Ana Patrícia da Nave Spencer
Licenciada em Bioquímica

New Fully Biodegradable PEG-Dendrimers: Synthesis and evaluation as siRNA vectors

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientadora: Victoria Leiro Rodríguez, Doutora, i3S
Co-orientadora: Ana Paula Pêgo, Doutora, i3S

Júri:

Presidente: Professora Doutora Margarida Castro Caldas Braga
Arguente: Professor Doutor João Paulo Borges
Vogal: Doutora Victoria Leiro Rodríguez

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2017

Ana Patrícia da Nave Spencer
Licenciada em Bioquímica

New Fully Biodegradable PEG-Dendrimers: Synthesis and evaluation as siRNA vectors

Dissertação para obtenção do Grau de Mestre
em Genética Molecular e Biomedicina pela
Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologias

Orientadora:
Victoria Leiro Rodríguez, Doutora, i3S

Co-orientadora:
Ana Paula Pêgo, Doutora, i3S

Setembro de 2017

New Fully Biodegradable PEG-Dendrimers: Synthesis and evaluation as siRNA vectors

Agradecimentos

Terminar uma importante etapa nunca é tão gratificante se não for acompanhada por pessoas "gigantes" e por isso não podia deixar de agradecer àquelas que o foram aos longo dos anos.

Em primeiro lugar queria muito agradecer à minha orientadora, Victoria Leiro, por me ter dado a oportunidade de trabalhar com ela e por me ter dado um ano recheado de aprendizagem. Um enorme obrigada também por todas as palavras de motivação e de força.

À Ana Paula Pêgo por me ter dado a oportunidade de trabalhar neste grupo fantástico no qual tanto cresci, em todos os aspetos.

A todo o grupo nBTT, o meu gigante obrigada por terem recebido tão bem esta moura e por me terem feito sentir em casa durante este ano, nunca negando qualquer ajuda e nunca poupando sorrisos e boa disposição, mesmo quando o Benfica ganhava. São todos um exemplo para mim, e não só a nível profissional. Quando for grande quero ser como vocês!

À Natália, Marília, Ana Sofia, Débora e Simão um muito obrigada por tudo! Pelos longos dias de trabalho na vossa companhia, às sessões musicais no laboratório, aos infinitos almoços, aos serões à "beira" da maquina do café, às tentativas de apanhar sol e até aos momentos de tensão.

Ao meu Vasquinho e Danielzinho, ainda bem que atravessei meio Portugal para ter a oportunidade de conhecer estas pérolas. Estão no coração!

Aos meus colegas de casa que se tornaram grandes amigos, um obrigada pela companhia, jantares, festas e por todos os serões na sala. Podemos estar separados pelo mundo, mas continuamos juntos.

Ao meu peepz da margem sul, nem há palavras para tudo o que nos une. Crescemos juntos e assim continuaremos, longe ou perto. Obrigada por estarem sempre lá.

Ao David, um enorme obrigada. E também aos pais, Gabriela e Duarte, por todos os bons momentos que pude passar com vocês.

À minha família, a quem devo tudo, especialmente à minha mãe, irmão, avó e tio, um muito obrigada por todo o apoio e ajuda para tornarem tudo isto possível. Vocês são os maiores. Espero vos deixar orgulhosos!

Resumo

A terapia genética tem sido proposta como uma abordagem poderosa para tratar / prevenir várias doenças. O conceito original consistia na inserção de DNA exógeno em células com o objetivo de corrigir doenças genéticas. Atualmente, o conceito é mais abrangente e outras estratégias são consideradas, como o uso promissor de pequenos RNA de interferência (siRNA) para a inibição de curto prazo da expressão proteica. Contudo, esta terapia requer o desenvolvimento de vetores clinicamente adequados, eficazes e biocompatíveis para transportar o ácido nucleico (NA).

Os dendrímeros são vetores de NA promissores devido à sua estrutura altamente ramificada, globular e bem definida, baixa polidispersão, à presença de grupos terminais que podem ser funcionalizados com diferentes ligandos e à sua capacidade de complexar e proteger NA em nanoestruturas ("dendriplexos"). Porém, uma desvantagem da maioria dos dendrímeros aplicados atualmente em biomedicina é a sua não degradabilidade em condições fisiológicas que pode resultar em citotoxicidade induzida pela acumulação de materiais sintéticos não degradáveis nas células e tecidos. Para além disso, a biodegradabilidade pode ser uma característica muito útil uma vez que favorece a libertação de siRNA, originando maiores eficiências de transfeção.

Aqui, apresenta-se uma nova família totalmente biodegradável, biocompatível e não tóxica de PEG-dendrímeros para atuarem como vetores de NA. Estes novos PEG-dendrímeros totalmente biodegradáveis permitiram a complexação e mediação eficiente da internalização de siRNA, mostrando excelentes eficiências de transfeção.

Termos chave

Dendrímeros, Terapia Genética, Biodegradabilidade, siRNA, Nanomedicina, Nanopartículas

Abstract

Gene therapy has been proposed as a powerful approach to treat/prevent several diseases. The original concept consisted in the insertion of exogenous DNA in cells to correct genetic diseases. Currently, the concept has been broadened and other strategies have been reported, such as the promising use of small interfering RNA (siRNA) for the short-term down-regulation of protein expression. However, gene therapy requires the development of clinically suitable, effective, and biocompatible vectors to carry the nucleic acid (NA).

Dendrimers are promising NA vectors due to their globular, well-defined, and highly branched, low polydispersity, the presence of terminal groups that can be multifunctionalized with different ligands, and their ability to complex and protect NAs in nanostructures ("dendriplexes"). However, one important drawback of most used dendrimers applied biomedically is their non-degradability under physiological conditions that can result in cytotoxicity induced by non-degradable synthetic materials in the organism. Moreover, biodegradability can be useful since it will favour the siRNA release, leading to higher transfection efficiencies.

Here, we report a new family of fully biodegradable, biocompatible, and non-toxic PEG-dendritic block copolymers to act as NA vectors. These novel fully biodegradable PEG-dendrimers allowed the efficient complexation and mediation of siRNA internalization, showing excellent transfection efficiencies.

Keywords

Dendrimers, Gene therapy, Biodegradability, siRNA, Nanomedicine, Nanoparticles

List of Contents

Agradecimentos vii
Resumo ix
Abstract xi
List of tables xvi
List of abbreviations xvii
1. Introduction 2
1.1. Gene therapy 2
1.1.1. Gene Therapy Strategies 3
1.2. Gene Delivery Vectors 6
1.2.1. Viral vectors 7
1.2.2. Non-viral vectors 8
1.2.2.1. Dendrimers 12
1.2.2.2. Dendrimers Toxicity 16
1.2.2.3. Biodegradable Dendrimers 17
2. Aim of the Project 20
3. Experimental Methods 22
3.1. Materials and instrumentation 22
3.2. Synthesis 23
3.2.1. tert-Butyl gallate 23
3.2.2. 2-[2-(2-Azidoethoxy)ethoxy]ethanol 23
3.2.3. 2-[2-(2-Azidoethoxy)ethoxy]ethyl 4-bromobutanoate 23
3.2.4. 3,4,5-Tris(4-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}-4-oxobutoxy)benzoic acid (the repeating unit) 23
3.2.5. Fully Biodegradable Dendrimer 1st generation (PEG-fbG1-N_3) 24
3.2.6. Fully Biodegradable G2 Dendrimer (PEG-fbG2-N_3) 24
3.2.7. Fully Biodegradable G3 Dendrimer (PEG-fbG3-N_3) 25
3.2.8. Functionalization of PEG dendritic block copolymers (PEG-fbG3-N_3) with positively charged amine ligands by Click Chemistry 25
Diamine-terminated fully biodegradable G3 dendrimer (fbD) 25
Benzylamine-terminated fully biodegradable G3 dendrimer (fbB) 26
3.3. Dendriplex preparation 26
"Small interfering" DNA 26
3.4. Polyacrylamide gel electrophoresis shift assay 27
3.5. SYBRGold® intercalation assay 27
3.6. Size and zeta potential measurements 27
3.7. Transmission electron microscopy (TEM) 28
3.8. Cell culture 28
3.9. Cytotoxicity studies 28

3.10. Cellular uptake 28
Flow cytometry 28
Confocal microscopy 29
3.11. Silencing studies 29
Flow cytometry 29
3.12. Statistical analysis 30
4. Results and discussion 32
4.1. Design, synthesis, and characterization of fully biodegradable PEG-GATGE dendritic block copolymers 32
4.2. Multivalent functionalization of fully biodegradable dendrimers with unprotected amines by “click” chemistry 36
4.3. Preparation and characterization of dendriplexes 38
4.3.1. siRNA Binding ability 38
4.3.2. Size and morphology 39
4.3.3 Zeta Potential 40
4.4. Biological performance evaluation 42
4.4.1. Cellular toxicity 42
4.4.2. Cellular association and uptake 43
4.4.3. Transfection efficiency 45
5. Concluding Remarks 48
6. Future perspectives 50
References 52
Supplementary information 58

List of figures

Figure 1.1 The current model for the biogenesis and post-transcriptional suppression of micro RNAs and small interfering RNA. 5
Figure 1.2 Barriers for non-viral gene delivery. 12
Figure 1.3 Schematic illustration of a third-generation dendrimer. 13
Figure 1.4 Methods of dendrimers synthesis. 14
Figure 1.5 Chemical structure of selected dendrimers 16
Figure 2.6 Schematic representation of the preparation of siRNA dendriplexes with amine-terminated fully biodegradable PEG-GATGE block copolymers (PEG-GATGE). 20
Figure 4.7 Synthesis of biodegradable repeating unit (5) 33
Figure 4.8 Synthesis of fully biodegradable PEG-GATGE dendrimer upon G3. 35
Figure 4.9 FTIR transmittance spectra (KBr) of azide-terminated first-, second- and third-generation dendrimer, G1-N_3, G2-N_3 and G3-N_3 and amine-terminated first- and second-generation, G1-NH_3^+ and G2-NH_3^+. 36
Figure 4.10 FTIR transmittance spectra of diamine-terminated third-generation dendrimer and benzylamine-terminated third-generation dendrimer. 37
Figure 4.11 Binding ability. 39
Figure 4.12 Size, surface charge and morphology of fully biodegradable dendrimer/siDNA complexes. 41
Figure 4.13 Relative metabolic activity (resazurin-based assay) using untreated cells as a reference determined after 24 h incubation of U2OS cells 43
Figure 4.14 Cellular association of dendriplexes. 44
Figure 4.15 Percentage of fluorescence intensity upon 72 h post-transfection of anti-eGFP siRNA dendriplexes and different N/P, and L2k. 46

List of tables

Table 1.1 Some advantages and disadvantages of several viral vectors for gene therapy.....8

List of abbreviations

Bis-HMPA	2,2-bis(hydroxymethyl)propionic acid
Cy5	Cyanine 5
fbB	Fully Biodegradable Benzylamine-terminated PEG-dendrimer
fbD	Fully Biodegradable Diamine-terminated PEG-dendrimer
FL	Fluorescence
FTIR	Fourier-transform infrared spectroscopy
GATGE	Gallic Acid Triethylene Glycol Ester
GFP	Green Fluorescence Protein
G*n*	Dendrimer Generation (n =1,2,3,…)
L2K	Lipofectamine® 2000
miRNA	MicroRNA
mRNA	Messenger RNA
NA	Nucleic Acid
NMR	Nuclear Magnetic Resonance
N/P	Amine-to-phosphate ratio
PAMAM	Poly(amido amine)
PBS	Phosphate Buffer Saline
PDI	Polydispersity Index
pDNA	Plasmid DNA
PEG	Poly(ethylene glycol)
PEI	Poly(ethylene imine)
PLL	Poly-*L*-lysine
PPI	Poly(propylene imine)
Pri-miRNA	Primary pri-miRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT	Room Temperature
SD	Standard deviation
siDNA	“Small interfering” DNA
siRNA	Small interfering RNA
TEM	Transmission Electron Microscopy

Chapter 1

Introduction

1.1. Gene therapy

In 1944, Avery, Macleod and McCarty proved that DNA encoded human genetic information, and since that year much other genetic information has been published until 1958, when Watson and Crick published an article with the double helix structure of DNA. This discovery gave rise to a genetic revolution explaining the mechanism of various diseases and the development of new therapeutic methods, such as gene therapy.[1]

In April 2003, the field of therapy changed with the conclusion of the world's largest collaborative biological project. The Human Genome Project is considered an international scientific project that, since 1990, aimed to identify and to determine the nucleotide sequence of all the genes present in the human genome. The knowledge of approximately 3.3 billion base pairs combined with the understanding of the molecular pathways of diseases revolutionised the therapy field.[2]

Gene therapy has gained significant attention over the past decades as a promising therapeutic option for the treatment of a wide variety of diseases.[3–7] This therapy consists in the introduction of exogenous nucleic acids into specific cells for therapeutic benefits.[8–11] The effect achieved on the cells will depend on the 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. On the other hand, the insertion of small interfering RNA (siRNA) has the intent of down-regulating a defective protein.[2,6,12]

When administered intravenously, nucleic acids are vulnerable to endogenous nuclease degradation and they are incapable of crossing the plasma membrane unassisted due to their negative charge and hydrophilicity. If they can reach the interior of the cell, naked nucleic acids are again subjected to degradation in the cytoplasm or in endosomes/lysosomes. Therefore, therapeutic molecules need to be carried to their specific sites (nucleus for pDNA or cytoplasm for siRNA) protected from degradation to produce biological effects. They are typically packaged into a larger molecule generally called "vector" that must overcome a series of extracellular and intracellular barriers.

