phosphate groups presents in the nucleic acid backbone. The resulting complexes/nanoparticles between nucleic acid and cationic lipids, polymers and dendrimers are designated lipoplexes, polyplexes and dendriplexes, respectively.[11]

Unlike viruses, non-viral vectors are not designed to naturally enter cells and deliver nucleic acids. Therefore, the main challenges and barriers that these delivery systems must overcome for a successful therapeutic application are: 1) protection of the nucleic acid from endonuclease degradation; 2) cellular internalization by endocytosis; 3) endosome escape; 4) nucleic acid release so it reaches the cytoplasm or nucleus; and, moreover, 5) the vector accumulation intra- and extracellularly must to be avoid (Figure 1.2).[13] The first obstacle can be avoided through the complexation with a positively charged material that compacts and protects nucleic acids by preventing the access of nucleases to them.[28, 50] However, non-specific electrostatic interactions with anionic extracellular proteins present in the organism, like serum albumin or glycosaminoglycans, reduce the amount of genetic material carried due to the competition for interaction with the cationic vector and promotes aggregation, which

results in a rapid clearance by macrophages. Functionalization of the surface of the material with hydrophilic and anti-biofouling polymers, such as poly(ethylene glycol) (PEG), is a widely adopted approach since it increases solubility and the blood residence time preventing nonspecific interactions with proteins through its ability to mask the positive charge of non-viral vectors.[41-42] However, PEGylation also led to several problems, like lower cellular uptake, difficult endosomal escape and reduction transfection efficiency. So, there is a "PEG dilemma". Thus, the PEG percentage used in the development of non-viral vectors should be well thinking.[28]

After systemic application by intravenous route, siRNA enters the bloodstream, distributing by different organs, which decreases the susceptibility of siRNA for be degraded by endonucleases and elimination through the kidneys.[28] So, once in the organs, siRNA should be disseminated through blood vessels for interstitial space, overcoming the endothelial barrier.[37] Like the RNAi machinery is located in the cytoplasm, the successful siRNA delivery is dependent of nanoparticles capacity to enter the cell, which is normally made by endocytosis.[65-67]

The endocytosis is known like a major pathway for siRNA uptake and although the most of the cells can internalize the vector, only a small percentage (around 2%) of nucleic acid that scape of endosomes have access to cytoplasm.[6, 28] Depending of nanoparticles properties, namely size, shape, surface charge and the type of cell, the endocytosis of nanoparticles can occur for many pathways and can be framed in two big groups: phagocytosis (specialized phagocytes like macrophages and dendritic cells) and macropinocytosis (any cell type). Depending of proteins involved, the pinocytosis can occur through pathways mediate by clathrin or pathways independents of clathrin.[41-42] For instance, large particles (above 1 μm) enter through macropinocytosis whereas by clathrin-mediated endocytosis the particles have sizes between 10 nm and 300 nm. Unfortunately, it is thought that the mechanisms occur simultaneously making it difficult to identify which mediates the internalization of the complex. Size is very important in cellular interactions and so important is the shape of nanoparticles. It has been observed that spherical complexes internalized more efficiently than others with different morphology.[51]

Once internalized, the complexes are loaded into endocytic vesicles formed during the endocytosis and eventually fuse with early endosomes. This endosome decides the fate of the internalized nanoparticles, they can be recycled to the plasma membrane or degraded in the lysosome.[40-42] Following the endocytic pathway, early endosomes slowly mature to late endosomes through the fusion of vesicles from the

Golgi apparatus which results in the accumulation of protons in the vesicle, leading to an rapid acidification (pH 5-6) of ATPase proton-pump enzyme. Subsequently, the late endosome reaches the lysosome leading to a further acidification (pH ~4.5) and the activation of several degradative enzymes. It has been demonstrated in numerous studies that the release of the complex from endosomes is one of the major barriers to efficient delivery presumably as result of their trafficking via late endosomes to lysosomal compartments, where the nucleic acid is degraded. Although, the efficiency can be improved by the design of delivery vectors that are capable of escaping from the endocytic pathway.[31] For the cationic lipids to escape from the endosome, the fusion and exchange of lipids with the membrane of the compartment has been tested, which theoretically promotes the destabilization and possibly the release of the nanoparticle. For the case of cationic polymers and dendrimers, there are some mechanisms proposed to promote the escape of the endosomal compartment.[11, 51] One of the mechanisms, called “proton sponge”, was proposed in 1997 by Behr which describes that protonation of the amines of the polymer/dendrimer generates a flow of hydrogens ions in lysosomes. To compensate the increase in absorption of ions, the vesicles introduce additional water molecules, leading to the osmotic swelling and eventual membrane rupture with consequently the release of the cellular content into the cytoplasm. In 2014, Park et al. reported the conjugation of poly(amido amine) (PAMAM) dendrimer derivates with basic amino acids with high buffering capacity and possibly this was the justification for the remarkable transfection efficiency achieved.[68-70]

Based in computational models, researches know that siRNA that flight of the endosome occurs with 1-2% of efficiency and only for a short time when nanoparticles reside in a vesicle that shares characteristics of later and early endosome.[5] Later, was developed other strategies to overcome endosomal barrier such as the incorporation of the chloroquine into the complex nucleic acid/vector. Chloroquine is well-known for its ability to raise pH the lysosomal environment by inhibiting the enzymes responsible for lysosomal degradation. However, this type of modifications is not transitive to the clinic due the toxicity of chloroquine. Although it is possible make the addiction of other molecules like peptides to promote the escape of the endosome.[6]

After the endosomal escape of the polyplex/dendriplex into the cytoplasm, a new barrier arises, the possible entrapment of the nucleic acid into the non-viral vector. Sometimes, when the complexes formed are very stable can occurs imprisonment of nucleic acids with non-viral vector and the connection between vector and nucleic acid doesn’t broken, leading that the nucleic acid will not perform the intended function.[11] In this sense, scientists reported some studies that indicate the introduction of degradable

bonds increased the release of the nucleic acid and therefore increased the transfection efficiency.[6, 29, 45]

When the nucleic acid is released from its delivery system it must access its target site. Some nucleic acids, such as siRNA, act directly on the cytoplasm. In the opposite, nucleic acids like pDNA act on the nucleus and therefore they need to travel across the cytoplasm and overcome the nuclear membrane, which constitutes an additional barrier to surpass.[51]

Finally, after the very useful and efficient performance of the non-viral vectors, they remain in the cytoplasm and may result in accumulation and consequent toxicity to the cell. Polycation/DNA complexes were usually found to be less toxic to cells than the uncomplexed positively charged delivery systems because they have the positive groups interacting with the nucleic acid, i.e. they are not available to interact with the cell components.[44-46] However, the exact toxicity mechanism is not known. Polymer and dendrimer-based systems most frequently used for assays include poly(ethylene imine) (PEI), PAMAM and poly(propylene imine) (PPI) dendrimers. Although widely used, these systems have conflicting evidence about their biologic safety because these systems are non-biodegradable, which presuppose a huge risk of bioaccumulation inside the cells, especially after many administrations. This type of macromolecules interacts with cellular membrane, causing destabilization of lipid bilayer, leading to the permeabilization, loss of cellular content and finally cellular lysis.[19] Cationic structures are internalized well than anionic or neutral due to the high affinity for negatively charged proteoglycans expressed on the surface of most cells. Nevertheless, cationic systems have been reported as cytotoxic. Besides that, toxicity may also associate with the size and morphology. To prevent the devastating effect from occurring, there is a need to develop biodegradable non-viral vectors capable of efficiently delivering the nucleic acid without causing any damage to the cell. Ideally, the vector upon reaching the target site and releasing the nucleic acid which compacted, protected, and delivered efficiently should degrade into smaller fragments to be easily removed from the cell by exocytosis and subsequently excreted from the body. Moreover, the mechanisms of biodegradability may also mean an aid to increase the nucleic acid release into the cytosol.[71-74]

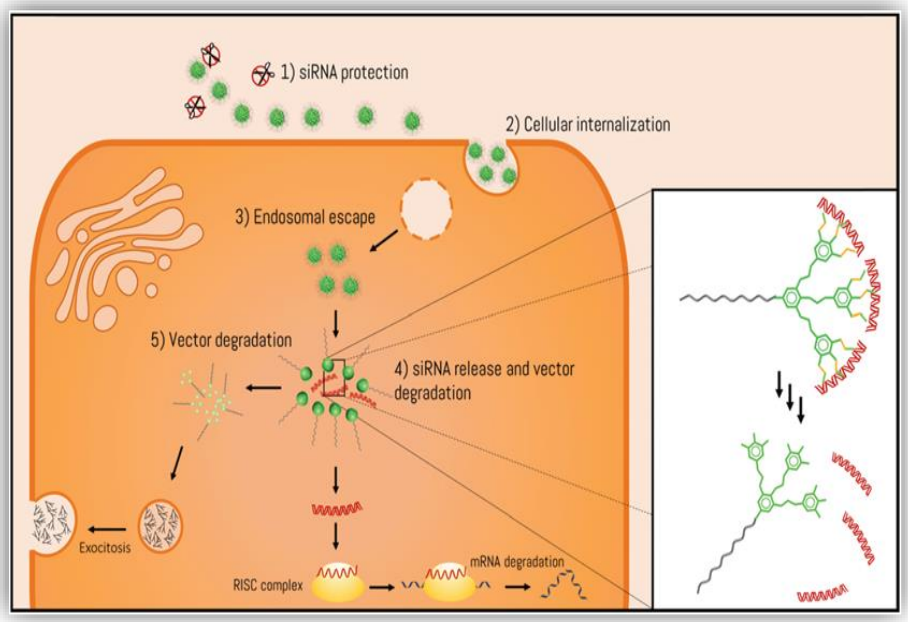


Figure 1. 2 - Extra and intracellular barriers for non-viral siRNA delivery, which are responsible for the poor siRNA delivery efficiency. 1) siRNA protection against degradation by endonucleases, 2) cellular internalization, 3) endosomal escape, 4) *siRNA* release from the vector and access to the cytoplasm and 5) vector degradation. Adapted from: Leiro et al., Journal of Materials Chemistry (2017).[45]

1.3 Dendrimers

Dendrimers are considerate very attractive vehicles by different bioactive molecules.[45, 58, 67] Dendritic structures emerged of a highly branched molecules ("cascade molecules"), firstly synthetized by Voëgtle et al. in 1978.[29, 48, 63] Later, Denkewalter, Tomalia, Newkome, Frechet and colleagues increased the complexity of these hyperbranched molecules and rename them to "dendrimers".[64] The word arises from the Greek *Dendron* = tree and *meros* = parts. These polymers are composed by tree main parts 1) a central core with one or more functional groups, 2) repeated units (monomers) covalently attached to the central core, organized in concentric layers, called "generations" (G), and 3) a high number of terminal functional groups on their surface (Figure1.3).[65]

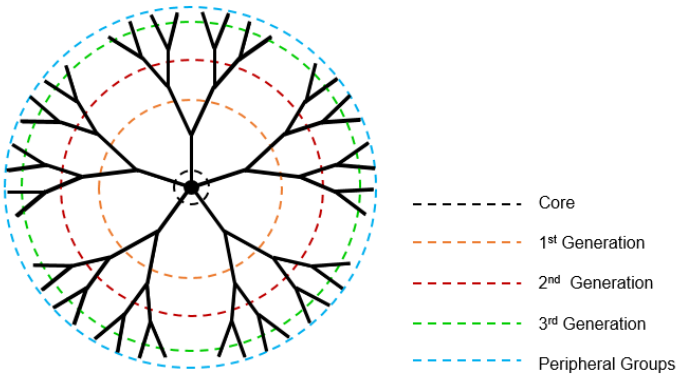


Figure 1. 3 - Schematic representation of a third-generation dendrimer structure. Broken lines represent dendrimer's components (core, generations and peripheral groups). Adapted from: Leiro et al., Current Gene Therapy (2017).[4, 31]

The branching from the core gives their semi-globular or globular structures. Depending the number of branches points, length of branches and dendrimer generation, these molecules can reach several nanometers. An increase in generation causes an increase in the nanoparticle diameter and consequently the number of terminal functional groups increases exponentially. Their physical-chemical properties are intrinsically dependent of the generation number and terminal groups.[66-67]

The size and shape of dendrimers are similar to those of proteins, making it an efficient nanocarriers for many therapeutic compounds,[68] with several applications in nanomecidine.[66]

Dendrimers present unique structural characteristics, such as a globular, well-defined, and highly branched structure, hydrophobic or hydrophilic cavities in the interior, adjustable nanosize and molecular weight[6, 29, 68-69] and low polydispersity, which enables a high level of reproducibility of pharmacological effects.[63] Their very branched structure result in one of the more attractive characteristics of dendrimers: their multivalency, thanks to their high number of peripheral groups. This multivalency allows the specific anchorage of different (bioactive) molecules.[63] Moreover, they have capacity to encapsulate and protect nucleic acids, in compact nanostructures (dendriplexes).[68]