The success of this therapy is mostly dependent on the designing of a sophisticated carrier vector that can protect the nucleic acid from degradation in *in vivo* environment, provide a

sufficient circulation time by avoiding premature clearance and internalize into the target cells to deliver the therapeutic agent.[8,13–15] Initially, researchers were focused on using viral carriers genetically altered, as retroviruses and adenoviruses, in clinical trials and laboratory studies due to their high efficiency in gene delivery. However, viral vector systems demonstrated several problems such as inflammatory effects, insertional mutagenesis, immune response and safety issues for cells and tissues (Table 1.1).[8,14,16]

These limitations in viral vector systems have given rise to the field of non-viral vectors. The development of a safe, efficient, specific, and non-pathogenic vehicle for gene delivery is highly attractive. Over the past several years, many attempts have been made to develop non-viral vectors with gene transfer efficiency similar to the viral vector. [17,18]

Since the primary human gene therapy test by Rosenberg in 1989, more than 1300 clinical trials have been concluded, are ongoing or have been accepted in 28 countries, using more than 100 genes. [19,20] In August 2017, the Food and Drug Administration (FDA) approved the first gene therapy in the USA. This therapy will be used in patients, mostly children, who suffer from acute lymphoblastic leukemia. It was the inauguration of a new approach in the treatment of patients in a country that is one of the world's greatest powers.

1.1.1. Gene Therapy Strategies

Gene therapies are divided into three major strategies. The classic approach in gene therapy is based on the introduction of pDNA, encoding a therapeutic gene sequence, into the nuclei of the specific cells resulting in the expression of functional proteins and compensate their absence. [13,21] Plasmids are high molecular weight double-stranded DNA constructs which when inside cells are expressed using the machinery of the cell. The access of the plasmid molecule into the nucleus, after entering the cytoplasm, is mediated by the nuclear pore complexes. [21] To control the gene expression, the pDNA sequence can contain regulatory signals as promoters and enhancers. The first federally approved human gene therapy protocol was started in 1990 to adenosine deaminase deficiency. Since then, over 500 gene therapy protocols have been approved or implemented. Nowadays, disorders with complex etiologies like cancer and neurodegenerative diseases are being targeted.[13,22–27]

Another approach is the down-regulation of a particular protein and such is achieved with antisense oligonucleotides and siRNA. Antisense oligonucleotides are single nucleic acid sequences with 13-25 pairs in length that bind to the target RNA through Watson-Crick base pairing. The first *in vitro* application was performed by Paul Zamecnik in 1978. The strand is synthesized knowing the sequence of the disease-causing gene and will bind to the messenger RNA (mRNA) and inactivate it resulting in the silencing of the protein expression.[28–31]

Unmodified oligonucleotides have low stability, whereby novel chemically modified chains have been shown. The first modification was suggested in the late 1980s with the introduction of phosphorothioate linkage between the nucleotides and modification of the 3'- and 5'-terminal with 2'-O-methoxyethyl or 2'-O-methyl.[29] The change at each end protects the molecule from degradation by nucleases, increasing the time of circulation inside the organism. These modified nucleotides also enhance binding to the target mRNA and reduce the associated side effects. The oligonucleotides remain intact in the cleavage process and then they are available to bind to another mRNA sequence.[29] The first antisense drug was approved in 1998 for the treatment of cytomegalovirus retinitis in AIDS patient.[21] Since then, antisense oligonucleotides have been studied as potential treatment for different diseases such as cancer, amyotrophic lateral sclerosis, and Parkinson's disease.[31–33]

In 1990, Napoli and Jorgensen first described a RNAi type of phenomenon in petunias and a few years later the mechanism was elucidated in *Caenorhabditis elegans*[34–36] The mechanism involves the degradation of double-stranded RNA (dsRNA) into shorter fragments, micro RNA (miRNA) or siRNA. Micro RNAs are non-coding fragments of 21-25 nucleotides that arise naturally from the transcription of a gene inside the nucleus that forms imperfect stem-loop structures of around 80 nucleotides (pri-miRNAs) being cleaved by Drosha into precursors of approximately 70 nucleotides (pre-miRNAs). These are exported to the cytoplasm with the aid of Exportin 5 and are cleaved by Dicer into small and imperfect miRNA duplexes that contain the mature miRNA strand and its complementary strand (miRNA*). The last one is discarded and the other one interacts with the RNA-induced silencing complex (RISC) to bind partially to the complementary mRNA causing its cleavage or expression repression. (Figure 1.1).[21,34,37,38] They were first described in 1993 and so far more than 1000 miRNAs have been found in the human genome and have important cellular functions like development, differentiation, metabolism, and growth.[39,40]

The approach of gene therapy that involves the delivery of siRNA is used as a post-transcriptional mechanism of gene silencing. Small interfering RNAs are described as double-stranded RNA segments with around 25 nucleotides.[17] It is now clear that one strand of siRNA is selectively incorporated into an effector complex, the RISC located in the cell cytoplasm. Within the RISC, the sense strand is discarded, and the antisense binds and essentially stimulate the cleavage of complementary endogenous mRNA, that lead to the silencing of the gene. The structure and functions of RISC are not completely elucidated yet. In mammalian cells, duplexes siRNAs are produced from the cleavage of foreign double-stranded RNA (dsRNA), carried by several vectors to target cytoplasm as duplexes of ~500 bp, through the action of RNase III endonuclease Dicer. The response to exogenous dsRNA reflect an endogenous defence mechanism against double-stranded RNA viruses.[13,21,26,29,34–36] Another way to trigger this mechanism is through the introduction of siRNA fragments that mimic Dicer-cleaved,

endogenous microRNAs, and interact directly with RISC.[41] In this case, it is not necessary the action of Exportin 5 to help the nucleic acid to leave the nucleus and as it acts directly on the cytoplasm, it is unlikely to interfere with the natural pathway of miRNA. Furthermore, unlike pDNA, siRNAs do not have to transfer through the nuclear membrane for their activity and then promise faster development and higher efficiencies.[26] These RNA segments can be used for downregulation of disease-causing genes through RNA interference being investigated as an option to inhibit hepatitis and influenza infection.[21,42]

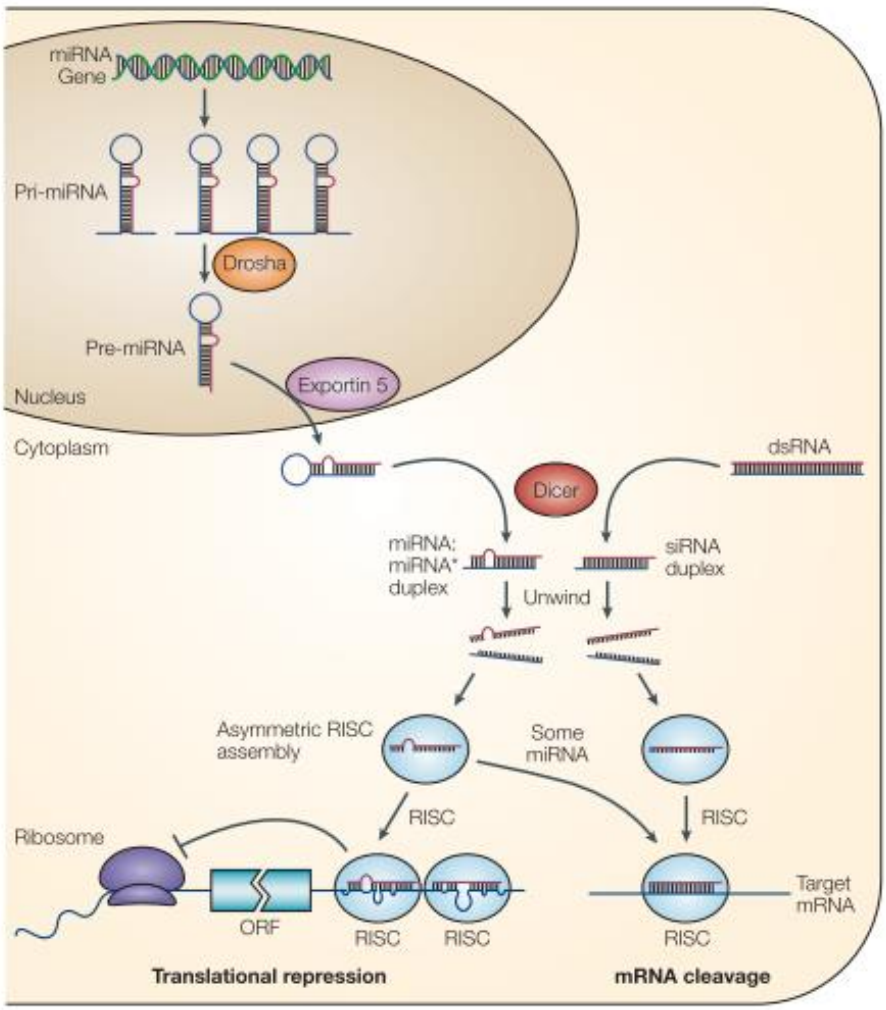

Figure 1.1 The current model for the biogenesis and post-transcriptional suppression of micro RNAs and small interfering RNA. Pri-miRNAs are transcribed from a gene by RNA polymerases and they are processed by the Drosha protein into precursors of ~70 nucleotides (pre-miRNA) within the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and cleaved by Dicer, obtaining miRNA duplexes. Dicer also cleaves the synthetic double stranded RNA (dsRNA) forming siRNA duplexes. Only one strand from each duplex bind to the RNA-induced silencing complex (RISC), which ultimately acts on the target triggering translation repression or mRNA cleavage. Adapted from: He et al., Nature reviews. Genetics (2004). [34]

However, despite the great advances made in recent years for the development of new drugs, there are still obstacles to overcome to reach its clinical application. SiRNA-based therapies have shown undesirable effects, such as the silencing of non-target protein expression. Synthetic siRNAs mimic miRNAs and may interfere with their effects. They may interact with RISC and bind to mRNAs that were not the intended target leading to the off-target silencing gene. Moreover, siRNA is able to activate innate immune response which gives rise to side effects such as the induction of pro-inflammatory cytokines or interferons.[29,43]

In the past, these effects have discredited siRNA for therapeutic application, but over the last years many modifications have been reported, reducing or eliminating all unintended events and improve potency, serum stability and specificity.[36,44–47] The proposed changes have shown lower side effects without compromising the silencing efficiency of the target mRNA. The siRNA sequence must be designed correctly so that the hybridization with the target mRNA occurs completely and thus avoiding unintended effects. It is necessary to combine the bioinformatic knowledge that through computer algorithms allows to rationally design sequences completely specific and complementary to its target. Inappropriate selection of the nucleic acid carrier vector may also contribute to the increase in off-target effects.[48]

Among the different mechanisms to silence protein expression, siRNAs are a really good choice because it is easy to discover unmodified siRNAs that work with high potency. As this nucleic acid has two strands, it is more resistant to nuclease degradation leading to a simpler delivery compared to antisense oligonucleotides. Moreover, the therapeutic effect is more potent and prolonged when siRNA is applied. This nucleic acid has its antisense strand fully complementary to the target mRNA whereas, for instance, dsRNA is only partially complementary to the target RNA. The full knowledge of siRNA synthesis methodology allows its design and preparation depending on the target gene to be silenced. There are already many commercially available ones with the ability to silence almost any gene in several different animals. For these reasons, the use of small interfering RNA has been considered as the most promising tool to be applied biomedically.[49]

1.2. Gene Delivery Vectors

The process of intentional introduction of nucleic acids into the correct target cells represents a critical step in gene therapy. Consequently, the development of clinically suitable, safe, and effective delivery vehicles for gene transport has been a major focus of research.

Previous studies reported that, when intravenously administered, nucleic acids need to overcome different barriers before they reach the target cells. The unprotected genetic material can be degraded by nucleases or aggregate with serum proteins, like albumin. Moreover, the mammalian cells were not designed to naturally uptake foreign genetic material and therefore,

after the internalization, the nucleic acid is subjected to degradation in endosomal/lysosomal compartments. The small amount of genetic material that can escape from these compartments is subsequently exposed to further cytoplasmic degradation. In some therapeutic cases, the nuclear entry is required and in this situation, there is an additional barrier to cross, the nuclear membrane. These are some of the impediments that result in the low internalization and consequently low transfection efficiency.[10]

To overcome the delivery barriers many systems have been developed and improved around the world.[10,50] A successful nucleic acid delivery carrier is desired to increase the transfection efficiency.[5] To achieve the goals, biocompatible and non-immunogenic nucleic acid delivery vectors should have the ability to compact and protect the genetic material, resist the premature degradation and be able to surpass the cellular barriers, both extracellular and intracellular. [51]

Current nucleic acid carriers are divided into two categories: viral and non-viral vectors.

1.2.1. Viral vectors

Viral vectors are derived from viruses which possess the natural ability to penetrate the host cells, deliver and exploit the cellular machinery to express its own genetic material. The ideal viral carrier would be obtained by maintaining all its internalization, delivery, and replication capabilities but expressing the intended genetic material rather than the viral material, which results in toxicity. This is achieved by genetic engineering through the removal of the viral components responsible for the pathogenicity while leaving intact all the necessary components for the packaging of the nucleic acid within the capsid or the integration into the host genome.[52,53] The resulting non-pathogenic virus carrying the therapeutic gene is called a viral vector. Several types of viruses, including retrovirus, adenovirus, adeno-associated virus, and herpes simplex virus have been modified for use in gene therapy applications.[7,52,54]

Viral vectors have aroused much interest due to their high transfection efficiency, being able to stably transfect close to 100% of target cells. These vectors have also the ability to permanently integrate the transferred nucleic acid into the host genome.[7,16,53]

However, these vectors have drawbacks such as gene carrying capacity, the difficult and expensive production in large quantities and residual viral elements that can potentially cause inflammatory response, cytotoxicity, immunogenicity and insertional mutagenesis (Table 1.1).[7,8,10,53,55] In 1999, an adverse reaction in a treated 18-year-old patient with viral vectors demonstrated that interactions with the human immune system could have fatal consequences.[52] The year 2000, brought the first success of this therapy in France with the cure of children suffering from a rare and fatal immunodeficiency, in which the effective insertion of a gene in

defective cells was achieved.[56] But by the end of 2002, almost three years after treatment, an alarming new emerged remarking that two treated children developed leukemia-like disease which has been shown to be associated with insertional mutagenesis of the gene near an oncogene.[20,52,57] Subsequently, new cases of leukemia appeared in treated patients and for safety reasons this treatment was put on hold until improvements were developed.[52]

Table 1.1 Some advantages and disadvantages of several viral vectors for gene therapy. Adapted from: Touchefeu et al., Alimentary Pharmacology and Therapeutics (2010). [125]

Vector	Advantages	Disadvantages
Viral vectors		
Adenovirus	Large transgene insert capacity Biologically safe	High immunogenicity
Herpes simplex virus	Large transgene insert capacity Availability of anti-herpetic drugs	Immunogenicity
Adeno-associated viruses	Stable transgene expression Reduced immunogenicity	Small transgene insert size capacity Need for helper viruses during manufacture
Retrovirus	Stable transgene expression Only infects dividing cells	Small transgene insert size capacity Possible insertional mutagenesis (integration)
Lentivirus	Infects both dividing and nondividing cells	Possible insertional mutagenesis (integration)
Vaccinia virus	Long history of safe human use Large transgene insert capacity	High immunogenicity Productive infection in immune suppressed patients Replication in skin lesions (eczema, psoriasis)
Vesicular stomatitis virus	Selective replication-competence in cells with defective interferon response (tumour cells)	High immunogenicity Animal pathogen (safety/environmental concerns)
Measles virus	Long history of safe human use (Edmonston vaccine strain)	Most adults are immune Wild-type virus is immunosuppressive Rare measles-like illness with vaccine strains

1.2.2. Non-viral vectors

These disadvantages of viral vectors have led scientists to find safer alternatives, such as the development of non-viral vectors.[10,11,53] The use of non-viral vector has obvious safety advantages as they present minimal toxic and immunological problems, easier to produce on a large scale through innovative synthesis schemes and they can transfer higher payloads of nucleic acids comparing with a viral delivery vector.[53,55,58,59] However, the non-viral vectors developed so far have low transfer efficiency, primarily as a result of the inability to overcome the numerous barriers encountered between the site of administration and the target cell.[50,53,59,60] Three major classes of non-viral systems can be distinguished, namely those based on lipids, polymers and dendrimers.[61] These non-viral carriers are cationic, favouring the complexation with nucleic acid through electrostatic connections with the anionic phosphate groups of nucleic acid backbone.[14] The nanoparticles formed between nucleic acids and cationic lipids, polymers and dendrimers are designated lipoplexes, polyplexes, and dendriplexes, respectively.

Unlike viruses, non-viral vectors are not designed to naturally enter cells and deliver nucleic acids. The main barriers that this kind of delivery system must overcome for successful therapeutic application are: 1) degradation of the nucleic acid by nucleases present in the extracellular medium; 2) internalization in cells 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 be avoided (Figure 1.2).[51,53,62] 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.[53] Furthermore, the interaction 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 anionic vector and promotes aggregation which results in a rapid clearance by macrophages.[10,11,51,53,63,64] 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.[60,64–66] Recent studies consistently show that PEGylated nanoparticles below 100 nm have reduced plasma protein adsorption to their surface having increased circulation time in the bloodstream.[64]

The properties of nanoparticles can influence their internalization in cells. Properties that stand out are zeta potential, size and, shape.[67] Neutral and anionic nanoparticles are internalized less efficiently than cationic ones due to the high affinity for negatively charged proteoglycans expressed on the surface of most cells.[67,68] However, it is important to note that most cationic systems have been reported as cytotoxic. After the interaction with the plasma membrane, the entry of the complex can occur through several mechanisms: clathrin- and caveolar-mediated endocytosis, phagocytosis, macropinocytosis and both clathrin- and caveolae-independent mechanisms. Some of these mechanisms are very specific in particle size, each of which is generally associated with a specific size range. 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.[67,69] Unfortunately, it is thought that the mechanisms occur simultaneously making it difficult to identify which mediates the internalization of the complex.[53,67] 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.[67]

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.[46,47] Following the endocytic pathway, early endosomes slowly mature to late endosomes through the fusion of vesicles from the Golgi apparatus which result in rapid

acidification (pH 5-6) due to the action of ATPase proton-pump enzyme.[10,53] 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.[10,53] 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. One of the mechanisms, called "proton sponge", was proposed in 1997 by Behr which describes that certain unprotonated amines of the polymer/dendrimer can absorb the protons existing in the endosome. To the natural acidification occur, more protons are transported into the vesicle leading to an increased influx of chlorine anions and water. A combination of the buffering effect delaying the acidification and osmotic swelling causes membrane rupture and consequently the release of the content into the cytoplasm.[10,14,53,72] 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.[59] Another approach is the incorporation of the chloroquine into the complex nucleic acid/vector. Chloroquine is well-known for its ability to raise pH of lysosomal environment by inhibiting the enzymes responsible for lysosomal degradation. Other molecules, such as peptides, have been studied to promote the escape of the endosome.[14,53]

The ideal PEG percentage covering the vector is also a key step. Despite the conjugation is indicated to improve the circulation time inside the organism, the high presence reduces the endosomal escape ability because it masks the positive charges of the dendritic structure reducing the possibility of these provoking the vesicle lysis.[73] Thus, hydrolysing the PEG chain binding upon arrival at the target site may be considered the indicated option. This chain release may enhance the endosomal escape capacity.

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. If the formed complex is very stable, the electrostatic bonds between the two elements are not broken and the nucleic acid will not perform the intended function. Therefore, vectors with bonds that after a stimulus trigger the degradation have been studied, external stimuli that can be variations of pH, temperature or light.[74] Some studies report that the introduction of degradable bonds increased the release of the nucleic acid and consequently increased the transfection efficiency.[74–77]

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 contrast, nucleic acids like pDNA act on the nucleus so they need to travel across the cytoplasm and overcome the nuclear membrane.[53]

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. However, the exact toxicity mechanism is not known.[53,78] Polymer and dendrimer-based systems most frequently used for assays include poly(ethylenimine) (PEI), PAMAM and poly(propylene imine) (PPI) dendrimers.[61] Although widely used, these systems have conflicting evidence regarding their biological safety because these systems are not biodegradable so there is a huge risk of accumulating inside the cells, especially after repeated administrations.[78,79] The free vectors can interact with the cell membrane and cause the destabilization of the lipid bilayer promoting the permeabilization that can finally originate lysis cells.[78] In addition to the system charge, toxicity may also be associated with the size and morphology.[79,80] 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.[61]

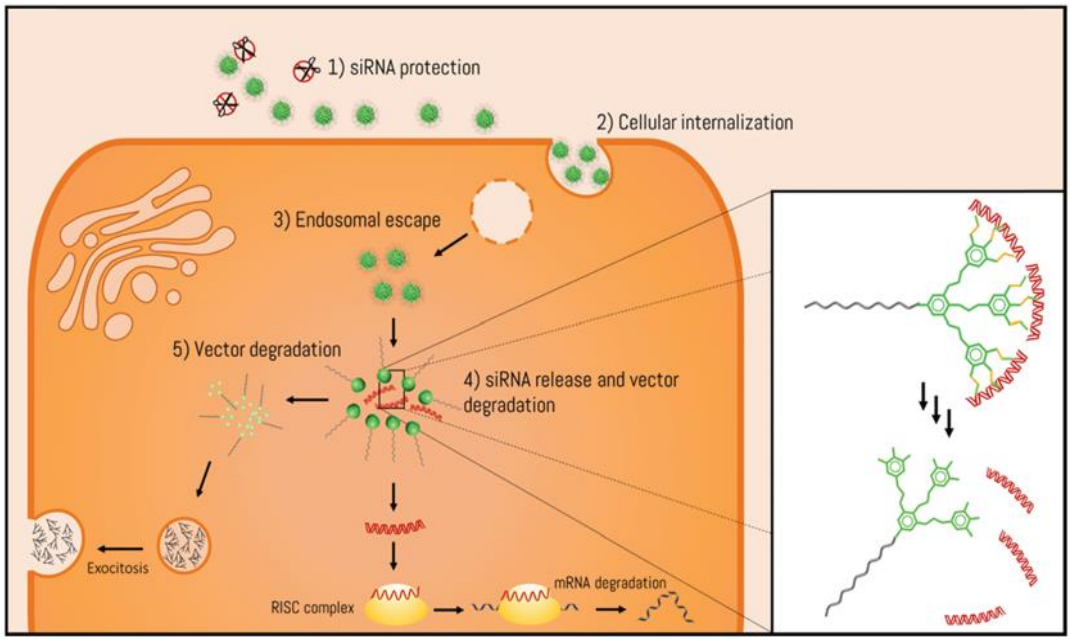

Figure 1.2 Barriers for non-viral gene delivery. 1) Protection against nucleic acid degradation by nucleases, 2) cellular internalization, 3) endosomal escape, 4) nucleic acid release from the vector and access to the cytoplasmic or nuclear target and 5) vector degradation. Adapted from: Leiro et al., Journal of Materials Chemistry (2017).[111]

1.2.2.1. Dendrimers

Very promising classes of non-viral vectors that have been developed are dendrimers. Historically, the polymers' chemistry was principally focused on linear-shaped molecules. However, innovative molecules have emerged differing from this initial idea. One of the compounds that revolutionized this field are globular macromolecules called 'dendrimers'. The word arises from the Greek *Dendron* = tree and *meros* = parts.[81–83]

Dendrimer-like structures were first synthesized in 1978 by Fritz Voëgtle and colleagues and called "cascade molecules".[82–84] In the next decade, Donald Tomalia, George Newkome, independently, with their co-workers, increased the complexity of these hyperbranched molecules and renamed them to "dendrimers". [83,85] Since then, numerous reports emerged to enrich this field with new dendritic structures developed, pioneering synthesis schemes and diverse applications.

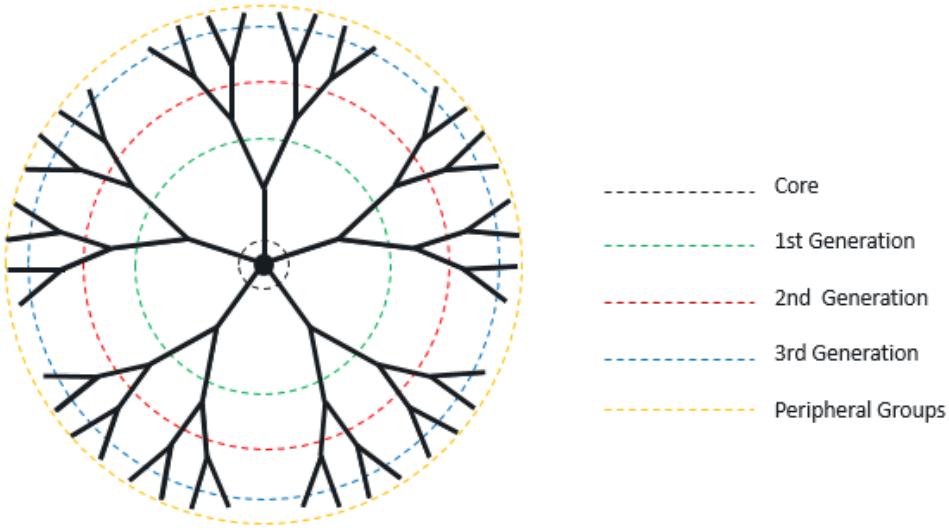

Figure 1.3 Schematic illustration of a third-generation dendrimer. Broken lines represent dendrimer's components (core, generations, and peripheral groups).