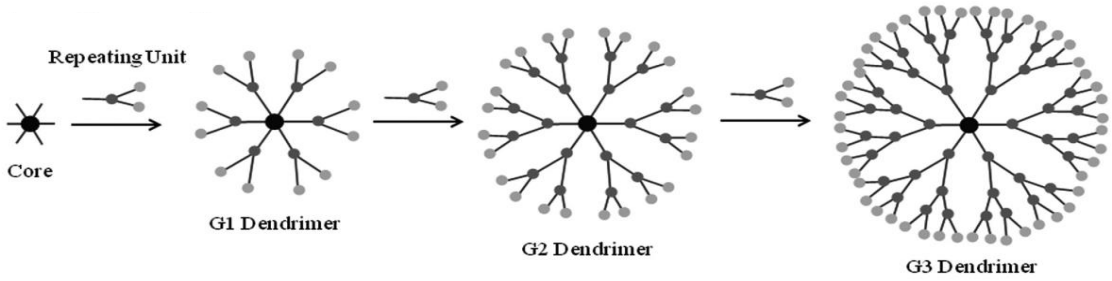
1.3.1 Dendrimers: from synthesis to application

The synthesis of dendrimers can be performed mainly in a divergent or convergent way through conventional methods.[30] The strategy to be used depends of structural constituent's nature and generation.[45]

The divergent synthesis (Figure 1.4), introduced by Tomalia, Newkome and Voëgtle allows the formation of the dendrimer from the inner core. It is a stepwise method beginning from the multifunctional core, which is extended outward through several coupling reactions. The addiction of repeating units (monomers) which bind to active sites of the core, produce a dendrimer of first generation. Then, the peripheral group is activated by reaction with more repeating units. This process is repeated many times until obtain dendrimers of a desired generation, creating the dendrimer layer by layer.[29] Each repetition forms a new layer of branched units (generation) that results in an increased molecular weight and number of groups in the periphery of the molecule. The first dendrimer synthesized by divergent methods was PAMAM and currently, this method is the most favored commercial strategy used by international producers.[70-71] However, divergent synthesis presents some disadvantages, such as non-continuous growth events and side reactions that can result in dendrimers without uniform size because the number of reactions increase exponentially in each step.[72] So, it is important that all reaction steps occur completely to avoid that dendrimers presents incomplete growth or irregular growth of the branches. This situation can be solved by the addiction of excessive amounts of the reagent to force the reactions to completion.[53]

To overcome these limitations, Fréchet et al. developed the convergent method (Figure1.4).[28, 73] This method starts on the perfected branched dendrons, which are attached to a multifunctional core after activation/deprotection of their focal point.[72] In convergent synthesis only a limited number of groups are activated by reaction which reduces the probability of structural failures. However, is difficult to synthesize dendrimers of high generations, due to steric hindrance between the corresponding dendrons. Nevertheless, a selection of an adequate core (size and multivalency) can help to reduce these obstacles.[29]

a) Divergent synthesis



b) Convergent synthesis

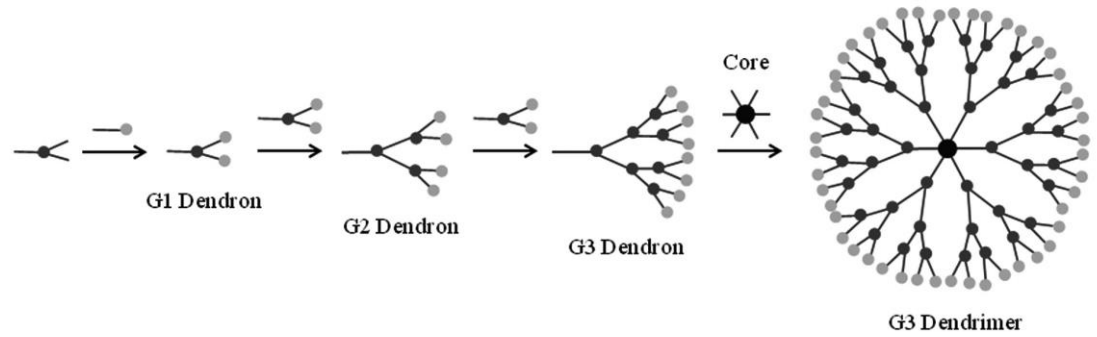


Figure 1. 4 - Methods of dendrimers synthesis. a) Divergent synthesis begins from the central core and grows until reach the periphery. **b)** Convergent synthesis begins in the branches and finally attach to the core. Adapted from: Leiro et al., Advanced Functional Materials (2017).[45]

Although the number of synthetic steps is similar in two approaches, the convergent synthesis entails less non-ideal growth events which leaves to a higher monodispersity of dendrimers.[72]

The limitations of these methods, have given the development of other strategies, as orthogonal methods and accelerated synthesis, which enables the synthesis of dendritic structures through more efficiency reactions with lower number of steps, being, therefore, more favorable to the user and environment.[53]

All of these strategies allow the control chemistry and size of dendritic structures, make dendrimers into ideal structures for obtaining nanoparticles with suitable characteristics for drug and nucleic acid delivery. In fact, a wide range of different dendrimer families there are with great potential for biomedical applications.[51] Probably, the most known and used families of dendrimers are PPI (1978), poly-*L*-lysine (PLL) (1981) and PAMAM (1985) dendrimers, which have been used like DNA and siRNA promisor vectors.[7, 73] Moreover, these polyamine dendrimers used at low concentrations, were able to rapidly remove infectious proteins (prions) from affected cells.[11]

PPI dendrimers are based on tertiary amines and amine group on their surface and they have applications in many areas, mainly in gene delivery.[74]

PLL dendrimers is a well-known family of dendrimers that have the amino-acid L-lysine as the repeating unit, which present positive charge at physiological pH. They are attractive carriers for biomedical applications, especially for gene therapy. However, they have lower transfection capacity when applied alone or unmodified because they remain, mainly, in the lysosomal pathway. Moreover, when administrated intravenously, PLL dendrimers interacts quickly with serum proteins and removed from the circulation.[31]

PAMAM dendrimers are relatively easy to synthesize and commercially available up to generation 10. As previously commented, they are very used as nucleic acid delivery vectors.[74] However, the transfection efficiencies obtained was low in many cases. Because of this, some alterations were suggested to improve the performance of PAMAM dendrimers, namely chemical modifications like those conducting to partially degraded PAMAM dendrimers in their surface.[7] Moreover, other modifications are also described, like functionalization with cyclodextrines, amino acids, peptides, proteins, glucocorticoids, among others.[8-9] Even nowadays, transfection kits based on PAMAM for *in vitro* assays are currently marketed, like Qiagen's SuperFect and Starpharma's ProfectTM.[74-76]

The families mentioned above are the most used but there are other families like poly(ether) copoly(ester) (PEPE),[77] phosphorus,[78] poly(ether imine) (PETIM),[79] carbosilane,[80] and gallic acid-triethylene glycol (GATG) dendrimers, among others.[81]

There are some dendritic agents in phase of clinic trails, namely: DEP ™ docetaxel, which already stay in studies of phase II for treatment of solid tumors like breast, lung, prostate; VivaGel®, a G4 PLL based-dendrimer, that acts like a antimicrobial in treatment/ prevention of wide range of sexual transmitted diseases and now it is in testes of phase III and granted as Qualified Infectious Disease Product and Fast Track by FDA; Gadomer-17®, a PLL- based dendrimer bearing 24 DOTA-Gd chelates, at this moment it is in trails of phase II like a dendritic contrast agent for diagnosis in magnetic resonance imaging (MRI).[11]

1.3. 2 Dendrimers toxicity

Most of these dendrimers have been used in *in vitro* assays for possible future biomedical applications. However, problems related to cytotoxicity have been reported recently and therefore the use of dendrimers in a biological media is constrained.[76]

Dendrimers toxicity is related to dose, exposure time, dendrimer generation/size and nature of their groups, especially their terminal groups.[82-83] A high dose or various administrations with short time intervals can induce lysosomal storage problems and in the case of non-biodegradable dendrimers, they can accumulate in the organelles. So, it is clear the necessity to ponder the ration between amount of the dendrimer administrated and the possibly effect.[29]

The high number of positive charges belonging to the surface groups causes cationic dendrimers to be much more cytotoxic than the anionic ones, since the cationic surface may interact with the negatively charged cell membrane and cause its disruption.[84] Then, Mecke et al. proposed another mechanism that considers the interaction of the dendrimer with membrane can result in membranes break due the formation of nanoholes, membrane thinning and erosion which consequently leaves to the loss of intracellular content, cell lysis, necrosis/ non-apoptotic cell death.[76] In addition to the membrane destabilization, there are another causes that can contribute for toxicity such as the abnormal oxidative metabolism resulting of mitochondrial dysfunction and changes in endogenous gene expression that eventually can leave to apoptosis.[29]

Presently, already there are some studies to reduce the cytotoxicity of cationic dendrimers due to the modifications on their cationic surface, such as acetylation or the conjugation with biocompatible molecules as PEG chains that partially protects the positive charges of the dendritic structure surface. Namely, in 2009, Wang et al. obtained a powerful decrease in PAMAM dendrimers cytotoxicity when it was modified with PEG. When the concentration is superior to 0.5 mg/mL, PAMAM dendrimers leaves to the death of the major cells. However, at the same concentration, the PEGylation of PAMAM-dendrimers, showed that the number of cells in apoptosis reduced 40%.[82]

In addition, non-biodegradability of dendrimers is also responsible for the possible toxicity observed. The most of dendritic families are very stable under physiological conditions and this may result in toxicity induced by the accumulation of non-degradable synthetic materials inside the cells or tissues. Some biodegradable dendrimers that have been used as nucleic acid vectors were evaluated about their cytotoxicity.[11, 51] For example, in 2011, Barnard et al. reported the synthesis of second-generation of 2,2-bis(hydroxymethyl)propionic acid (bis-HMPA) dendrimers

functionalized with cholesterol as nucleic acid delivery systems.[29] These modified dendrimers were found to be toxic to cells above 20 µg/mL. Movellan et al. reported in 2015 a study comparing the biocompatibility of bis-HMPA and PAMAM dendrimers. Until highly concentration tested, 0,5 mg/mL, the bis-HMPA dendrimers presents low cytotoxicity with cellular viability above 80% while PAMAM dendrimers has highly toxicity with cellular viability around 10 to 68%.[13]

1.3.3 Biodegradable dendrimers

Although dendrimers have applications well defined and suitable in many areas, they have some limitations due to cytotoxicity problems. To overcome these problems of conventional non-degradable dendrimers, many teams around the world are focused on developing biodegradable dendrimers.[66] The use of biodegradable dendrimers appears like an approach to produce desirable large molecular weight vectors that reach target tissues in large quantities and later degrades into smaller fragments that can be eliminated easily through metabolic pathways or excreted in the urine.[85] The biodegradability in dendrimers can be achieved through the inclusion on their structure of labile bonds to be broken due the action of a stimulus or a specific biological activity. Presently, most efforts have been focused on the development of dendritic architectures with hydrolysable bonds.[45, 86] The functional groups more propitious to hydrolysis are based on anhydrides, esters, phosphoesters, hydrazones, among others.[87]

The degradation rate of dendritic structures is controlled by the chemical linkages present in their subunits as well as their hydrophobicity, verifying that hydrophilic subunits presents higher degradation rates than hydrophobic ones. Moreover, dendrimers size has consequence in degradation rate, this is, the bigger dendrimers degrades slower than the smaller ones due to the bigger packing.[88-92] Finally, another preponderant factor in degradation rate is the localization of the cleavage connections because the hydrolysis of inner bonds leads to faster degradation of the entire dendrimer. So, taking into account these factors, it is possible modulate the biodegradability and achieve desired degradations rates. The degradation can occur through the removal of dendritic branches, the core or peripheric groups.[93] The cleavage of certain parts is enough to lead to the total degradation of the dendritic structure. However, is important to refer that in some polymeric materials as aliphatic polyesters, the degradation can be accelerate due the presence of superoxide ions, presents in corporal fluids during the inflammatory response to an abnormal situation from cells.[94-95]

The most attractive biodegradable dendrimers to degrade under physiological conditions are based on esters bonds, which are very interesting because they present a good relation between their biodegradability under physiological conditions and their possibility of relative easy synthetic manipulation. Nevertheless, the preparation of nanocarriers based in esters linkages is a challenging task because of the undesirable and/or premature degradation observed during synthesis, purification, subsequent steps of functionalization and/or application. All these challenges explain the reduced number of biodegradable ester-based dendritic structures developed for specific biomedical applications.[45]

Chapter 2 – Aim of the project

In the context of this thesis we have carried out the synthesis of biocompatible and fully biodegradable azide-terminated G3 PEG-GATGE (Gallic Acid-Triethylene Glycol-Ester) dendrimers and their evaluation as vehicles of siRNA to neuronal cells.