Dendrimers are highly branched macromolecules with controllable molecular weight, size, and spherical shape. [86] A typical dendrimer is composed by three main parts: the central core which may be based on one or several functional groups, repeated units or monomers covalently linked to the core organized in branching layers (generations), and the surface with multiple functional groups (or peripheral groups) which play a key role in their properties (Figure 1.3).[81–83,87] This multivalent surface can be functionalized according to its application.[82]

The branching from the core that defines the material gives it semi-globular or globular structures. Most of the dendrimers proposed so far have globular structures with diameters less than 10 nm, which can be modulated by changing dendrimer generations.[82] An increase in generation causes the nanoparticle diameter to increase and the number of terminal groups increases exponentially.[82,87] The size and shape of dendritic structures are similar to those of proteins and other biomolecules, making it an ideal biomimetic.[82]

Dendrimers have shown many advantages, such as their high solubility in different media, enhanced stability, hydrophobic or hydrophilic cavities in the interior, high compatibility, and low immunogenicity.[82,86,87] Due to the attractive chemical and physical characteristics presented by dendritic structures, several applications, mainly as carriers of bioactives molecules, have been presented in biotechnology, medicine, and in the materials' field.[82,86]

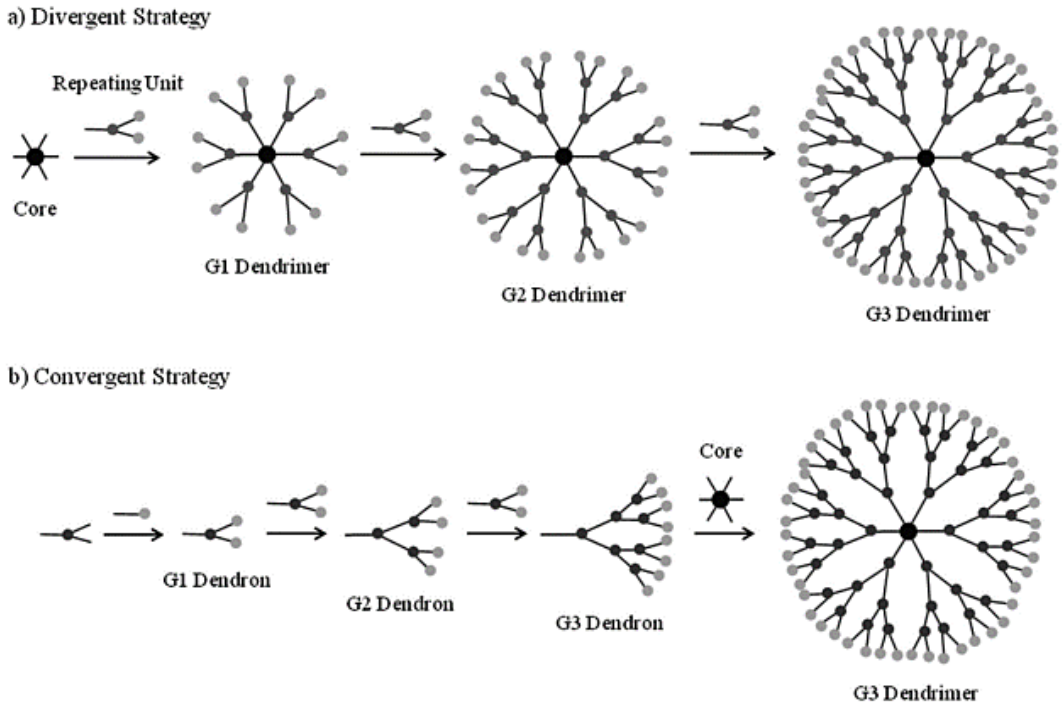

Figure 1.4 Methods of dendrimers synthesis. a) Divergent synthesizes initiates from the central core and grow until reach the periphery. **b)** Convergent synthesis begins in the branches and finally attach to the core. Adapted from: Leiro et al., Advanced Functional Materials (2017).[89]

There are mainly two conventional methods of synthesizing dendrimers. The first method to be described is known as 'divergent method'. Donald Tomalia defined it by 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 addition of repeating units, or monomers, which bind to active sites of the core, gives rise to first generation dendrimer. The peripheric group is then activated to react with the next set of repeating units creating the dendrimer layer by layer (Figure 1.4).[86,88] By repeating this process, it is possible to obtain dendrimers of a desired size. Each repetition forms a new layer of branched units (generation) that results in a molecular weight and number of groups in the periphery of the molecule increase. PAMAM dendrimers were the first synthesized by the divergent method and, currently, this method is the most favoured commercial strategy used by international producers.[81] Some disadvantages of this synthetic method are the possibility of incomplete growth and side reactions leading to defective dendrimers. Uniformity of sizes is difficult to obtain since the number of reaction increases exponentially in each step. It is very important that all reaction steps occur completely to prevent errors in the dendrimer, such as changes in its size due to the incomplete or irregular growth of the branches. This drawback can be solved by adding excessive amounts of the reagent to force the reactions to completion.[86,88]

As a solution to some of these limitations, Hawker and Fréchet developed the convergent method in 1990. In this approach, synthesis is initiated on the perfect branched dendrons (dendrimer wedges), which are attached to a multifunctional core after activation/deprotetion of their focal point.[89] This method solves the problem of purity, enhancing the monodispersion. However, some problems have been reported in the production of high generations dendrimers due to steric hindrance that branches experienced.[86,88] Although and appropriated selection of the size and multivalency of central core could reduce these effects.[89]

Due to the *in vitro* and *in vivo* behaviour, a wide range of different dendrimer families exists with great potential for biomedical applications like gene delivery, drug delivery, and magnetic resonance imaging. [82,83,90–92]

The controllable chemistry and size of dendritic structures are optimal for obtaining nanoparticles with the ideal characteristics for drug and nucleic acid delivery. [90] One of the first successful trials as achieved with a fourth-generation carboxylate-terminated PAMAM dendrimer. The dendrimer was conjugated with a poorly water-soluble anti-tumour drug.[91] There are even dendrimers, suitably modified, that act as drugs by themselves. Dendrimers with polyamines, such as PAMAM and PPI, at low cytotoxic concentrations, were able to rapidly remove infectious proteins (prions) from affected cells.[93]

Imaging is a very important tool in biomedicine for providing much information without invasive methods. The first *in vivo* applications of dendrimers were as macromolecular magnetic resonance imaging contrast reagents.

Gene delivery was achieved using a wide variety of positively charged dendrimers, such as PAMAM, which complexes with DNA and transfect cells. Kits with PAMAM-based technology for *in vitro* assays are currently marketed, like Qiagen's SuperFect and Starpharma's ProfectTM.[89]

Cascade molecules synthesized in 1978, allowed the development of PPI dendrimers that were the first family of dendrimers, and in the next decade other important, and widely used, families were reported: poly-*L*-lysine (PLL) (1981) and PAMAM (1985) dendrimers (Figure 1.5).[87,94]

PLL first prepared in the early 1980s, is a well-established family of dendrimers that have the amino acid *L*-lysine as the repeating unit.[15,95] This amino acid is positively charged at physiological pH and therefore PLL dendrimers are studied for biomedical applications. They have poor transfection ability when applied alone or unmodified as they are mostly retained in the lysosomal pathway. When administered intravenously, it binds rapidly to serum proteins causing them to be removed from the circulation.[15]

PAMAM dendrimers are relatively easy to synthesize and commercially available up to generation 10 (G10).[96] One of the major structural differences is observed in its central core, where molecules of ammonia or ethylenediamine are usually found, from which the process of

dendrimer growth begins. Its synthesis was first reported in 1985 by Tomalia's group and nowadays it is one of the most used and studied dendrimer. In 1993, Haensler and Szoka first reported the PAMAM dendrimers-based gene transfection ability.[97] However, the transfection efficiency was comparatively low. The increase in the number of terminal groups, which is proportional to the generation of the dendrimer, improves the transfection efficiency to a certain degree. With each new generation formed, the number of surface groups doubles and the molecular mass more than doubles.[15,98]

PPI dendrimers inaugurated a very promising class of biomaterials with possible applications in several fields. As their name suggest, they are based on tertiary amines and, generally, also present amine groups on their surface.[99]

Although the families mentioned above are the most used, there are other large families such as poly(ether) copoly(ester) (PEPE)[100], phosphorus[101] and gallic acid-triethylene glycol (GATG)[102] dendrimers, among others.

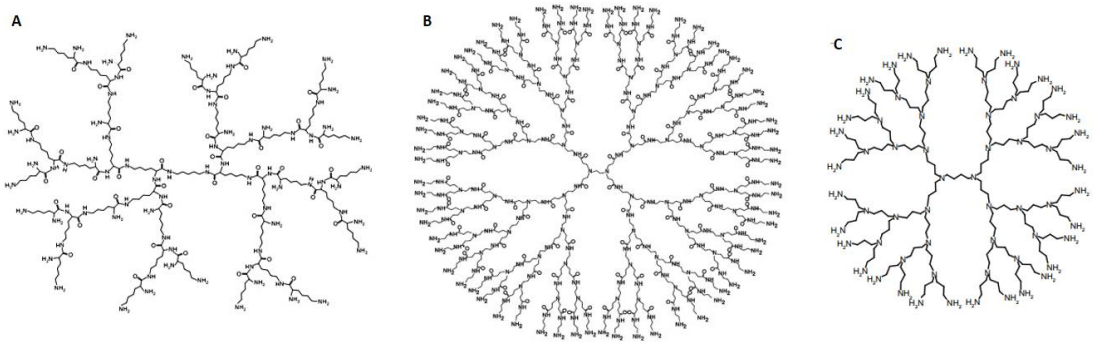

Figure 1.5 Chemical structure of selected dendrimers: (**A**) Second generation PLL[95], (B) fourth generation PAMAM[97] and (**C**) fourth generation poly(propylene imine) (PPI)[99] dendrimer.

1.2.2.2. Dendrimers Toxicity

Several of these dendrimers have been used in the *in vitro* trials for possible future biomedical applications in the fields of diagnostics, drug delivery, and gene delivery.[103] However, problems related to cytotoxicity have been reported recently and therefore the use of dendrimers in a biological system is constrained.[98] Toxicity is generally dependent on the dose, exposure time, dendrimer generation/size and nature of the groups.[98] Moreover, it is known that the toxicity is especially influenced by the characteristics of the groups with which the surface is functionalized.[15] The high number of positive charges belonging to the surface groups causes cationic dendrimers to be much more cytotoxic than the anionic ones. The cationic surface may

interact with the negatively charged cell membrane and cause its disruption.[98] Yet, many studies have already shown less cytotoxicity due to modifications on this cationic surface, such as acetylation[104] or the conjugation of PEG chains that partially shield the positive charges of the dendritic structure surface.[15,79,103–108] For instance, in 2009, Wang *et al.* had a dramatic decrease in PAMAM dendrimer cytotoxicity when it was modified with PEG. When the concentration is above 0.5 mg mL^{-1}, PAMAM caused death to most cells. At the same concentration, the number of cells in apoptosis reduced 40% when incubated with PEGylated PAMAM-dendrimers.[107]

Undoubtedly, another characteristic also responsible for dendrimers toxicity is their non-biodegradable nature.[109, 110] The majority 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 in tissues.[111] Some studies of biodegradable dendrimers that have been used as nucleic acid vectors have arisen, but few have effectively evaluated cytotoxicity.[112,113] In 2011, Barnard *et al.* reported the synthesis of second-generation (G2) 2,2-bis(hydroxymethyl)propionic acid (bis-HMPA)-based dendrimers functionalized with cholesterol as nucleic acid delivery systems. These modified dendrimers were found to be toxic to cells (50 % cell viability) above 20 µg mL^{-1}.[112] Movellan *et al.* reported in 2015 a study comparing the biocompatibility of bis-HMPA and PAMAM dendrimers. Up to the highest concentration tested, 0.5 mg mL^{-1}, the bis-HMPA-based dendrimers showed low cytotoxicity (cell viability above 80 %), while with PAMAM the cell toxicity was more elevated (cell viability between 10 and 68 %).[113]

1.2.2.3. Biodegradable Dendrimers

To overcome the cytotoxic drawback, many teams around the world are focused on developing biodegradable dendritic structures.[114] Materials that degrade under physiological conditions are ideal to overcome the risk of long-term accumulation, which could happen especially in cases of several administrations.[111] The use of biodegradable dendrimers emerged as an approach to produce desirable large molecular weight vectors that reach the target tissues in large quantities but degrade in time into smaller fragments that can be eliminated through metabolic pathways or excreted in the urine.[114,115]

The most common process of endowing a dendritic system with biodegradability is through the introduction of labile bonds to be broken due to a specific biological activity or stimulus.[111,114] Most approaches in the development of dendritic structures focus on the inclusion of hydrolysable bonds within the structure.[114] The interactions more susceptible to hydrolysis are based on anhydrides, esters, phosphoesters, hydrazones, among others. The rate of dendritic structures

degradation is controlled by different factor: 1) chemical linkages present on or connecting the repeating units; 2) the repeating units hydrophobicity – the more hydrophilic ones are degraded faster than hydrophobic ones; 3) size of dendrimers – bigger dendrimers degrade slower than the smaller ones due to the higher packaging; and 4) the location of the cleavable bonds – the hydrolysis of inner bonds leads to faster degradation of the entire dendrimer. The degradation can occur through the removal of dendritic branches, the core or peripheric groups. The cleavage of certain parts is enough to lead to the total degradation of the dendritic structure.[114]

The majority of biodegradable dendrimers reported so far are based on ester bonds. These linkages are one of those that have stood out due to their degradation rate under the conditions of the organism and their relatively easy manipulation.[109] Moreover, polyester dendrimers constitute an attractive class of compounds due to their non-toxicity and biocompatibility. These dendrimers have been shown to be associated with various novel applications. Whenever they have been tested, they have been found to have low toxicity and this is extremely important if these molecules will be used in biological systems.[115] However, the synthesis of biodegradable dendritic nanocarriers is challenging because of the undesired and/or premature degradation observed during their synthesis, purification, functionalization and/or application[116,117], therefore very few works have been reported so far where biodegradable dendritic structures are applied for a specific biomedical application.[114] Particularly in the area of nucleic acid delivery, very few examples of degradable bis-HMPA polyester dendrimers to encapsulate DNA are found.[110,111,114] Because of this, we have recently proposed a new family of water-soluble, biocompatible and hybrid-biodegradable PEG-GATGE (Gallic Acid-Triethylene Glycol-Ester) dendritic molecule, until G2, consisting on a non-biodegradable core and a biodegradable dendritic shell presenting ester bonds localized at its branches, and we have evaluated its functionality as siRNA vectors.[111] The developed macromolecule had a good biological performance without causing cytotoxic effects. It efficiently complexed the siRNA and mediated its internalization, but the transfection efficiency was low due to poor endosomal escape. To overcome this hurdle, now we propose the development of fully biodegradable dendrimers of the GATGE family.