This G3 GATGE dendritic structure is completely based on the biodegradable GATGE repeating unit. The biodegradable trait is given by aliphatic ester bonds localized at the branches of the GATGE repeating unit, which are degradable under physiological conditions. Moreover, with the aim of increasing the biocompatibility and circulation time of these systems, a 5kDa PEG chain was attached to the focal point of the dendritic part. So, we obtained PEG-GATGE dendritic block copolymers. The functionalization of their peripheral azides with different amine moeties was achieved by means of the Coper (I)-catalyzed azide-alkyne cycloaddition (CuAAC, “click” chemistry). These terminal amine groups will remain positively charged at physiological pH, allowing the complexation of nucleic acids by electrostatic interactions and evaluate the performance of these dendrimers as nucleic acid vectors.

Therefore, the aim of this work was the development of a non-toxic and efficient nanoparticle system able to deliver siRNA to the neuronal cells. So, the first step was the synthesis of the dendritic vectors. Then, the evaluation of their ability to complex and protect siRNA. Finally, we studied the capacity of these fully biodegradable nanocarriers to mediate siRNA transfer and down-regulate the protein expression in neuronal cell lines.

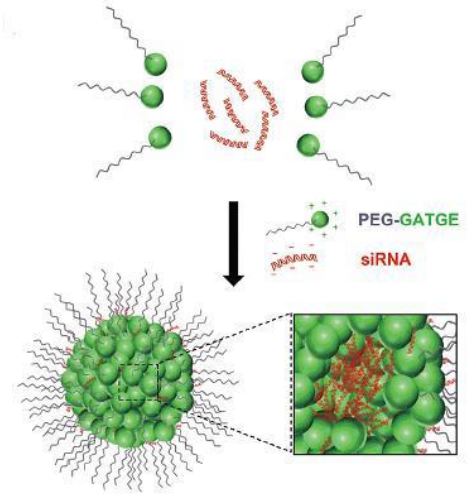


Figure 2. 1 - Schematic representation of a siRNA dendriplexe formation and nanostructure.

Chapter 3 – Experimental Methods

3.1 Materials and Instrumentation

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDC·HCl), gallic acid, *tert*-butanol, oxalic acid, 4-bromobutiric acid, N,N'-dicyclohexylcarbodiimide (DCC), 2-[2-(2-chloroethoxy)ethoxy]ethanol, 4-dimethylaminopyridine (DMAP), potassium carbonate, 18-crown-6, dimethylformamide (DMF), palladium on carbon (Pd/C), sodium azide, hydroxybenzotriazole (HOBt), sodium ascorbate and poly(ethylene glycol) methyl ether were purchased from Sigma-Aldrich. Dry dichloromethane and HCl were purchased from VWR and Amatek Chemical Co Limited, respectively. N-2-propyn-1-yl-1,3-propanadiamine·2HCl and 4-ethynyl-benzenemethanamineCopper (II) sulphate pentahydrate were purchased from Riedel-de-Haen. All solvents were HPLC grade and were purchased from Fluka, Prolabo, and Sigma-Aldrich. Column chromatography was performed with 230-400 mesh silica gel and the thin-layer chromatography was performed on silica 60/F-254 aluminium-backed plates from Merck Millipore. Ultrafiltration was done on Amicon stirred cells with Ultracel® 1 and 3 kDa membranes from Merck Millipore. Nanopure water (18MΩ cm) was obtained from a Milli-Q water filtration system (Merck Millipore) and nuclease free (NF) water was purchased from Qiagen. Filter PTE 0.45 µM for filtering the dendrimer solution were purchased from VWR.

Non-labelled *siDNA*/siRNA and the *siDNA* labelled at the 5' end of the sense strand was purchased from Integrated DNA Technologies. SYBR Gold Nucleic Acid Stain was purchased from Life Technologies. Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM and fetal bovine serum (FBS) were purchased from Gibco. Trypsin and Penicillin-Streptomycin were purchased from Sigma-Aldrich. Cell culture plates were purchased from BD Biosciences.

ND7/23-GFPLuc is a cell line made to stably express the fusion protein GFP-Luciferase. The cells were previously made by transfecting a plasmid DNA encoding the GFP-Luciferase gene driven by a CMV promoter and the neomycin resistance gene expression cassette. The neomycin gene was then used to select for cells that stably integrated the GFP-Luc and Neo gene through selection with the geneticin antibiotic. Later cells were further selected for GFP-Luc expression by FACS sorting and further cell culture expansion.

3.2 Synthesis

3.2.1 *tert*-Butyl gallate

EDC·HCl (1180 mg, 6.17 mmol) was gradually added, in small portions, to a suspension of gallic acid (1000 mg, 5.88 mmol) in dry *tert*-butanol (35 mL) and the reaction was vigorously stirred at room temperature (RT) for 48 h. Then, oxalic acid (970 mg, 0.29 mmol) and diethyl ether (25-40 mL) were added and finally the resulting solution was filtered and the filtrate was washed to give *tert*-Butyl gallate (980 mg, 74%).

3.2.2 2-[2-(2-Azidoethoxy)ethoxy]ethanol

2-[2-(2-chloroethoxy)ethoxy]ethanol (5020 mg, 29.8 mmol) and sodium azide (3870 mg, 59.6 mmol) were dissolved in water (14.9 mL) and the reaction was strongly stirred for 48 h at 75 ºC. After, the reaction mixture was cooled down to RT and then it was concentrated under reduced pressure. Finally, the resulting white residue was filtered with diethyl ether to give 2-[2-(2-azidoethoxy)ethoxy]ethanol (5120 mg, 98%), as a yellow oil.

3.2.3 2-[2-(2-Azidoethoxy)ethoxy]ethyl 4-bromobutanoate

2-[2-(2-cloroethoxy)ethoxy]ethanol (1500 mg, 8.56 mmol), 4-bromobutyric acid (2144 mg, 12.84 mmol), DCC (2649 mg, 12.84 mmol) and DMAP (104 mg, 856 mmol) were dissolved in dry dichloromethane (17.12 mL). The suspension was continuously stirred for 24 h under inert atmosphere and when all of the 2-[2-(2-azidoethoxy)ethoxy]ethanol had reacted, trimethylamine was added (2.96 mL, 21.50 mmol) and stirred for 1 h. Then, the solution was evaporated and the resulting crude was filtered in diethyl ether and acetonitrile to remove the urea. Finally, the resulting yellow oil was purified by column chromatography (hexane/ethyl acetate [2:1]) to obtain 2-[2-(2-azidoethoxy)ethoxy]ethyl-4-bromobutanoate (2325mg, 84%).

3.2.4 3,4,5-Tris(4-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}-4-oxobutoxy)benzoic acid (the repeating unit)

tert-Butyl gallate (250 mg, 1.15 mmol), dry potassium carbonate (1527 mg, 11.05 mmol) and 18-crown-6 (29.21 mg, 0.11 mmol) were added to 2-[2-(2-azidoethoxy)ethoxy]ethyl-4-bromobutanoate (1433 mg, 4.42 mmol) in dry DMF (2.21 mL) under inert atmosphere. The reaction mixture was vigorously stirred for 12 h at 80 ºC. When reaction was complete, the mixture was cooled down to room temperature, the solvent was evaporated and the resulting crude was filtered with dichloromethane to remove solid residues. Later, the filtrate was purified by column chromatography (hexane/ethyl acetate [1:2]) to yield tris{2-[2-(2-azidoethoxy)ethoxy]ethyl}-4,4',4''-{[5-(*tert*-butoxycarbonyl)benzene-1,2,3-triyl]tris(oxy)}tributanoate (782 mg, 74%) as a yellow oil.

Tris{2-[2-(2-azidoethoxy)ethoxy]ethyl}-4,4',4''-{[5-(*tert*-butoxycarbonyl)benzene-1,2,3-triyl]tris(oxy)}tributanoate (244 mg, 0.26 mmol) was dissolved in a mixture of dried dichloromethane/TFA (2:1) and continuously stirred under inert atmosphere for 3 h. After, the solvents were evaporated to yield a yellow oil, the 3,4,5-tris(4-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}-4-oxobutoxy)benzoic acid (229.7 mg, 100%).

3.2.5 Fully Biodegradable G1 Dendrimer (PEG-fbG1-N_3)

Poly(ethylene glycol) methyl ether (300 mg, 0.06 mmol), the repeating unit (108 mg, 0.12 mmol), EDC·HCl (23 mg, 0.12 mmol) and DMAP (0.7 mg, 0.006 mmol) were dissolved in dry dichloromethane (1.8 mL). The resulting solution was strongly stirred under inert atmosphere for 12 h at RT. Then, it was concentrated and precipitated with dichloromethane/isopropyl alcohol to give PEG-fbG1-N_3 as a white powder (347 mg, 98%). ^{1}H NMR (400 MHz, CD_2Cl_2, Me_4Si) δ: 1.96-2.14 (dm, 6H), 2.56 (dt, *J*= 23.2, *J*= 7.4, 6H), 3.32 (s, 3H), 3.34 (t, *J*= 5.0, 6H), 3.39-3.79 (m, ~552H), 4.04 (m, 6H), 4.19 (m, 6H), 4.40 (dd, *J*= 5.1, *J*= 4.8, 2H), 7.25 (s, 2H).

3.2.6 Fully Biodegradable G2 Dendrimer (PEG-fbG2-N_3)

Pd/C (68 mg) and 1 M hydrochloric acid in methanol (3,49µL, 0.35 mmol) were added to a solution of PEG-fbG1-N_3 (344 mg, 0.059 mmol) in methanol (14.68 mL). The mixture reaction was magnetically stirred under H_2 for 4 h. When reaction was complete, the catalyst was removed by filtration with methanol and the filtrate was concentrated and dried to obtain the reduced form (PEG-fbG1-$NH_3^+Cl^-$) in 100 % yield's reaction.

HOBt (93 mg, 0.698 mmol), EDC·HCl (131 mg, 0.698 mmol) and triethylamine (48µL, 0.349 mmol) were added to PEG-fbG1-$NH_3^+Cl^-$ and the repeating unit (232 mg, 0.261 mmol) in dry dichloromethane (1,72 mL). The resulting solution was continuously stirred at RT for 48 h under inert atmosphere. After, it was concentrated and precipitated in dichloromethane/isopropyl alcohol to give PEG-fbG2-N_3 as a white powder (79 mg, 74 %).^{1}H NMR (400 MHz, CD_2Cl_2) δ: 1.94-2.13 (dm, 24H), 2.56 (dt, *J*= 25.6, *J*= 7.3, 24H), 3.32 (s, 3H), 3.34 (m, 18H), 3.39-3.78 (m, ~552H), 4.02 (m, 24H), 4.19 (m, 24H), 4.38-4.41 (m, 2H), 6.69-6.79 (m, 3H), 7.01 (s, 4H), 7.25 (s, 4H).

3.2.7 Fully Biodegradable G3 Dendrimer (PEG-fbG3-N_3)

Pd/C (36 mg) and 1 M hydrochloric acid in methanol (383µL, 0.382 mmol) were added to a solution of PEG-fbG2-N_3 (177mg, 0.021 mmol) in methanol (4.93 mL). The mixture reaction was vigorously stirred under H_2 for 8 h. After, to remove the catalyst, we filtered the solution with methanol to obtain PEG-fbG2-$NH_3^+Cl^-$ with 100 % yield.

HOBt (74 mg, 0.54 mmol), EDC·HCl (104 mg, 0.54 mmol) and triethylamine (75µL, 0.54 mmol) were added to PEG-fbG2-$NH_3^+Cl^-$ (173 mg, 0.020 mmol) and the repeating unit (245 mg, 0.27 mmol) in dry dichloromethane (1.84 mL). The resulting solution was continuously stirred at RT for 48 h under inert atmosphere. Then, it was concentrated and precipitated with dichloromethane/isopropyl alcohol. Later, to remove all impurities that remained in solution, the final compound was purified by ultrafiltration (Ultracell ® 3000 MWCO) after washing with a solution of 30% methanol/ water, to give pure PEG-fbG3-N_3, as a white-yellow powder (210.6 mg, 63%). ^{1}H NMR (400 MHz, CD_2Cl_2) δ: 1.95-2.12 (dm, 78H), 2.50-2.60 (dm, 78H), 3.32 (s, 3H), 3.32-3.35 (m, 54H), 3.39-3.78 (m, ~552H), 3.95-4.05 (m, 78H), 4.17-4.21 (m, 78H), 4.38-4.40 (m, 2H), 6.58 (br s, 8H), 6.82 (br s, 4H), 7.02 (s, 16H), 7.24 (s, 10H).