Chapter 2

Aim of the Project

With the aim of improving the transfection efficiency results obtained with the previously mentioned hybrid-biodegradable G2 of PEG-GATGE dendrimers recently proposed by us, in this work we report the synthesis and functionalization of new fully biodegradable and biocompatible G3 of PEG-GATGE dendrimers, as well as their evaluation as a siRNA delivery system.

This new G3 dendritic structure is completely based on the biodegradable GATGE repeating unit recently proposed by us.[111] In order to combine, the already mentioned the favourable characteristics of PEG and those of dendrimers, a 5 kDa PEG chain was attached, also thought a degradable bond, to the focal point of the dendritic part, obtaining PEG-GATGE dendritic block copolymers. They present azides as terminal groups allowing the easy functionalization with different functional groups through the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC, "click" chemistry)[118,119]. In this case, their functionalization with several amine groups, that will remain positively charged at physiological pH, enables complexation of nucleic acids and explore them as nucleic acid vectors (siRNA in this work) (Figure 2.6).

Therefore, the main objective of this work was the synthesis and evaluation of these new fully biodegradable PEG-GATGE dendritic block copolymers as siRNA vectors. Firstly, PEG-GATGE copolymers were synthesized until G3 and characterized. After that, dendriplexes were prepared/produced between the synthesized G3 dendrimers and siRNA, and characterized physicochemically. Finally, their biological performance was gauged.

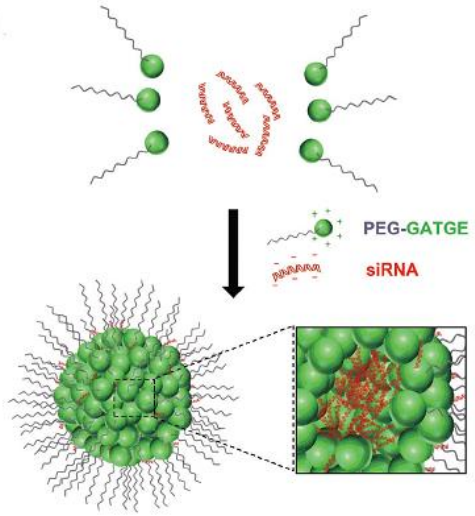

Figure 2.6 Schematic representation of the preparation of siRNA dendriplexes with amine-terminated fully biodegradable PEG-GATGE block copolymers (PEG-GATGE).

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 was purchased from VWR. N-2-propyn-1-yl-1,3-propanadiamine·2HCl and 4-ethynyl-benzenemethanamine·HCl were purchased from Amatek Chemical Co Limited. Copper (II) sulphate pentahydrate was purchased from Riedel-de-Haen. All solvents were HPLC grade and were purchased from Fluka, Prolabo, and Sigma-Aldrich and used without further purification. Column chromatography was performed with 230-400 mesh silica gel (Merck Millipore). Thin-layer chromatography was performed on silica 60/F-254 aluminium-backed plates (Merck Millipore). Ultrafiltration was done on Amicon stirred cells with Ultracel® 1 kDa membranes. Both stirred cells and membranes were purchased from Merck Millipore. Nanopure water (18MΩ cm) was obtained from a Milli-Q water filtration system (Merck Millipore).

Non-labelled *siDNA*/siRNA and the *siDNA* labelled at the 5' end of the sense strand were purchased from Integrated DNA Technologies. The nuclease free (NF) water used was purchased from Qiagen. SYBRGold Nucleic Acid Stain was purchased from Life Technologies. Dulbecco's Modified Eagle's Medium (DMEM), Opti-MEM, 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. U2OS cells stably expressing the fusion protein GFP-luciferase (U2OS/GFPLuc cells) were kindly gifted by Prof. Edvard Smith (Karolinska Institute, Sweden).

Infrared (IR) spectra were recorded using a FTIR-RAMAN Perkin Elmer 2000 spectrometer through the potassium bromide technique. Each pellet was prepared by blending 1.5 mg of the compound (vacuum dried for 24 h at 40 ºC) with 200 mg of potassium bromide. IR spectra were recorded by accumulation of 200 scans at 4 cm^{-1} spectral resolution over the range from 400 to 4000 cm^{-1} with background deduction.

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). The reaction mixture was continuously stirred at room temperature (RT) for 48 h under inert atmosphere (Ar). Oxalic acid (970 mg, 0.29 mmol) and diethyl ether (25-40 mL) were added. The resulting solution was filtered and the filtrate was washed to give *tert*-Butyl gallate (980 mg, 74%).[111]

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). The mixture was vigorously stirred for 48 h at 75 ºC. Then, the mixture was cooled down to RT and it was concentrated under reduced pressure. The resulting white residue was suspended in diethyl ether and filtered to give 2-[2-(2-azidoethoxy)ethoxy]ethanol (5120 mg, 98%), as a yellow oil.[120]

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

2-[2-(2-azidoethoxy)ethoxy]ethanol (1505 mg, 8.59 mmol), 4-bromobutyric acid (2152 mg, 12.88 mmol), DCC (2658 mg, 12.88 mmol) and DMAP were dissolved in dry dichloromethane (17.20 mL). The suspension was continuously stirred for 24 h under inert atmosphere (Ar). Triethylamine (2.80 mL, 21.50 mmol) was added and stirred for 1 h. Then, the suspension was evaporated and the resulting crude was resuspended in diethyl ether. To remove solid residues, filtration was done and the resulting yellow oil was purified by column chromatography (hexane/ethyl acetate [2:1]) to yield 2-[2-(2-azidoethoxy)ethoxy]ethyl-4-bromobutanoate (2290 mg, 82%) as a yellow oil.[111]

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, 1.11 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 heated at 80 ºC for 12 h under continuous stirring.[120] Completed the reaction time, the mixture was cooled down and the resulting residue was filtered. The filtrate was

concentrated and the resulting crude 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. [111]

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 (1:1) (5.1 mL) and magnetically stirred under inert atmosphere for 1.5 h. Then, 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 (216.3 mg, 94%).[111]

3.2.5. Fully Biodegradable Dendrimer 1st generation (PEG-fbG1-N_3)

Poly(ethylene glycol) methyl ether (100 mg, 0.02 mmol), the repeating unit (36 mg, 0.04 mmol), EDC·HCl (7.67 mg, 0.04 mmol) and DMAP were dissolved in dry dichloromethane (1.2 mL). [121] The resulting solution was magnetically stirred for 12 h at RT under an inert atmosphere (Ar), then it was concentrated and precipitated with dichloromethane/isopropyl alcohol to give PEG-fbG1-N_3 as a white powder (101 mg, 86%). ^{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 (18.3 mg, 10% w/w) and 1 M hydrochloric acid in methanol (92 µL, 0.092 mmol) were added to a solution of PEG-fbG1-N_3 (92 mg, 0.016 mmol) in methanol (3.9 mL). The resulting solution was vigorously stirred under H_2 (1 atm) for 4 h. Then, a filtration was done to remove the catalyst and the filtrate was concentrated and dried.[111,121] The resulting PEG-fbG1-$NH_3^+Cl^-$ was obtained with 100 % yield.

HOBt (10.3 mg, 0.076 mmol), EDC·HCl (14.6 mg, 0.076 mmol) and triethylamine (10.6 µL, 0.076 mmol) were added to a solution of the above product (75 mg, 0.013 mmol) and the repeating unit (68.4 mg, 0.076 mmol) in dry dichloromethane (380 µL). The resulting solution was continuously stirred at RT for 48 h under inert atmosphere. Then it was concentrated and precipitated in dichloromethane/isopropyl alcohol to give PEG-fbG2-N_3 (79 mg, 74 %).[111,121] ^{1}H NMR (400 MHz, CD_2Cl_2) δ: 1.95-2.13 (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 (14.5 mg, 10% w/w) and 1 M hydrochloric acid in methanol (153.1 µL, 0.15 mmol) were added to a solution of PEG-fbG2-N_3 (72.3 mg, 9 µmol) in methanol (3.0 mL). The resulting solution was vigorously stirred under H_2 (1 atm) for 7 h. Then, to remove the catalyst a filtration was done and the filtrate was concentrated and dried.[111,121] The resulting PEG-fbG2-$NH_3^+Cl^-$ was obtained with 100 % yield.

Hydroxybenzotriazole (33.91 mg, 0.20 mmol), EDC·HCl (48.1 mg, 0.20 mmol) and triethylamine (35.0 µL, 0.20 mmol) were added to a solution of the above product (79.5 mg, 0.011 mmol) and the repeating unit (150.3 mg, 0.20 mmol) in dry dichloromethane (835.9 µL). The resulting solution was continuously stirred at RT for 48 h under inert atmosphere. Then it was concentrated and precipitated with dichloromethane/isopropyl alcohol to give pure PEG-fbG3-N_3, as a white-yellow powder (95 mg, 63%).[111,121] ^{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

Fully biodegradable PEG dendritic block copolymer (PEG-fbG3-N_3) was dissolved in dimethylformamide/water (1:1) to give a 0.1 M final concentration of terminal azides. Alkynated ammonium salts (N-2-propyn-1-yl-1,3-propanadiamine·2HCl and 4-ethynyl-benzenemethanamine·HCl) (200 mol % per terminal azide), aqueous 0.1 M copper (II) sulfate pentahydrate (5 mol % equiv per azide) and 0.1 M sodium ascorbate (25 mol % per azide) were added and magnetically stirred for 48 h, at RT.[121] The final solution was purified by ultrafiltration (Ultracell ® 1000 MWCO) after washing with 0.1 M EDTA (pH 6), 0.6 M sodium chloride and water.

Diamine-terminated fully biodegradable G3 dendrimer (fbD)

PEG-fbG3-N_3 (51.7 mg, 3.2 µmol) and N-2-propyn-1-yl-1,3-propanadiamine·2HCl (32.0 mg, 0.17 mmol) were dissolved in dimethylformamide (431.67 µL)/water (136.7 µL) and 0.1 M sodium ascorbate (215.8 µL) and aqueous 0.1 M copper (II) sulfate pentahydrate (43.17 µL). Following the procedure above fbD was obtained as a pale yellow powder (65.3 mg, 97 %). ^{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), 7.12-7.26 (m, 26H), 8.21-8.22 (m, 27H).

Benzylamine-terminated fully biodegradable G3 dendrimer (fbB)

PEG-fbG3-N_3 (42.2 mg, 2.7 μmol) and 4-ethynyl-benzenemethanamine·2HCl (23.6 mg, 0.15 mmol) were dissolved in dimethylformamide (352.4 μL)/water (141.0 μL) and 0.1 M sodium ascorbate (176.2 μL) and aqueous 0.1 M copper (II) sulfate pentahydrate (35.24 μL). Following the procedure above fbB was obtained as a yellow powder (52.7 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 several *N/P* ratios (where *N* is the number of primary amines in the dendritic copolymer and *P* is the number of phosphate groups in the siRNA backbone) between 5 and 80 by adding siRNA (20 μM) to different volumes of dendritic copolymer solution (6 mg mL^{-1}) in Nuclease- Free (NF) water. The samples were vortexed for 10 seconds and incubated for 30 minutes at RT before experiments.

For the assays where biological activity is not measured, a double stranded DNA with the same sequence was used to mimic siRNA, as DNA oligos are easier to synthesize and obtain in higher yields and purity. siRNA sequence: sense 5'-GCUGACCCUGAAGUUCAUCUGCACC-3'.