3.2.8 Functionalization of PEG dendritic block copolymers (PEG-fbG3-N_3) with positively charged amine ligands by "Click" Chemistry

3.2.8.1 Diamine-terminated fully biodegradable G3 dendrimer (fbD)

PEG-fbG3-N_3 (73 mg, 4.51μmol) and N-2-propyn-1-yl-1,3-propanadiamine·2HCl (45 mg, 0.24 mmol) were dissolved in dimethylformamide (1.21 mL)/water (913μL) and 0.1 M sodium ascorbate (305 μL) and aqueous 0.1 M copper (II) sulfate pentahydrate (61μL) were added and continuously stirred for 24 h, at RT. The final solution was purified by ultrafiltration (Ultracell ® 1000 MWCO) after washing with 0.1 M EDTA (pH 6), 0.6 M sodium chloride (pH 7.2) and water to obtain PEG-fbG3-D as a pale-yellow powder (86 mg, 90 %). ^{1}H NMR (400 MHz, D_2O) δ: 1.97-2.19 (m, 132H), 2.46-2.66 (m, 78H), 3.04-3.25 (~108H), 3.43 (s, 3H), 3.56-3.79 (m, ~552H), 3.92-4.10 (m, ~132H), 4.21-4.25 (m, ~78H), 4.40-4.43 (m, 54H), 4.64-4.68 (m, ~54H), 4.64-4.68 (m, ~54H), 7.12-7.26 (m, 26H), 8.21-8.22 (m, 27H).

3.2.8.2 Benzylamine-terminated fully biodegradable G3 dendrimer (fbB)

PEG-fbG3-N_3 (73 mg, 45.15μmol) and 4-ethynyl-benzenemethanamine·2HCl (41 mg, 0.24 mmol) were dissolved in dimethylformamide (1.21 mL)/water (913μL) and 0.1 M sodium ascorbate (305μL) and aqueous 0.1 M copper (II) sulfate pentahydrate (61μL) were added and continuously stirred for 24 h, at RT. The final solution was purified by ultrafiltration (Ultracell ® 1000 MWCO) after washing with 0.1 M EDTA (pH 6), 0.6 M sodium chloride (pH 7.2) and water to obtain PEG-fbG3-D as a yellow powder (91.6 mg, 98 %). ^{1}H NMR (400 MHz, D_2O) δ: 1.75-1.94 (m, 78H), 2.33-2.45 (m, 78H), 3.42 (s, ~3H), 3.42-3.93 (m, ~684H), 4.06-4.17 (m, ~132H), 4.50-4.56 (m, ~54H), 6.93-7.06 (m, ~26H), 7.43-7.47 (m, ~54H), 7.65-7.72 (m, ~54H), 8.19-8.26 (m, ~27H).

3.3 Dendriplex preparation

Dendrimer/ siRNA complexes were prepared at different *N/P* ratios, where N means the number of primary amines in the dendritic copolymer and P the number of phosphate groups in the siRNA backbone, between 5 and 80. Firstly, we dissolved the

dendrimer in nuclease free water to obtain a dendritic copolymer solution of 6 mg/mL. Then, we filtered the solution with filter PTE 0.45 µM for remove the possible aggregates and finally we proceed to the preparation of dendriplexes by adding siRNA (20 µM) to different volumes of dendritic solution, water and buffer (PB 10 mM for diamine-terminated dendrimers and Hepes 20mM + 5% glucose for benzylamine-terminated dendrimers). Later, the samples were vortexed for 10 seconds at maximum velocity and incubated for 30 minutes at RT prior to experiments. For the experiments where biological activity is not measured, an annealed sense and antisense DNA strand with the same sequence was used for mimic siRNA, as DNA oligos are easier to synthesize and obtain in higher yields and purity.

"Small interfering" DNA

To replace the use of siRNA in assays that biological activity is not assessed, two complementary DNA sequences (sense and antisense) were designed which we named small interfering DNA (*siDNA*). These sequences have not any known biological function (sequences shown in Table 1).

Table 1 - *siDNA* and siRNA sequences used for the assays.

Nucleic acid	Sequence 5'-3'
siDNA	S: GCT GAC CCT GAA GTT CAT CTG CAC C AS: GGT GCA GAT GAA CTT CAG GGT CAG CTT
siRNA	S: GCU GAC CCU GAA GUU CAU CUG CAC C AS: GGU GCA GAU GAA CUU CAG GGU CAG CUU

3.4 Polyacrylamide gel electrophoresis shift assay

Polyacrylamide gels, with 4% stacking and 20% resolving gel, were prepared in Tris/borate/EDTA (TBE buffer). Then, when the gels were done, we added the dendriplexes that were prepared at various *N/P* ratios as previously described using *siDNA* instead of siRNA. The volume of dendriplex solution corresponding to 12 pmol of *siDNA* was mixed with 6 µL of loading buffer and the electrophoresis run was at 100 V, 30 minutes. The gels were stained with SYBRGold® nucleic acid stain, diluted in TBE buffer (1:10000), for 30 minutes and visualized using GelDoc XR imager (BioRad). Dendriplex/*siDNA* binding was shown by a lack of migration of the *siDNA* in the electrophoretic field.

3.5 SYBRGold® intercalation assay

Dendriplexes were prepared as previously described and then incubated in NF water and buffer for 10 minutes at RT in a 96-well black plate with 2 µL of a 1:100 SYBRGold solution (in TAE buffer). After incubation, fluorescence was measured (λexc = 485 nm, λem = 540 nm) using a microplate reader (SynergyMx, Biotek).

The results are shown as a percentage of complexation, where 100% represents the complete *siDNA* complexation. The presented data are expressed as mean ± SD of two independent sample measurements.

3.6 Size and zeta potential measurements

Size, polydispersity index (PDI) and zeta potential of dendriplexes were measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK) following the manufacturer's instructions. Size and PDI were determined at RT with a scattering angle of 173º using ZEN0040 cells in the automatic mode (final volume 80 µL). The mean hydrodynamic diameters were determined by cumulative analysis (Z-average mean). Zeta potential measurements were performed with the same conditions mentioned above in capillary cells (DTS1070) (final volume 100 µL). The Smoluchowski model was applied for zeta potential determination, and cumulate analysis was used for mean particle size determination. The presented data are expressed as the mean ± SD of three independent sample measurements. The software used was Zetasizer Software version 7.12, supplied by the manufacturer (Malvern Instruments, UK).

3.7 Cell culture

The neuroblastoma ND7/23 cell lines were cultured in DMEM medium supplemented with 10% (v/v) heat inactivated FBS (56º C, 30 min) and 1% Penicillin-Streptomycin at 37 ºC, 5% CO2, in a cell incubator.

3.8 Cytotoxicity studies

Cell viability was evaluated as a function of dendritic copolymer/dendriplex type, concentration, and *N/P* ratio. ND7/23 cells were seeded in 96-well plates (final volume 200 µL) at a density of 3.75 x 10^4 viable cells per cm^2 and incubated for 24 h in supplemented DMEM medium at 37 ºC, 5% CO_2 and grow to reach 70-80% confluence prior transfection. Then, the medium was replaced for non-supplemented DMEM and dendrimers and dendriplexes were added. 24 h post transfection, the medium was

replaced with fresh medium containing 10% (v/v) FBS and 10% resazurin and incubated for another 3h.

Fluorescence was measured (λexc = 530 nm, λem = 590 nm), after incubation, using a multimode microplate reader (SynergyMx, Biotek). Untreated cells were used as reference and cells transfected with Lipofectamine® 2000 (Invitrogen) and Triton X 1% were used as negative and positive controls, respectively. Lipofectamine® 2000 was used according to the manufacturer instructions. The cells' viability exposed to the dendritic copolymer/dendriplexes was expressed as a percentage of viability of non-treated cells. The presented data are expressed as mean ± SD of three independent sample measurements.

3.9 Cellular uptake

Flow cytometry

ND7/23 cells were seeded in 24-well plates at a density of 2.5 x 10^4 viable cells per cm^2 and incubated for 24 h in supplemented DMEM medium at 37 ºC, 5% CO_2 and grown to reach 70-80% confluence prior transfection. Then, the medium was substituted by non-supplemented DMEM and dendriplexes were added (50 µL dendriplexes in a final volume of 300 µL). Dendriplexes were prepared with Cy-5 labeled *siDNA* as described above with *N/P* ratios of 5, 10, 20, 40 and 80 (*siDNA* concentration of 0.1 pmol/µL).

After 24 h incubation, cells were rinsed twice with 1x Phosphate Buffer Saline (PBS), trypsinized, centrifuged, resuspended in 1x PBS containing 2% FBS and analyzed by flow cytometry (FACSAccuri, BD Biosciences). Non-treated cells and cells transfected with Lipofectamine® 2000 were used as negative and positive controls, respectively. The resulting data was analyzed using FlowJo software (version 10, FLOWJO, LLC). The presented data are expressed as mean ± SD of three independent sample measurements.

3.10 Silencing studies

Flow cytometry

ND 7/23 /eGFPLuc cells were seeded in 24-well plates at a density of 2.5 x 10^4 viable cells per cm^2 and incubated for 24 h in supplemented DMEM medium at 37 ºC,

5% CO_2, and grown to reach 70-80% confluence prior transfection. Then, the medium was substituted by non-supplemented DMEM and dendriplexes were added (50 µL dendriplexes in a final volume of 300 µL). Dendriplexes were prepared with *N/P* ratios of 5, 10, 20, 40 and 80 as described above (siRNA concentration of 0.1 pmol/µL).

After 24 h incubation, the medium was replaced with fresh supplemented DMEM and incubated another 48 h. Then, cells were rinsed twice with 1x PBS, trypsinized, centrifuged, resuspended in 1x PBS containing 2% FBS and analyzed by flow cytometry (FACSAccuri, BD Biosciences). Non-treated cells and cells transfected with Lipofectamine® 2000 were used as negative and positive controls, respectively. The resulting data was analyzed using FlowJo software (version 10, FLOWJO, LLC). The presented data are expressed as mean ± SD of one independent sample measurements.

3.11 Statistical analysis

Data are given as mean ± standard deviation (SD) with n denoting the number of repeats. Significant differences were examined using one-way ANOVA. Turkey's multiple comparison test was further employed after one-way ANOVA for samples where homogeneity of variances was observed. Games-Howell multiple comparison test was employed after Welch ANOVA for samples violating homogeneity of variances. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using software SPSS version 23 for Windows.

Chapter 4 – Results and Discussion

4.1 Synthesis and FTIR characterization of fully biodegradable PEG-GATGE dendritic block copolymers

PEG–GATGE dendritic block copolymers are interesting nanocarriers for different biomedical applications since they have hydrophobic and hydrophilic character in the same building unit, due the presence of gallic acid and triethylene glycol butanoate arms, which conceives good capacity to encapsulate and delivery different therapeutic drugs, such as nucleic acids. Moreover, these G3 dendritic structures requiring a small amount of dendrimer to complex the nucleic acid due the presence of a high number of cationic terminal groups. This could leave to a not too high complex stability and, therefore, the siRNA release would be favored. Moreover, the dendritic vectors are fully biodegradable, so the breakdown of the dendrimer is expected to provide a high number of small charged fragments, resulting in the accumulation of counter ions inside endosomes which leaves to swelling, rupture and favoring the release of the endosomes contain to the cytoplasm. Also, the dendrimer degradation/breakdown is expected to favor the release of the transported siRNA.

Thus, fully biodegradable G3 PEG-GATGE dendrimers completely based on the biodegradable repeating unit GATGE were synthesized. These macromolecules have high multivalency (27 arms/terminal groups) and 40 different degradation points along the whole structure of the dendrimer, including an interesting degradation point between the PEG and the dendritic part.

GATGE repeating unit (**5**) is based on gallic acid (**1**) and triethylene glycol butanoate ester arms (**4**) (Figure 4.1). The repeating unit (**5**) was obtained in a very good yield from commercially available 2-(2-(2-cloroethoxy)ethoxy)ethanol (**3**), 4-bromobutyric acid and gallic acid (**1**). So, the first reaction required is the synthesis of *tert*-butyl gallate (**2**), which consists on the treatment of gallic acid with *tert*-butanol in the presence of EDC and DMAP, giving (**2**) in 74% yield. Then, 2-(2-(2-azidoethoxy)ethoxy)ethanol (easily obtained from (**3**) and NaN_3) reacts with 4-bromobutiric acid, DCC and DMAP, originating the triethylene glycol butanoate ester (**4**) in a good 82% yield. Finally, the coupling of *tert*-butyl gallate (**2**) with (**4**), in the presence of K_2CO_3, 18C6, and subsequent hydrolysis of the *tert*-butyl group, allows us to obtain the biodegradable GATGE repeating unit (**5**) in a very good 84% yield.

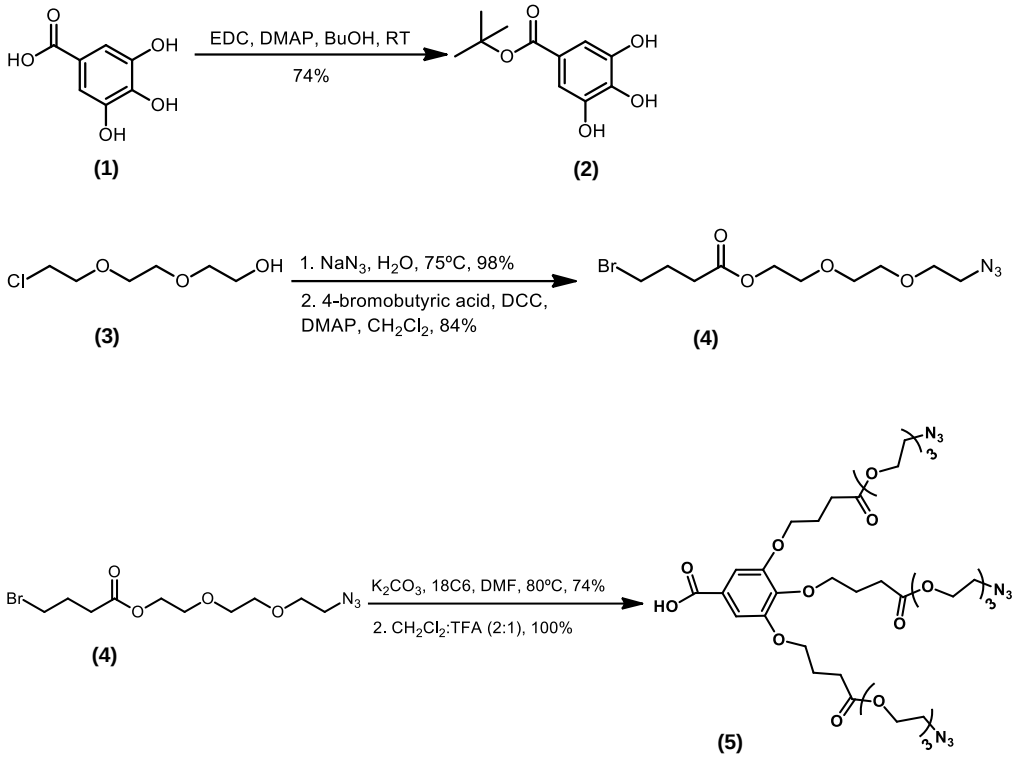


Figure 4. 1 - Synthesis of the biodegradable GATGE repeating unit 5.

Then, PEG methyl ether was added to a solution of biodegradable GATGE (**5**), EDC.HCl and DMAP to obtain the first generation of the fully biodegradable dendrimer (**6**) in an excellent 98% yield after purification by precipitation thanks to the solubility properties of PEG (Figure 4.2). The catalytic hydrogenation of the terminal azides at (**6**) under acid medium, followed by the reaction with another set of repeating units (**5**), under EDC.HCl, HOBt and Et_3N led to the formation of the second-generation dendrimer (**7**) in a good 74% yield after purification by precipitation. The same process was repeated until having the dendrimer of desired generation, in our case PEG-fbG3-N_3 (**8**) in 63% yield after purification by precipitation and ultrafiltration. Finally, amine functionalization of PEG-fbG3-N_3 (**8**) were achieved by CuAAC with the unprotected ammonium salts N-2-propyn-1-yl-1,3-propanadiamine·2HCl (**9**) and 4-ethynyl-benzenemethanamine·2HCl (**10**) to obtain fully biodegradable diamine-terminated PEG dendrimer (fbD, **11**) and fully biodegradable benzylamine-terminated PEG-dendrimer (fbB, **12**). CuAAC reaction was carried out under the presence of $CuSO_4$ (5 mol% per azide) as the source of cooper and sodium ascorbate (25 mol% per azide) as reducing agent, in DMF:H_2O 1:1 (RT, 24 h). After 24 h of reaction, the final solution was purified by ultrafiltration to obtain fbD (**11**) and fbB (**12**), in 90% and 98% yield, respectively.

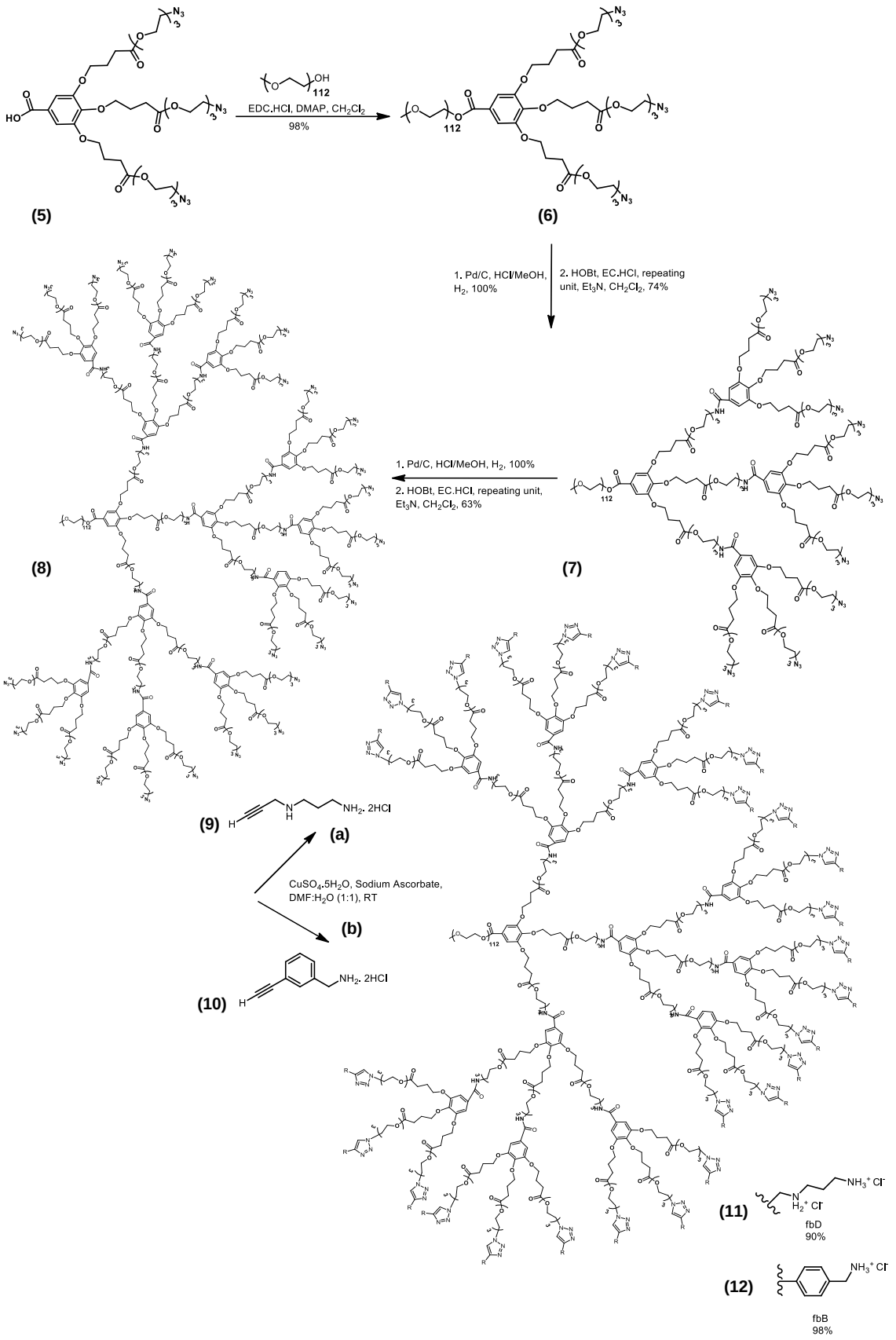


Figure 4. 2 - Synthesis of fully biodegradable amine-terminated PEG-fbG3-N_3 dendrimers. Synthesis of first-generation (G1) dendrimer (**6**), second-generation (G2) dendrimer (**7**), the final fully biodegradable third-generation (G3) dendrimer (**8**). Functionalization of fully biodegradable third-generation (G3) dendrimer (**8**) surface by "click" chemistry with diamine (fbD, **9**), and benzylamine (fbB, **10**).

All products were characterized by ^{1}H NMR (for compounds **2**, **4** and **5**, please see data in Leiro et al. J.Mat Chem B 2017; and, for dendritic copolymers, see Figure S1-S5, Suplementary Information) and Fourier-transform infrared spectroscopy (FTIR) spectroscopy (Figure 4.3). After catalytic hydrogenation of each azide-terminated dendrimer (G1 **6** and G2 **7**), the obtained amino-dendrimers were characterized by FITR to confirm the complete reduction of the terminal azides into the corresponding amine groups, which are required for the posterior coupling with the carboxylic acid of the GATGE repeating unit (**5**) and lead to the next dendrimer generation.

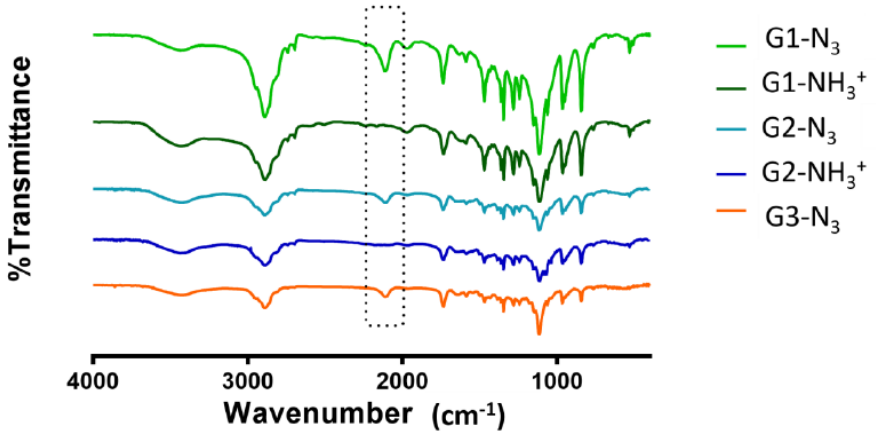


Figure 4. 3 - FTIR transmittance spectra of azide-terminated first-, second- and third- generation dendrimer (fbG1-N_3, fbG2-N_3 and fbG3-N_3) and amine-terminated first- and second-generation dendrimers (fbG1-NH_3^+ and fbG2-NH_3^+).

The azide group has a characteristic band at 2110 cm^1 and as shown in Figure 4.3, therefore after complete hydrogenation this band must disappear. As is can be seen, the hydrogenation reaction of each dendrimer was achieved in a successful manner (no presence of the azide band at 2110 cm^1 in the spectra corresponding to G1-NH_3^+ a G2-NH_3^+, Figure 4.3). Moreover, all spectra (for azide- and for amine-terminated dendrimers) clearly revealed the presence of characteristic peaks at 1741 cm^1 corresponding to the ester groups (Figure S6).

4.2 Multivalent functionalization of fully biodegradable dendrimers with unprotected amines by “click” chemistry

The cationic characteristics of amines under physiological pH is exploited to enable the binding and compaction of nucleic acids with non-viral vectors. Because of this, two different amine groups were proposed for modifying the surface of the synthesized dendritic structures: diamine group (N-2-propyn-1-yl-1,3-propanadiamine

9) and benzylamine group (4-ethynyl-benzenemethanamine **10,** Figure 4.2).[45] The functionalization of dendrimers with diamine groups, bearing two positive charges, increases the multivalency of the system without increasing the generation and size of the dendrimer, improving the binding strength of the dendrimer to the nucleic acid. Although electrostatic interactions are the major contributors to dendriplexes formation and stability, other interactions, such as hydrophobic ones, are also very important. Therefore, the functionalization with benzylamine groups aims to increase the hydrophobicity of the vector, which improves the stability of the dendriplexe.

Both amine-terminated dendrimers were characterized by ^{1}H NMR (Figure S4 and S5) and FTIR spectroscopy. The complete conjugation was verified by the disappearance of the characteristic azide band at 2110 cm^{1} (Figure 4.4).

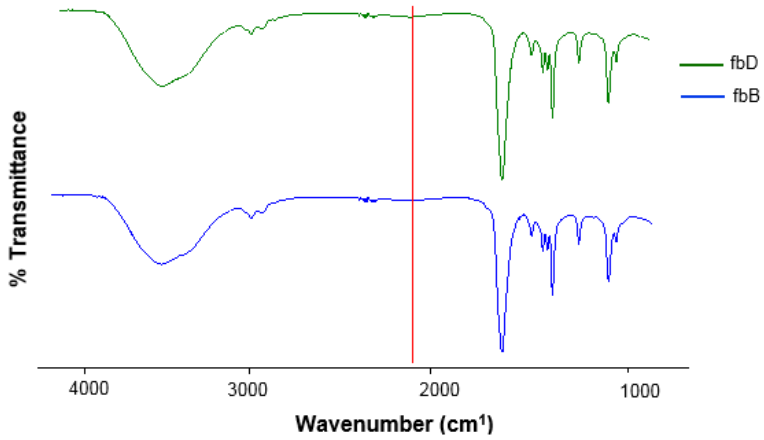


Figure 4. 4 - FTIR transmittance spectra of diamine- and benzylamine-terminated third-generation dendrimers (fbD and fbB).

4.3 Preparation and characterization of dendriplexes

Complexation between fully biodegradable amine-terminated dendrimers (fbD and fbB) and siRNA was studied and the physicochemical properties of the resulting dendriplexes were analyzed. In experiments where biological activity is not assessed, a double stranded DNA (*siDNA*) with the exactly same sequence as anti-enhanced green fluorescence protein siRNA (anti-eGFP siRNA) was used for mimic siRNA due to a easier synthesis and obtaining in higher yields and purity than their counterpart siRNA.