"Small interfering" DNA

Two complementary DNA sequences (sense and antisense) were designed to replace the use of siRNA in assays where biological activity is not assessed, which we named small interfering DNA (*siDNA)*. These sequences have not any known biological function (sequences shown in Table S1.).

The ideal ratio for *siDNA* single-stranded sense and antisense sequences annealing was determined through a polyacrylamide gel electrophoresis, with 4% stacking and 15% resolving, always before new *siDNA* annealed were prepared. Figure S1. shows a representative result of these gels. Annealing was performed at a final concentration of 20 μM by mixing both strands in

the presence of the annealing buffer (100 mM Potassium Acetate; 30 mM HEPES), followed by incubation at 94 ºC for 5 minutes. Annealed sequences were then left at RT for 30 minutes before being frozen at -20 ºC.

3.4. Polyacrylamide gel electrophoresis shift assay

Polyacrylamide gels, with 4% stacking and 15% resolving gel, were prepared in Tris/borate/EDTA (TBE buffer). Dendriplex solutions were prepared as previously described using *siDNA* instead of siRNA. The volume of dendriplex solution corresponding to 12 pmol of *siDNA* was loaded with 6 µL of loading buffer and the electrophoresis run was at 100 V. The gels were stained with SYBRGold® nucleic acid stain, diluted in TBE buffer (1:10000), for 10 minutes and visualized using GelDoc XR imager (BioRad). The bind between dendriplex and *siDNA* 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 for 10 minutes at RT in a 96-well black plate with 2 µL of a 1:100 SYBRGold solution (in TAE buffer) (final volume 200 µL). After incubation, using a microplate reader (SynergyMx, Biotek), the fluorescence was measured (λ_{exc} = 485 nm, λ_{em} = 540 nm).

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 three 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 scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK) following the manufacturer's instructions. Size and PDI were determined at RT with a detection angle of 173º using ZEN0040 cells in the automatic mode. The mean hydrodynamic diameters were determined by cumulative analysis (Z-average mean). Zeta potential measurements were performed with a detection angle of 173º. For size and PDI measurements, dendriplexes were prepared in a final volume of 80 µL. For zeta potential measurements, dendriplexes were prepared in a final volume of 250 µL and diluted to 750 µL in milli Q water prior to the measurements in capillary cells (DTS1070). The Smoluchowski model was applied for zeta potential determination, and cumulant analysis was used for mean particle size determination.

The presented data are expressed the mean ± SD of three independent sample

measurements. The software used was Zetasizer Software version 7.11, supplied by the manufacturer (Malvern Instruments, UK).

3.7. Transmission electron microscopy (TEM)

Dendriplexes were prepared as previously described at *N/P* of 10, 40 and 80. Samples were mounted on a 200-mesh Ni grid with Formvar and carbon supporting film (not glow discharged) and stained with 2% uranyl acetate solution. The stains in excess were removed with filter paper, and the grid was dried prior to imaging. Samples were imaged using a Jeol JEM 1400 operated at 80 kV. Images were processed using ImageJ software (NIH, USA).

3.8. Cell culture

The osteosarcoma cell line U2OS was cultured in DMEM medium supplemented with 10% (v/v) heat inactivated FBS (56º C, 30 min) and 1% Penicillin-Streptomycin at 37 ºC, 5% CO_2, in a cell incubator.

3.9. Cytotoxicity studies

Cell viability was evaluated as a function of dendrimer/dendriplex type, concentration, and N/P ratio. U2OS/eGFPLuc cells were seeded in 96-well plates 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 grown to reach 70-80% confluence prior transfection. The medium was substituted by non-supplemented DMEM 1 h at the time of transfection.

24 h post transfection, the medium was replaced with fresh medium containing 10% (v/v) FBS and 10% resazurin and incubated for further 3 h. After incubation, using a microplate reader (SynergyMx, Biotek), the fluorescence was measured (λ_{exc} = 530 nm, λ_{em} = 590 nm). Cells' viability exposed to the dendrimer was expressed as a percentage of viability of non-treated cells. The presented data are expressed as mean ± SD of three independent sample measurements.

3.10. Cellular uptake

Flow cytometry

U2OS 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. The medium was substituted by non-supplemented DMEM

1 h before transfection. Dendriplexes were prepared with Cy-5 labeled siDNA as previously described with *N/P* ratios of 5, 10, 20, 40 and 80. Transfection was performed using 50 µL dendriplexes in a final volume of 300 µL (*siDNA* concentration of 0.6 pmol μL^{-1}).

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

Confocal microscopy

U2OS 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. The medium was substituted by non-supplemented DMEM 1 h before transfection. Dendriplexes were prepared with Cy-5 labeled *siDNA* as previously described with *N/P* ratios of 5 and 10. Transfection was performed using 50 µL dendriplexes in a final volume of 300 µL (*siDNA* concentration of 0.6 pmol μL^{-1}). After 24 h, transfected cells were washed three times with PBS and incubated with PFA 4% (Sigma) for 15 minutes at RT for cell fixation. Cells were then washed three times with PBS and incubated for 10 minutes at RT with a 1:10 000 diluted solution of DAPI (Life Technologies) for nuclear staining. After this time, cells were then washed three times with PBS and incubated for 20 minutes at RT with a 1 : 100 diluted solution of Alexa Fluor 488 Phalloidin (ThermoFisher) for filamentous actin staining. Cells were then washed three times with PBS. Coverslips were then mounted with Fluoromount™ Aqueous Mounting Medium (Sigma) and samples were observed and photographed on Leica TCS SP5 Confocal Microscope (Leica Microsystems, Inc). Three-dimensional z-stacks at a resolution of 1024x1024 pixels were captured and processed using Leica Application Suite X 3.3.0.16799 software (Leica Microsystems).

3.11. Silencing studies

Flow cytometry

U2OS/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. The medium was substituted by non-supplemented

DMEM 1 h before transfection. Dendriplexes with *N/P* ratios of 5, 10, 20, 40 and 80 were prepared as previously described. Transfection was performed using 50 µL dendriplexes in a final volume of 300 µL (siRNA concentration of 0.6 pmol μL^{-1}).

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 analysed by flow cytometry (FACSCalibur, BD Biosciences). Untreated cells and cells transfected with Lipofectamine® 2000 (Invitrogen) were used as negative and positive controls, respectively. Lipofectamine® 2000 was used according to the manufacturer instructions. The resulting data was analysed using FlowJo software (version 10, FLOWJO, LLC) The presented data are expressed as mean ± SD of one independent sample measurements.

3.12. 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 applied after Welch ANOVA for samples violating homogeneity of variances. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla California USA.

Chapter 4

Results and discussion

4.1. Design, synthesis, and characterization of fully biodegradable PEG-GATGE dendritic block copolymers

As previously mentioned, the G2 of a hybrid-biodegradable PEG-GATGE dendrimer recently developed by our team[111] has been shown to be biocompatible, non-toxic, and capable of complexing, protecting, and transporting siRNA into the cells. However, low transfection efficiencies were obtained, which would be much better if the endosomal escape was not such a difficult challenge to overcome. The dendriplexes formed and internalized showed difficulty in endocytic vesicles leakage.

Since in dendriplexes formation, the primary responsible interaction is electrostatic and occurs between positively charged dendrimers and negatively charged nucleic acids, an increase in dendrimer generation results in higher positive charges density available to interact with nucleic acids (NA), usually leading to improved dendriplex stability and the delivery can happen in an improved manner, rendering higher transfection efficiencies.[2]

Thus, to improve the results and to overcome this limiting step (poor endosomal escape and low transfection efficiencies), we have proposed the development of fully biodegradable PEG-GATGE dendritic block copolymers of a higher generation (G3). This G3 dendritic structure provides a larger number of cationic terminal groups, requiring a smaller amount of dendrimer to complex the NA. Thus, the complex stability is expected to be lower and the siRNA release would be favoured. Moreover, the fully biodegradable nature will be advantageous in several ways. On one hand, is expected to provide a high number of small charged fragments after breakdown of the dendrimer, what will lead to an accumulation of counter ions inside endosomes, resulting in their swelling, rupture and favouring the release of the endosomes contain to the cytoplasm. On the other hand, the dendrimer degradation/breakdown will favour the release of the transported siRNA.

Therefore, both characteristics (third-generation and fully degradability) are expected to favour the intracellular release of the NA, leading to higher transfection efficiencies.

Thus, a fully biodegradable G3 PEG-GATGE dendrimer completely based on the biodegradable repeating unit GATGE[111] was synthesized. This new PEG-dendrimer has higher multivalency (27 arms/terminal groups) and 40 different degradation points, including the linkage between the PEG chain and the dendritic part. So, after degradative action, dendritic fragments

charges would be more exposed and this facilitate the endosomal vesicles escape as previously commented (Section 1.2.2).

The synthesis of the biodegradable GATGE repeating unit consisting of gallic acid and triethylene glycol ester arms is shown in Figure 4.7. Despite the presence of degradable ester bonds in the structure, which complicates the synthesis, the repeating unit (**5**) was obtained in very good yield from commercially available 2-(2-(2-azidoethoxy)ethoxy)ethanol (**3**), 4-bromobutyric acid and *tert*-butyl gallate (**2**). Firstly, the synthesis of *tert*-butyl gallate (**2**) was required. Gallic acid (**1**) was treated with *tert*-butanol in the presence of EDC and DMAP, giving **2** in 74% yield. Initial treatment of 2-(2-(2-azidoethoxy)ethoxy)ethanol (easily obtained from **3** and NaN_3)[111,120] with 4-bromobutiric acid, DCC and DMAP led to the ester **4** in 82% yield. Subsequently, the coupling of *tert*-butyl gallate (**2**) with **4** (K_2CO_3, 18C6), followed by hydrolysing the *tert*-butyl group, led to the wanted biodegradable GATGE repeating unit **5** in 74% yield.

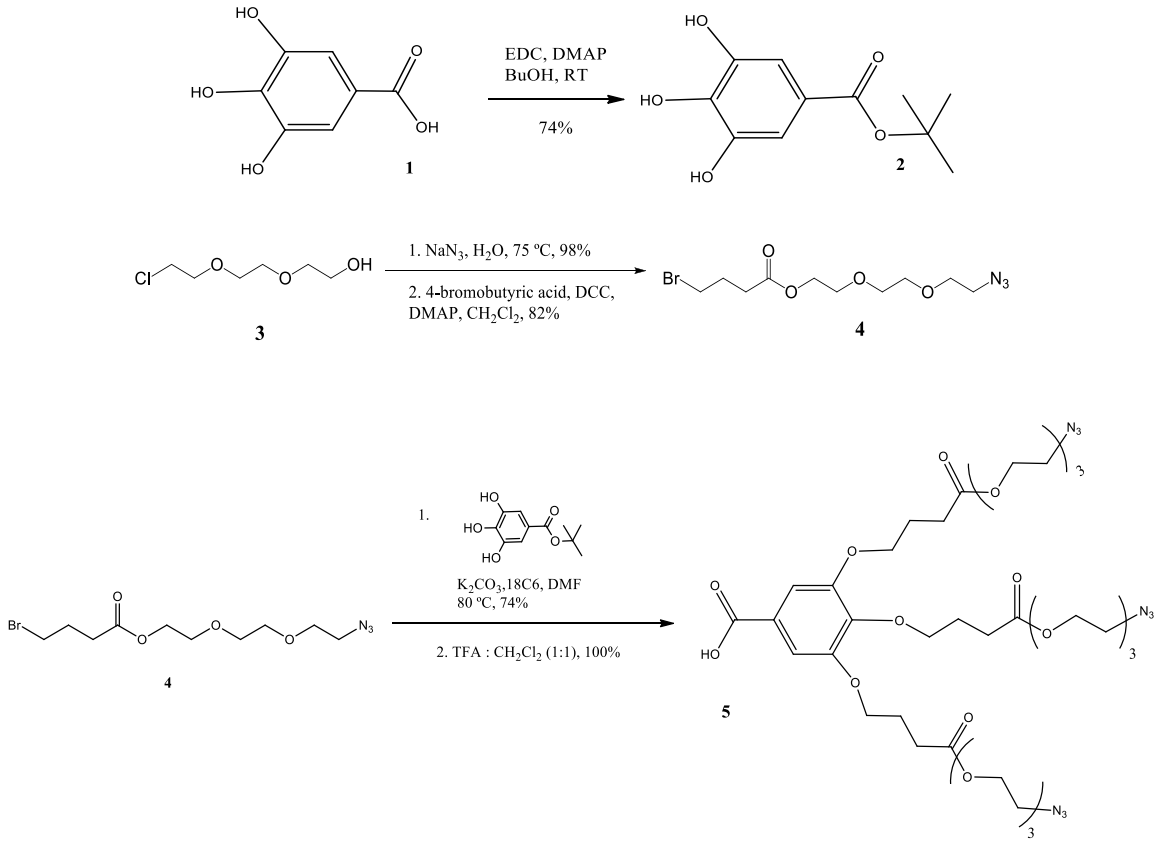

Figure 4.7 Synthesis of biodegradable repeating unit (**5**)

PEG methyl ether was added to a solution of **5** to give the fully biodegradable PEG-G1 dendrimer (**6**) (EDC, DMAP) in an excellent 86% yield after purification by precipitation, thanks to the solubility properties of PEG (Figure 4.7). The catalytic hydrogenation of the terminal azides at **6** under acid medium, followed by the reaction with **5** and triethylamine (EDC, HOBt, Et_3N) led to the formation of the G2 dendritic structure (**7**) in 74% yield, again after purification by precipitation (Figure 4.8).