4.3.1 siRNA binding ability

As previously commented, to apply a gene therapy strategy, we need to use carriers with ability to bind and protect the nucleic acid.

To evaluate the complexation strength of dendrimers with *siDNA,* a polyacrylamide gel retardation assay (PAGE) was carried out. Due to *siDNA* is a short nucleic acid, this type of gel offers more resolution and sensibility than agarose gels. The binding to the dendrimer is expected to reduce the electrophoretic mobility of *siDNA* along the gel. The weakly bound or unbound nucleotide sequences will migrate through the gel when subjected to electrophoresis, whereas strongly bound nucleic acid will remain in the wells with the dendrimer, as the positive charge and size of the complexes does not allow gel migration.

As shown in Figure 4.5a, the bind nucleic acid ability improves with the increasing of *N/P* ratio. Moreover, it can also seen that benzylamine-terminated dendrimers presents a higher capacity to retain *siDNA*, probably due to hydrophobicity interactions.

In addition, the complexation efficiency was evaluated by a nucleic acid dye (SYBRGold®) accessibility assay. SYBRGold® is a cationic dye and when binding to the nucleic acid has a large increase in the fluorescence intensity, which allows determining the amount of free *siDNA*. Is important to refer that when complexed to the dendrimer, the nucleic acid is inaccessible by the dye.

The amount/percentage of *siDNA* complexed increases with the increase of *N/P* ratio, ranging from 33-70% for fbD and 42-85% for fbB (Figure 4.5b). One more time, benzylamine-terminated dendrimers showed, in general, slight better results regarding siRNA complexation thanks to their extra hydrophobicity.

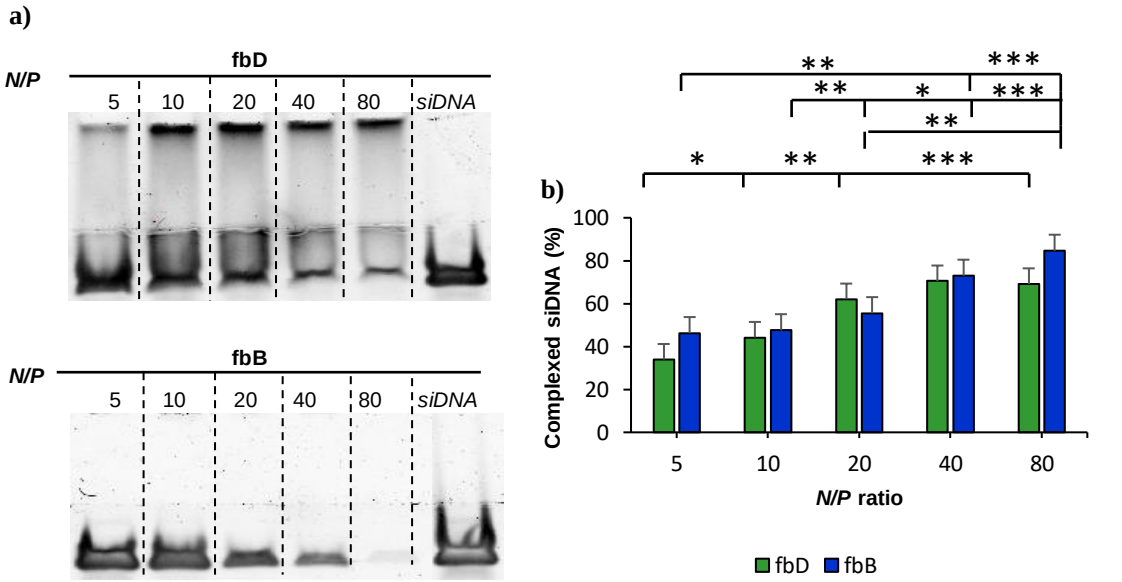


Figure 4. 5 - *siDNA* dendriplexes binding ability. a) Polyacrylamide gel retention assay (PAGE) from fully biodegradable dendrimer at different *N/P* ratios indicated above each column. In both gels, the last column corresponds to the free *siDNA*. **b)** SYBRGold® exclusion assay. Results are expressed as mean ± SD of two independent experiments. Significant differences *p<0.05, **p<0.01 and ***p<0.001. One-way ANOVA testes were used for statistical analysis.

4.3.2 Size

siDNA-dendriplexes were prepared at different *N/P* ratios for both fully biodegradable dendrimers and characterized in terms of size and polydispersity index (PDI) by Dynamic Light Scattering (DLS). The obtained dendriplexes sizes were similar for both dendrimers and for the different *N/P* ratios. Diamine-terminated dendrimers showed sizes between 70-88 nm and benzylamine-terminated dendrimers exhibited sizes ranges from 69-82 nm (Figure 4.6a). Therefore, our dendriplexes present suitable nanosizes for cellular uptake.

Both dendriplexes also showed similar PDI values (0.35 and 0.38 for diamine- and benzylamine-terminated, respectively; Figure 4.6b). The values are around 0.3, meeting the performance criteria of effective non-viral gene delivery system.

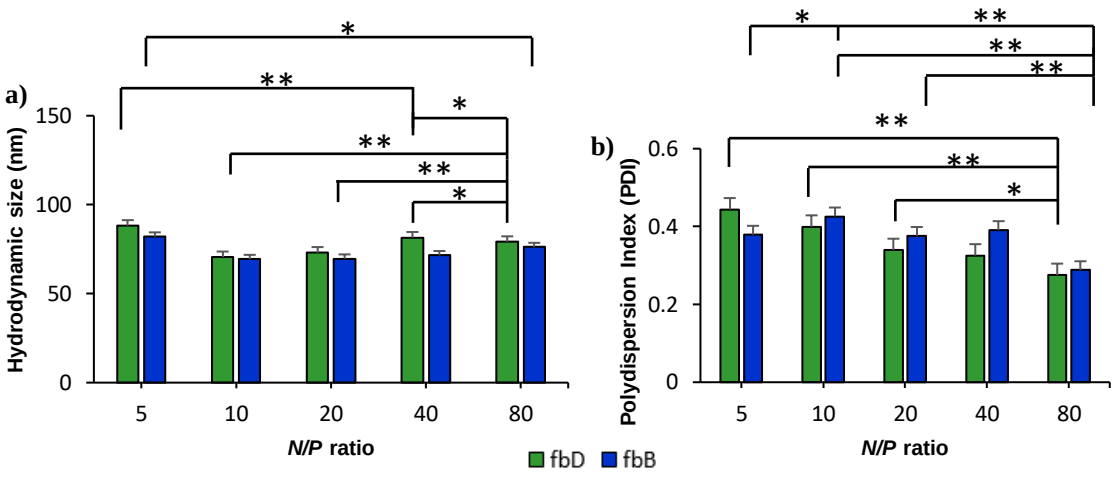


Figure 4. 6 - Physicochemical properties of diamine- (fbD) and benzylamine- (fbB) terminated dendrimers, evaluated through light dynamic scattering (DLS). a) Size distribution of *siDNA* dendriplexes at different *N/ P* ratios (n=3, mean ± SD). **b)** Polydispersity index (PDI) of *siDNA* dendriplexes at different *N/P* ratios (n=3, mean ±SD). Significant differences *p<0.05, **p<0.01 and ***p<0.001. One-way ANOVA testes were used for statistical analysis.

4.3.3 Zeta potential

Surface charges of *siDNA* dendriplexes were measured by laser Doopler electrophoresis (Figure 4.7). All dendriplexes at all *N/P* ratios showed positive charge on their surface, except *N/P* 5 for benzylamine dendrimers. Moreover, in general, the increase of *N/P* ratio leads to the increase of surface charge. Diamine-terminated dendriplexes have higher positive charge in their surface (range 1.7-2.4 mV) in comparison with benzylamine-terminated dendriplexes (range -0.49-0.85 mV). This difference in charge between both dendriplexes was expected because of higher density of positive charges associated with the diamine groups.

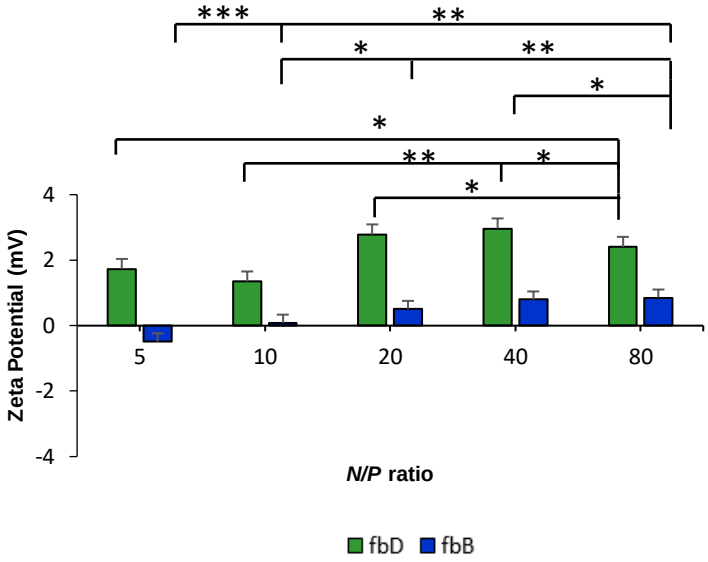


Figure 4. 7 - Zeta potential values of *siDNA* dendriplexes at diferente *N/P* ratios (n=3 ±SD). Significant differences *p<0.05, **p<0.01 and ***p<0.001. One-way ANOVA testes were used for statistical analysis.

The positive zeta potential reinforces the capacity of these nanostructures to act as gene delivery systems, since their positive charges can establish electrostatic interactions with negative charged membranes. Moreover, the values are not too high, which is an advantage because high positive charges number are associated with cytotoxic effects as we previously said.

As reported above, the zeta potential of fbB dendriplexes at *N/P* 5 was negative, presumably due to the low amount of dendrimer at this *N/P*, that together with different forms of packing of the nucleic acid, in which the *siDNA* can be more exposed to the surface of the dendriplexes can contribute for the negative ZP observed.

4.4 Biological performance evaluation

Diamine- and benzylamine-terminated fully biodegradable dendrimers were studied regarding their cytotoxicity, ability to mediate cellular uptake of siRNA and transfection efficiency in neuronal cell lines (ND7/23 cell line).

4.4.1 Cellular toxicity

Since one of our final objectives is to use these dendritic structures as gene delivery systems targeted to the nervous system, to evaluate their possible toxicity is a preponderant factor. Because of this, the toxicity of diamine- and benzylamine-terminated dendrimers and the corresponding dendriplexes were studied. Cytotoxicity studies were developed in neuroblastoma cell line (ND7/23) by metabolic changes of cells through a resazurin-based assay. The range concentrations tested was 0.04 to 2.56 mg/mL for dendrimers and the highest *N/P*s (20, 40 and 80) were tested for dendriplexes

which corresponding to the dendrimers concentrations of 0.125, 0,25 and 0.5 mg/mL, respectively. The concentrations that we have tested were very high in comparison to those that efficiently is administered in the clinic with the aim of determining the Lethal Dose (LD_{50}).

Lipofectamine and Triton X-100 were used as positive and negative controls, respectively. The metabolic activity of cells that were incubated 24 h with dendrimers was measured by comparing with untreated cells and showed values for fbD dendrimers ranging from 87 to 33% and benzylamine-terminated dendrimers from 92-43% (Figure 4.8). The diamine-terminated dendrimers showed to be more toxic to the cells than the dendrimers with benzylamine groups, probably due the high positive charge associated with diamine group. The LD_{50} for both amine-terminated dendrimers is between 1.28 and 2.56 mg/mL. Both fbD and fbB dendrimers showed a very good cytotoxic profile, probably due to the PEG chain that mask the positive charge of the amine groups presents in the periphery of the dendritic structure.

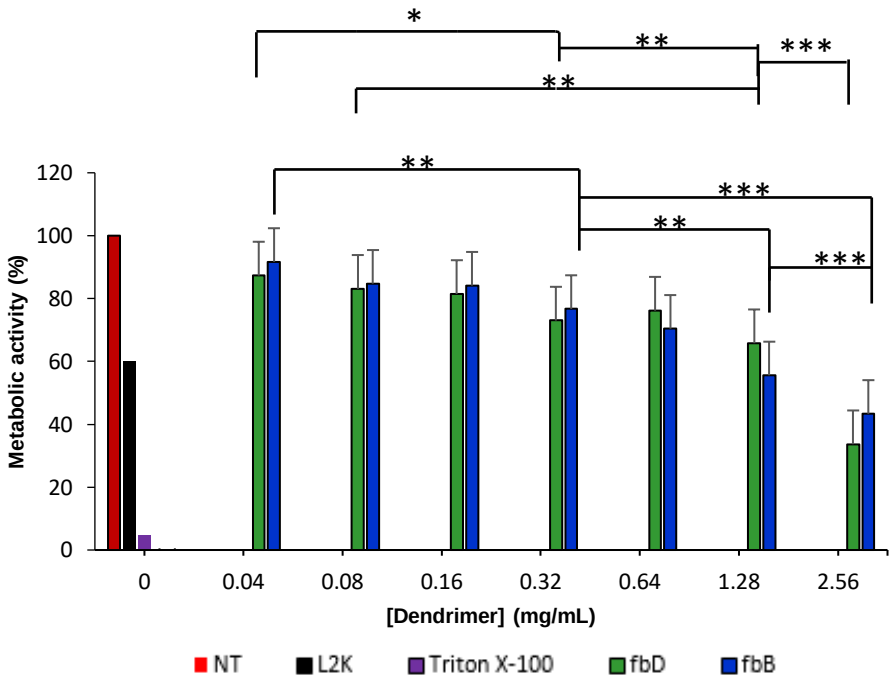


Figure 4. 8 - Relative metabolic activity, determinate through a resazurin-based assay, using untreated cells as reference, lipofectamine and triton X-100 as positive and negative controls, determined after 24 h incubation of ND7/23 cells with diamine- (fbD) and benzylamine-terminated (fbB) dendrimers (n=3 ± SD). Significant differences *p<0.05, **p<0.01 and ***p<0.001. One-way ANOVA testes were used for statistical analysis.