Repeating the previous process but this time in the dendritic compound of G2 (**7)**: acid catalytic hydrogenation of the terminal azides, and the subsequent reaction with **5** (EDC,HOBt, Et3N), the azide-terminated G3 dendrimer (**8**) is obtained in 63% yield, after purification by precipitation. (Figure 4.8)

All products were characterized by ^1H NMR (for compounds 2, 4 and 5, please see data in Leiro et al., J.Mat Chem B 2017;[111] and, for dendritic copolymers, see Figure S2-S4, Suplementary Information) and FTIR spectroscopy (Figure 4.9). 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.

The azide group has a characteristic band at 2110 cm^{-1} and as shown in Figure 4.9, so after complete hydrogenation this band must disappear. As it can be seen, the hydrogenation reaction of each dendrimer occurred in a successful manner with no band at 2110 cm^{-1}.

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 S2.).

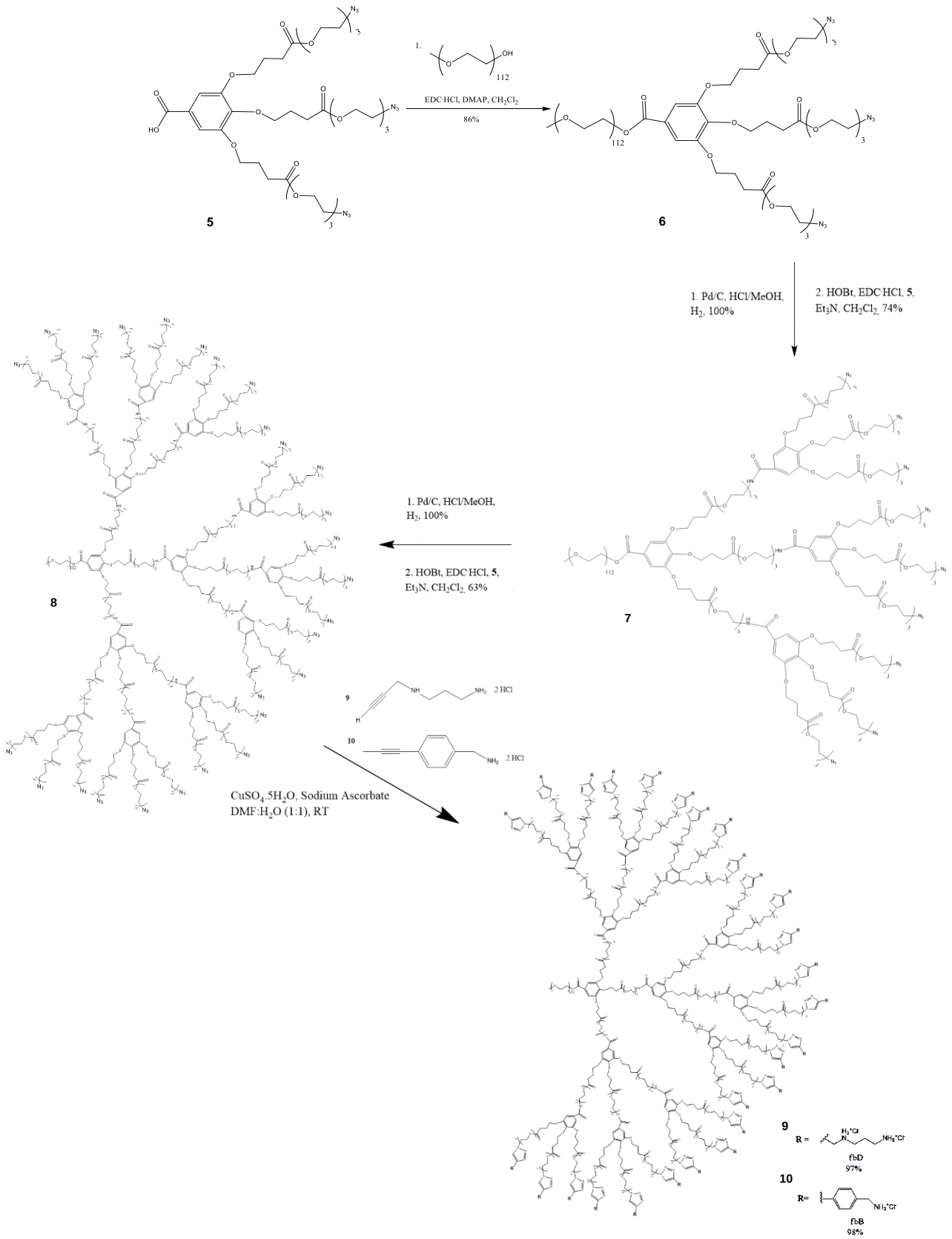

Figure 4.8 Synthesis of fully biodegradable PEG-GATGE dendrimer upon G3. Synthesis of first-generation (G1) dendrimer (**6**), **b)** second-generation (G2) dendrimer (**7**), the final fully biodegradable third-generation (G3) dendrimer (**8**). Functionalization of fully biodegradable third-generation (G3) dendrimer (**8**) surface functionalization by “click” chemistry with diamine (fbD **9**), and benzylamine (fbB **10**).

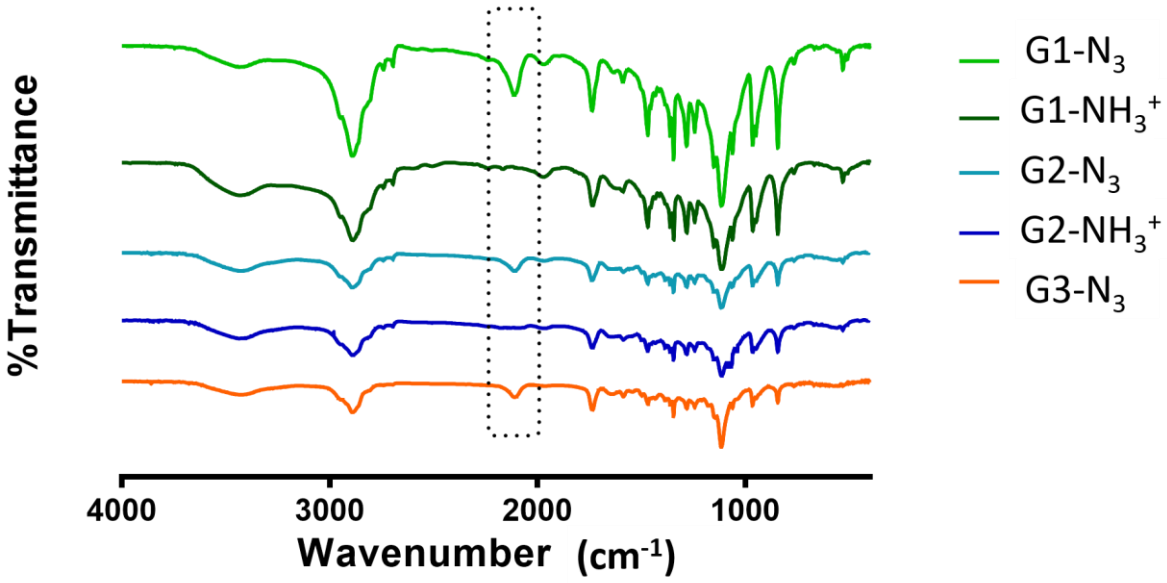

Figure 4.9 FTIR transmittance spectra (KBr) of azide-terminated first-, second- and third-generation dendrimer, G1-N_3, G2-N_3 and G3-N_3 and amine-terminated first- and second-generation, G1-NH_3^+ and G2-NH_3^+.

4.2. Multivalent functionalization of fully biodegradable dendrimers with unprotected amines by "click" chemistry

The cationic characteristic of amines at physiological pH is regularly exploited to enable the binding and compaction of NA with the several non-viral vectors being developed.[2]

Here, two different amine groups are proposed for modifying the surface of the synthesized dendritic structure: diamine group (N-2-propyn-1-yl-1,3-propanadiamine **9**) and benzylamine group (4-ethynyl-bensenemethanamine **10**). By functionalizing with the diamine groups, bearing two positive charges, it is desired to increase the multivalency of the system without increasing the generation and size of the dendrimer, and thus the binding strength of the dendrimer to the NA will be higher. Electrostatic interactions are the major contributors to complexes formation and stability, but other interactions are very important as well, namely the hydrophobic interactions. Thus, functionalization with benzylamine groups seeks to further increase the hydrophobicity of the vector which, besides the contribution to the complex stability, interact with the hydrophobic components of the cell membrane what may be useful in internalization.

As previously commented, the presence of azides as terminal groups allows the easy surface functionalization of this family of dendrimers by means of the Cu (II)-catalyzed Huisgen cycloaddition (CuAAC, "click" chemistry). Therefore, the dendrimer surface functionalization was performed using the Cu (II)-catalyzed Huisgen cycloaddition with the alkynated ammonium salts **9** and **10** (Figure 4.8). The reaction was carried out with $CuSO_4$ (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, 48 h) (Figure 4.8).

The resulting diamine- and benzylamine-terminated fully biodegradable dendrimers (fbD and fbB) were purified by ultrafiltration and obtained in excellent yields of 97 % and 98 % (Figure 4.8). Both products were characterized by ^1H NMR (D_2O) (Figure S5 and S6) and FTIR spectroscopy (KBr technique) (Figure 4.10).

The complete conjugation was verified by the disappearance of the characteristic azide band that arises at 2110 cm^{-1} (Figure 4.10).

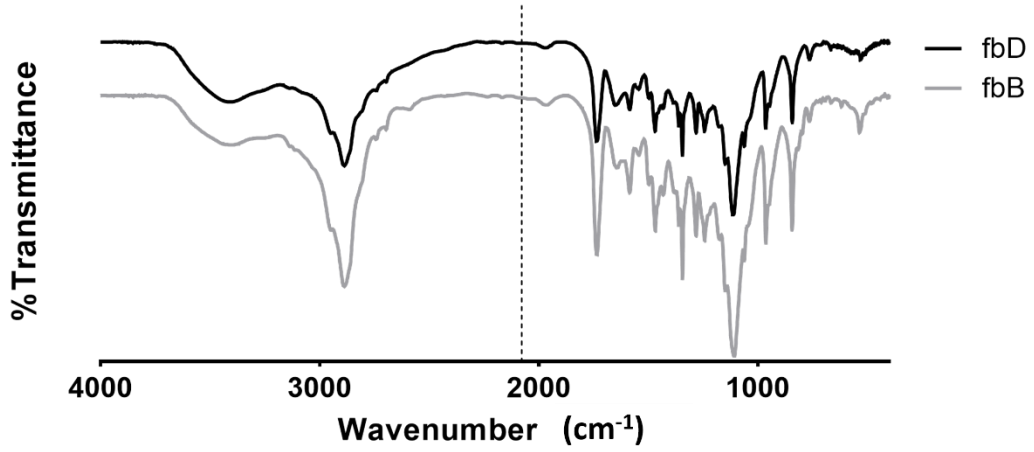

Figure 4.10 FTIR transmittance spectra of diamine-terminated third-generation dendrimer and benzylamine-terminated third-generation dendrimer. (KBr) Broken line at 2110 cm^{-1}.

4.3. Preparation and characterization of dendriplexes

Complexation between the fully biodegradable amine-terminated dendrimers (fbD and fbB) with siRNA was studied and the physicochemical properties of the resulting dendriplexes were evaluated. 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 as a mimic due to its easy synthesis and possibility of obtaining in high yields and purity.

4.3.1. siRNA Binding ability

The ability of a certain material to bind, compact and protect NA is imperative for efficient gene delivery.[2,14] Electrostatic interactions between negatively charged siRNA and cationic vectors forming complexes are the basis for the majority of non-viral vector-mediated delivery. However, the process of complexing siRNA is different from the already known of plasmid DNA, since siRNA is smaller, less flexible and has lower density of negative charges to which the cationic molecule can interact.[49]

In order to assess the interaction strength of dendrimers with the *siDNA*, a polyacrylamide gel retardation assay (PAGE) was performed. As the NA is short, this gel offers higher resolution than what would be achieved with an agarose gel. Here, 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 4.11a, the amount of free *siDNA* that migrated along the gel decreased with the increasing amount of dendrimer present (*N/P* ratio ranged from 5 to 80). Increasing the amount of dendritic structure per NA molecule promotes the complexation between the two.

Both the diamine- (fbD) and benzylamine-terminated (fbB) dendrimers were able to bind to *siDNA*. Benzylamine-terminated dendrimer showed higher binding capacity with only a little amount of free nucleotide sequence allowed to migrate at the *N/P* ratio of 20. The presence of extra hydrophobic groups may lead to different NA packaging and consequently may positively contribute to this interaction strength.