The toxicity of *siDNA* dendriplexes were tested at the highest *N/P*s because these ratios contain a higher amount of dendrimer, therefore these would potentially be more toxic to the cells. The metabolic activity of cells that were incubated 24 h with the *siDNA* dendriplexes, was higher than 80% for every *N/P* tested and for both dendrimers (Figure 4.9), which also indicates an excellent biocompatibility of these nanosystems.

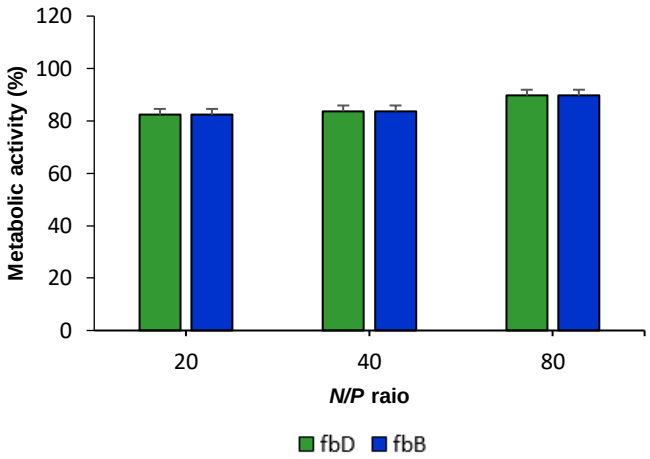


Figure 4. 9 - Relative metabolic activity, determinate through a resazurin-based assay, using untreated cells as reference, determined after 24 h incubation of ND7/23 cells with *siDNA* dendriplexes at *N/P*s 20, 40 and 80 (equivalent to a dendritic concentration of 0.125, 0.25 and 0.5 mg/mL, respectively) (n=3 ± SD). One-way ANOVA testes were used for statistical analysis. No significant differences were obtained.

As expected, the toxicity for dendriplexes was lower than for free dendrimers, because the *siDNA* present in the dendriplexes contribute to neutralize the positive groups of the amine-dendrimers, reducing the cytotoxicity level.

4.4.2 Cellular internalization

Dendriplexes ability to associate/internalize the cell membrane was evaluated by flow cytometry after incubation of ND7/23 cells at 37 ºC for 24 h with dendriplexes carrying Cy5-labeled *siDNA*. Cy5 is a cyanine fluorescence marker.

Cells treated with all developed dendriplexes showed a shift to higher fluorescence intensity compared to untreated cells (Figure 4.10). Moreover, for both cases (fbD and fbB dendriplexes) it was observed an increase of fluorescence intensity with the increasing of the *N/P* ratio, probably because of the increase of complexed siRNA percentage as well as a higher protection of this nucleic acid in the dendriplexes.

Both dendriplexes showed a very good ability to internalize the nucleic acid, especially fbD dendrimers, which showed an excellent ability to associate/internalize the *siDNA* with fluorescence intensity values similar or even higher than Lipofectamine® 2000 (a standard agent for *in vitro* transfections).

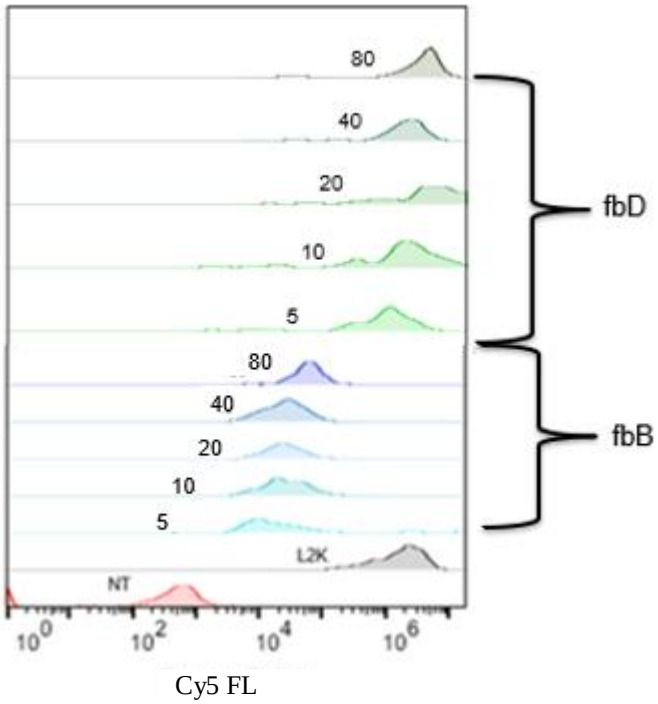


Figure 4. 10 - Cellular internalization of dendriplexes at different *N/P* ratios. Dendriplexes of Cy5 labeled *siDNA* were incubated for 24 h with ND7/23 cells at a final Cy5-*siDNA* concentration of 0.1 µM. Untreated cells and Lipofectamine® 2000 were used as negative and positive control (n=3 ± SD).

4.4.3 Transfection efficiency

The dendriplexes ability to mediate gene silencing was tested on ND7/23 cells stably expressing the fusion protein e-GFP Luciferase. Firstly, cells were incubated at 37 ºC with anti-eGFP siRNA dendriplexes with non-supplemented medium. After 24 h incubation, the medium was replaced with fresh supplemented DMEM and incubated another 48 h. Transfection efficiency was assessed by measuring the decrease of fluorescence intensity comparing with untreated cells.

As show in Figure 4.11, the fluorescence intensity of e-GFP siRNA dendriplexes decrease abruptly compared to the untreated cells. Both amine-terminated dendrimers silenced around 50% of GFP expression at every *N/P* tested. Moreover, the fluorescence intensity for dendrimers is very similar for those obtained by L2K (good standard control).

This excellent silencing effect obtained using our fbD and fbB dendrimers indicates that the dendriplexes, after internalization, and having been in the endosomal pathway, were able to escape from the vesicle to the cytoplasm. The biodegradability of dendrimers allows the breakdown of the structure, leading to the formation of many charged fragments that favors the endosomal vesicle disruption, which consequently leaves to the siRNA release to the cytoplasm and successful to fulfil its biological function.

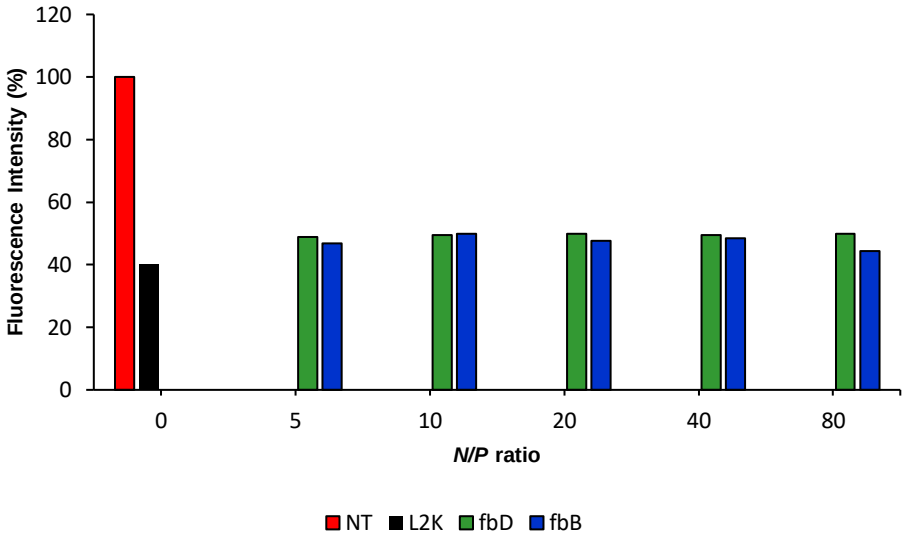


Figure 4. 11 - Percetage of fluorescence intensity upon 72 h post-transfection of anti-eGFP siRNA dendriplexes and diferent *N/P* and L2K (n=1).

Chapter 5 – Concluding Remarks

The number of people that are affected with nervous system diseases is increasing, therefore the development of new therapeutic methods, such as gene therapy, is an urgent issue. However, this type of therapy requires the development of suitable vectors with the ability to compact, protect and deliver the therapeutic nucleic acid with safety and a minimum toxicity, to the target cells. Dendrimers are very attractive non-viral vectors, due its globular, well-defined and very branched structure, controllable nanosize, and the presence of several terminal groups that allow the surface multifunctionalization according to the desired properties.

Although most of the dendrimers currently developed were effective as gene delivery systems *in vivo* and *in vitro*, they didn't were used into the clinical trials, mainly due at cytotoxicity problems. Because these structures can accumulate inside the cells/tissues, which can lead to toxicity, unleashing immune responses among other adverse effects. Therefore, very recently the interest in our research group has been focused on the development of biodegradable dendritic structures, which degrade into small fragments under physiological conditions that can be easily removed from the body. However, this is a challenging task due the undesirable/premature degradation of the vector during synthesis, purification, functionalization or even during subsequent application steps. Because of this, there are no many different structures of biodegradable dendrimers in the literature reported for specific biomedical functions.

In this work, we have synthesized the third-generation of our biocompatible, water-soluble and fully biodegradable azide-terminated PEG-GATGE dendrimers. Moreover, we have functionalized them by means of "click" chemistry with two types of amine moieties to evaluate them as siRNA delivery systems to the nervous systems, testing their ability to deliver siRNA to neuronal cell lines.

The amine-terminated PEG-GATGE dendrimers showed a good ability to bind, complex and protect siRNA. Moreover, since the physicochemical properties of the nucleic acid-vector complexes are important parameters to take into account during the development of a vector for gene delivery, the size and charge of the resulting nanoparticles (dendriplexes) were measured, showing sizes between 70-88 nm and positive surface charges, which are suitable physicochemical properties for cellular uptake.

Moreover, we have confirmed that both PEG-GATGE dendrimers and their corresponding siRNA dendriplexes showed a non-toxic profile in a neuronal cell line (ND7/23). Even we have tested high concentrations of both dendrimers in order to find the LD_{50}, that is between 1.28 and 2.56 mg/mL.

The cellular uptake was very good for both dendrimers, with higher values of internalization/association for dendrimers with diamine groups (fbD). Regarding the transfection efficiency, we have obtained an excellent silencing effect, which was similar for both dendrimers.

Our PEG-fully biodegradable dendrimers, which can be functionalized with different functional groups on the periphery by means of “click” chemistry, allows the design of a great variety of nanostructures with application in different biomedical fields, such as diagnosis, vaccines and drug delivery.

Chapter 6 – Future Perspectives

We have carried out flow cytometry experiments to evaluate the ability of the dendriplexes to associate/internalize the cell membrane. Shortly, some additional studies will be done to confirm the cellular internalization of the dendriplexes, which will be performed using confocal fluorescence microscopy assays and imaging flow cytometry. Furthermore, additional transfection replicates will be performed.

Later, to get selectivity to the desired neuronal cell/tissue, the dendritic systems will be functionalized with a suitable neuronal target molecule, which will be linked to the end of the PEG chain, in order to expose it to the surface of the dendriplexes.

Posteriorly, all biological performance of these neuronal-targeted dendriplexes will be assessed *in vitro* in neuronal cell lines, primary cultures and, finally, *in vivo* studies will be carried.

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Supplementary Information

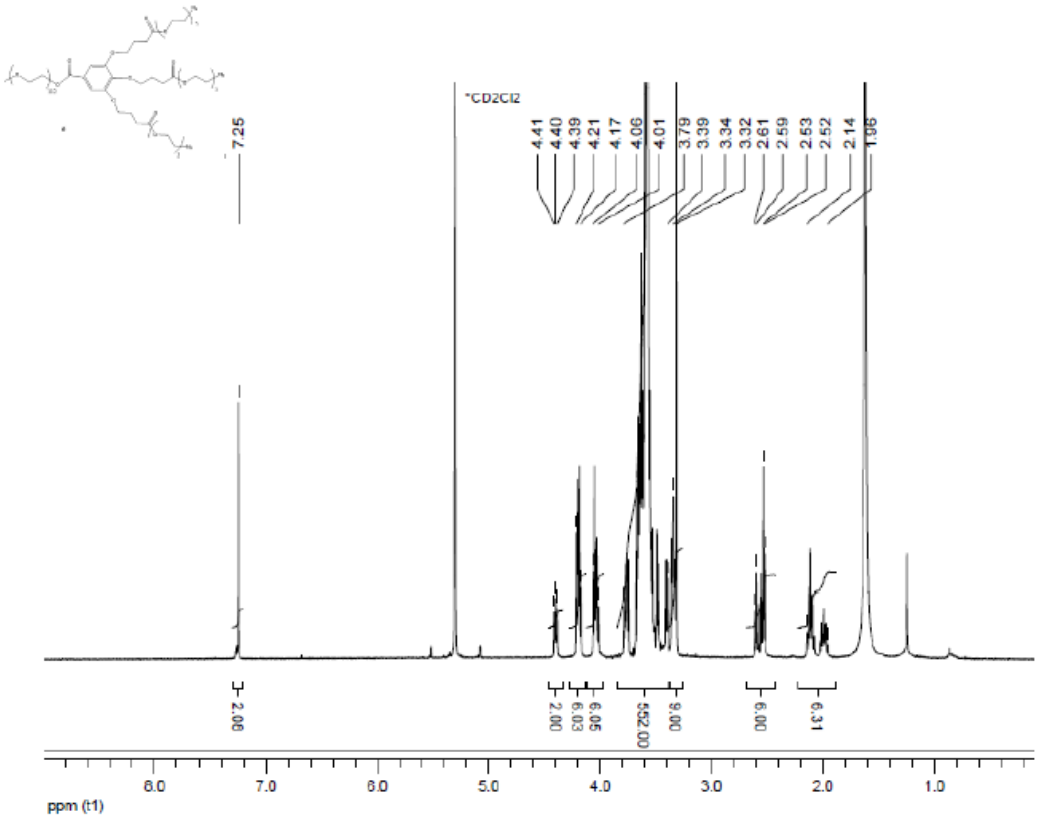

Figure S 1 - ^{1}H NMR Spectra of 6. Solvent peak labelled as * in spectra.

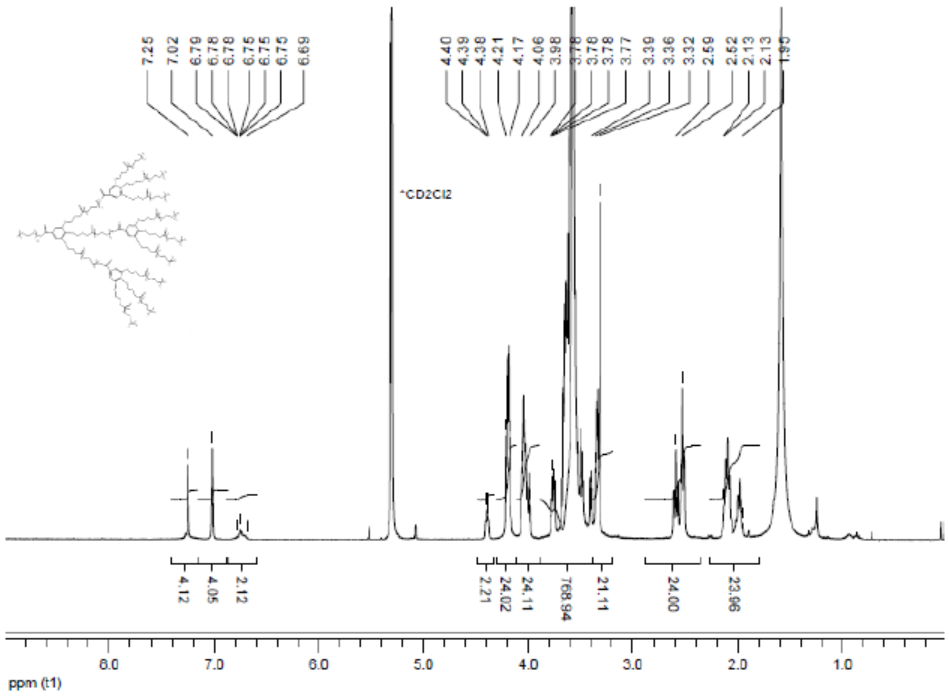

Figure S 2 - ^{1}H NMR Spectra of 7. Solvent peak labelled as * in spectra.

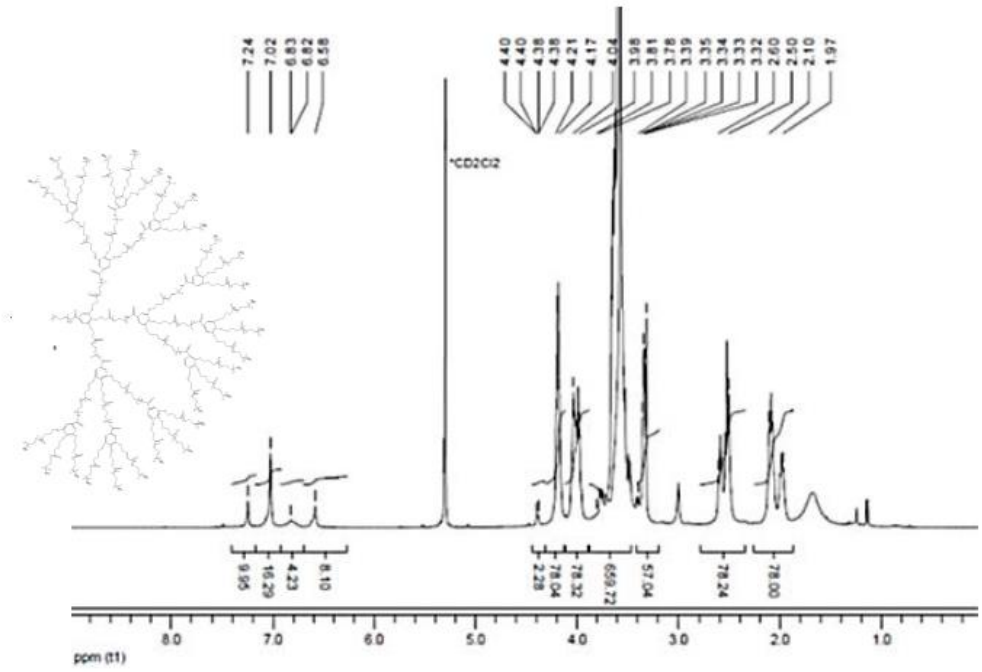

Figure S 3 - ^{1}H NMR Spectra of 8. Solvent peak labelled as * in spectra.

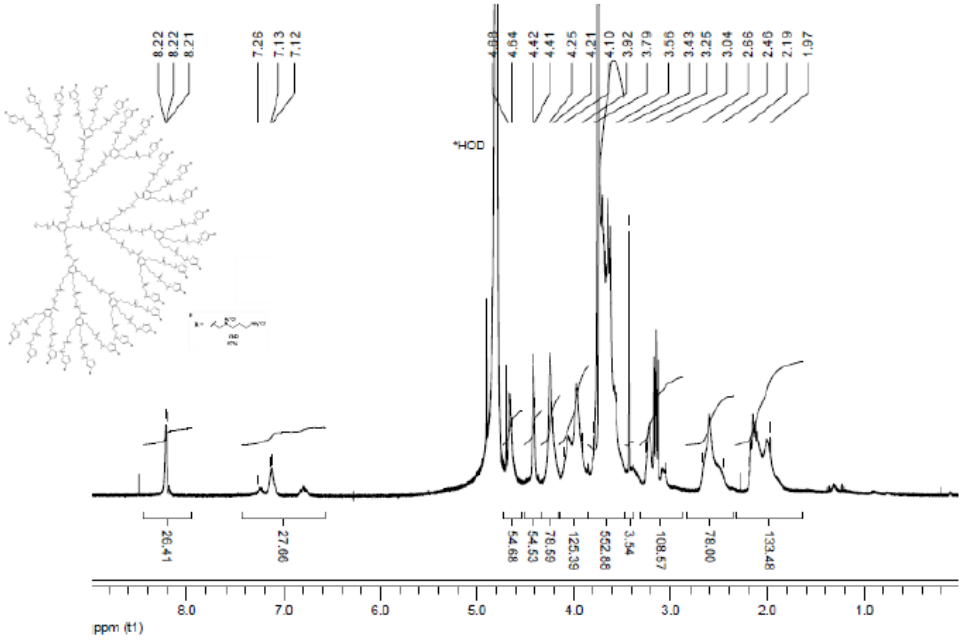

Figure S 4 - ^{1}H NMR Spectra of fbD. Solvent peak labelled as * in spectra.

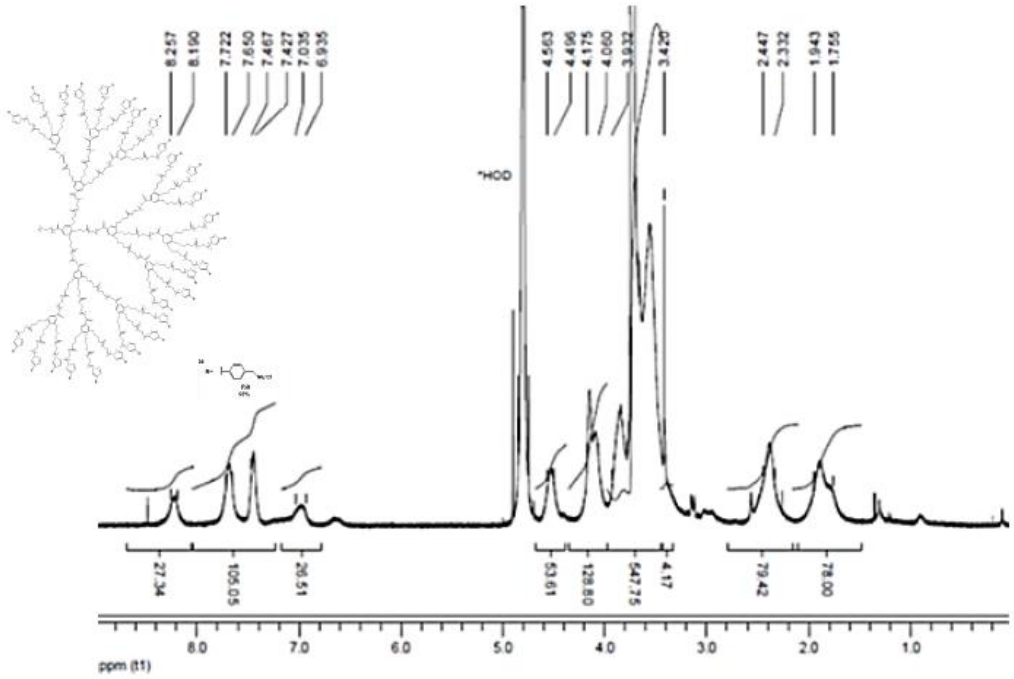

Figure S 5 - ^{1}H NMR Spectra of fbB. Solvent peak labelled as * in spectra.

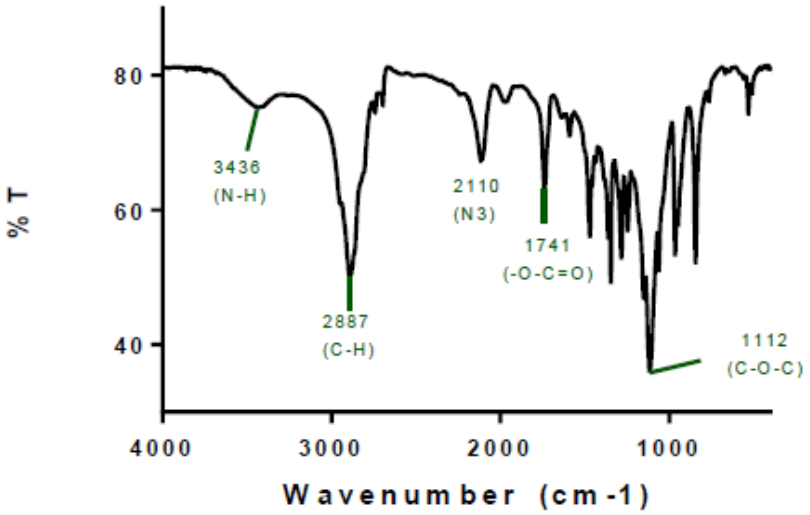

Figure S 6 - FTIR transmittance spectrum of fully biodegradable third-generation dendrimer (8) with functional groups and wavenumber identified in green. Adapted from: Leiro et al. Journal of Materials Chemistry (2017).[45]

Table S 1 - Percentage of ND7/23 cells with Cy5-*siDNA* fluorescence, after 24 h in contact with dendriplexes or Lipofectamine.

Sample	*N/P*	% of positive cells
Untreated cells (NT)		0.95
L2k		98.9
fbD	**5**	97.6
	10	94.7
	20	100
	40	97.5
	80	99
fbB	**5**	71.8
	10	90.7
	20	83.1
	40	92.5
	80	98.9