The complexation efficiency was evaluated by a nucleic acid dye (SYBRGold®) accessibility assay. SYBRGold® is cationic and when bound to NA there is a large increase in fluorescence intensity, which is ideal for estimating the amount of free *siDNA*. It should be noted that when complexed to the dendrimer, the nucleic acid is inaccessible to the dye.

As shown in Figure 4.11b, the amount of complexed nucleic acid increases with the *N/P* ratio. Complexation was over 60% even at the lowest *N/P* tested (*N/P* 5), with values greater than 80% above *N/P* 10.

Both PAGE and SYBRGold® assay showed that fbB are the most efficient dendrimers to retain and complex *siDNA*, possibly due to its further hydrophobic characteristics.

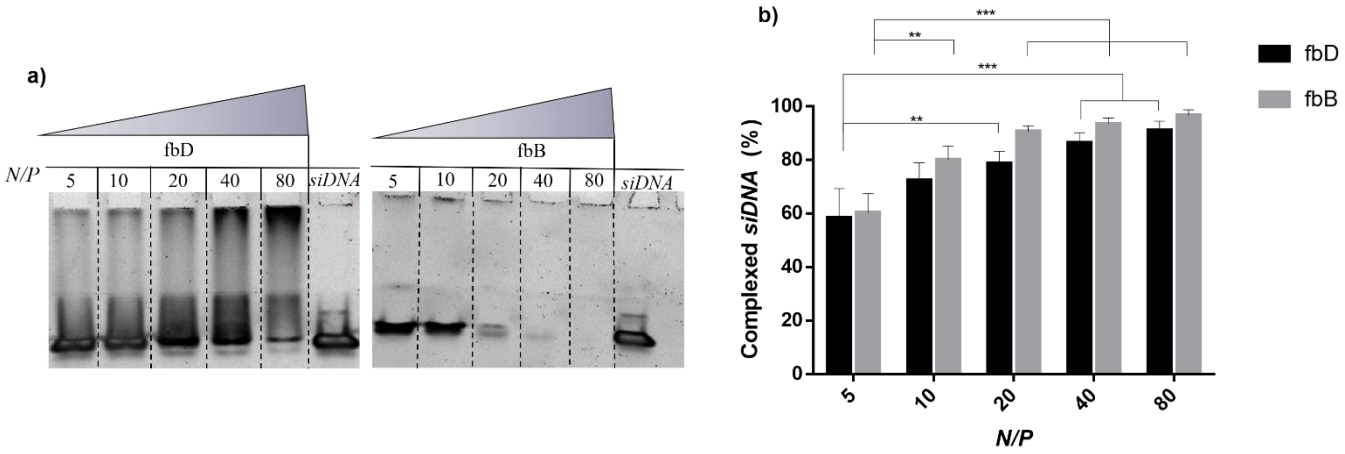

Figure 4.11 Binding ability. a) Polyacrylamide gel retention assay (PAGE) of *siDNA* dendriplexes from fully biodegradable dendrimer (fbD and fbB) at different *N/P* ratios indicated above each column. In both gels the last column corresponds to free *siDNA*. **b)** SYBRGold® exclusion assay at room temperature. Results are expressed as mean ± SD of three independent measurements (n=3). Significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. One-way ANOVA tests were used for statistical analysis.

4.3.2. Size and morphology

siRNA-dendriplexes were characterized in terms of size, polydispersity index (PDI) and morphology using dynamic light scattering (DLS) and electron transmission microscopy (TEM) (Figure 4.12a and 4.12b). In general, hydrodynamic diameters, measured by DLS, showed higher values with the increase of *N/P* ratio. The dendriplexes size formed with both amine-terminated dendrimers was similar, with diamine-terminated dendrimers sizes ranging from 78 nm to 97 nm, and benzylamine-terminated dendrimers from 74 to 90 nm (Figure 4.12a). As complexes have this range of nanoscale sizes, they have the possibility to enter the cells. These sizes are in accordance with those appropriate for cellular uptake.[14,67]

The population of dendriplexes with diamine groups were slightly more homogenous than those with benzylamine groups, with PDI around 0.25 and 0.3, respectively. (Figure 4.12c) The values are mostly inferior or equal to 0.3, meeting the performance criteria of effective non-viral gene delivery system.[122]

As shown by TEM all dendriplexes showed spherical morphologies regardless of the terminal amine group (diamine or benzylamine) and *N/P* ratio (Figure 4.12e), with

dendriplex/nanoparticle sizes similar to those obtained by DLS. It is worth mentioning that some size differences can be obtained due to the different method of particle analysis. Through the DLS, the complexes are analysed in solution giving the hydrodynamic diameter datum. On the other hand, analysing by TEM requires that the sample is dried previously, what usually can lead to lower diameters being obtained.

In 2002, Peterson *et al.* reported a possible polymeric gene delivery carrier that did not pack the nucleic acid so efficiently, resulting in polyplexes with different morphologies and sizes in the same sample.[65] Other cationic systems with PEG, such as PLL copolymer, presented similar morphological results.[105] Interestingly, our results show that functionalization of PEG-GATGE dendritic block copolymers with these groups (diamine and benzylamine) is fundamental for the successful encapsulation of siRNA in spherical and well-defined structures.

4.3.3 Zeta Potential

The surface charge of the dendriplexes in water was measured by laser Doppler electrophoresis (Figure 4.12d). All dendriplexes formed at different *N/P* tested showed positive charge on their surface, except for dendriplexes with benzylamine group at *N/P* 5. In all cases, the surface charge increased with the increasing *N/P*. In addition, the diamine group complexes have higher positive charge in their surface (range 8-26 mV) compared to complexes with the other dendrimer (range -6-16 mV). This difference in charge between both dendrimers was expected due to the higher density of positive charges attributed by diamine group.

The positive zeta potential results emphasize the potential of these dendritic vectors as carriers of siRNA for cellular delivery, as their corresponding positive charged dendriplexes will have the capability to interact with negatively charged membranes. Furthermore, the fact that these values are not far from zero can be an advantage regarding complexes cytotoxicity, once excessive positive charge is associated with cellular damage.[123]

As previously mentioned, dendriplexes prepared with benzylamine-terminated dendrimer at *N/P* 5 were negatively charged unlike all other samples. This result may be due to the insufficient amount of dendrimer present that, together with different forms of packaging the biological material than in the fbD *N/P* 5, it does not bind to all nucleic acid allowing free *siDNA* contribute to the negative charge recorded.

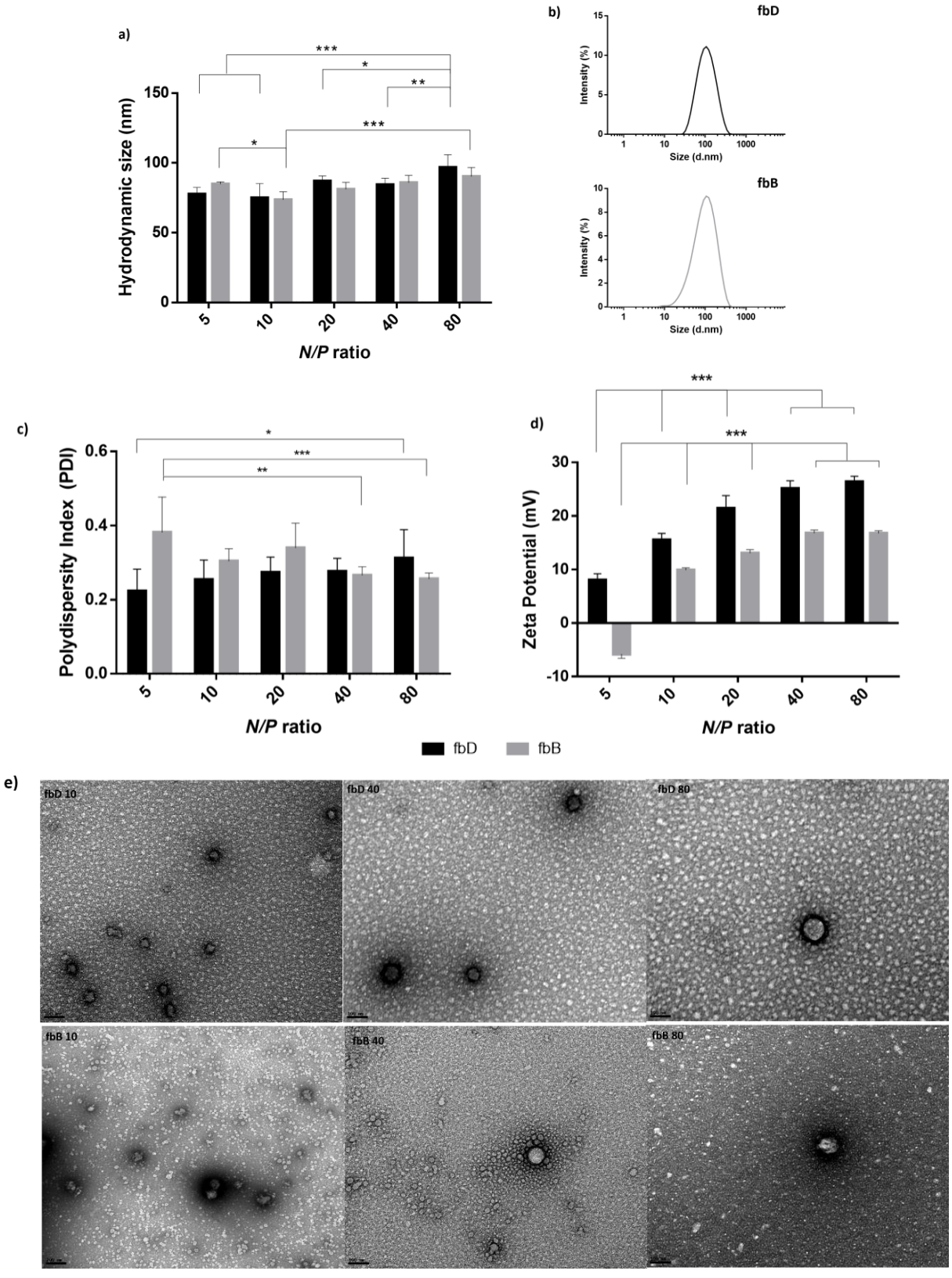

Figure 4.12 Size, surface charge and morphology of fully biodegradable dendrimer/*siDNA* complexes. a) Size distribution of *siDNA* dendriplexes measured by DLS at different *N/P* ratios (*n*=3, mean ± SD). **b)** Representative size measurements of *siDNA* dendriplexes (*N/P* 40) using DLS: fbD (Z-average: 89.97 nm, PDI: 0.228); fbB (Z-average: 80.88 nm, PDI: 0.260). **c)** Polydispersity index (PDI) of *siDNA* complexes were measured by DLS at different *N/P* ratios (*n*=3, mean ± SD). **d)** Zeta potential values of *siDNA* complexes at different *N/P* ratios. **e)** TEM images of *siDNA* dendriplexes at *N/P* 10, 40 and 80. Scale bar: 100 nm. All dendriplexes prepared in nuclease free water. Significant differences **p*<0.05, ***p*<0.01 and ****p*<0.001. One-way ANOVA testes were used for statistical analysis.

4.4. Biological performance evaluation

Both dendrimers were further evaluated regarding their cytotoxicity, ability to mediate cellular uptake of siRNA and transfection efficiency.

4.4.1. Cellular toxicity

In many cases, macromolecular systems toxicity to cells has been an obstacle to their implementation in biomedicine. Because of this, the toxicity of synthesized PEG-dendritic block copolymers and their corresponding dendriplexes was studied. Cytotoxicity was assessed in human osteosarcoma cell line (U2OS) by metabolic changes of cells through a resazurin-based assay. For the amine-terminated PEG-dendritic structures, concentrations of 0.25 to 1.5 mg mL^{-1} were tested, whereas *N/P*s 40 and 80 were tested with the dendriplexes, corresponding to 0.25 and 0.5 mg mL^{-1}, respectively (Figure 4.13). The concentrations tested were high when compared with the previously mentioned literature on biodegradable dendritic molecules for gene delivery.

For both PEG-dendrimers and every concentration evaluated, cell metabolic activity was equal or higher than 80 % after 24 h of contact (Figure 4.13a). The diamine-terminated dendrimer (fbD) showed to be more toxic to cells than the one terminated in benzylamine (fbB) (metabolic activity $\geq$ 90 %), probably due to the higher density of positive charges. In any way, the results were very good for both fbD and fbB, what was expected due to the presence of the PEG chain that can mask the positive charge of the amine groups present in the periphery of the dendritic structure. The toxicity of *siDNA* dendriplexes was tested only at the highest *N/P*s because at these ratios the amount of dendrimer present is higher and could potentially be more toxic to cells. The metabolic activity of cells, that were incubated for 24 hours with the dendriplexes, was above 85 % for all conditions tested (Figure 4.13b). As expected, both dendrimers, when complexed with the *siDNA*, showed less cytotoxicity, compared to the result obtained with the non-complexed PEG-dendrimer.

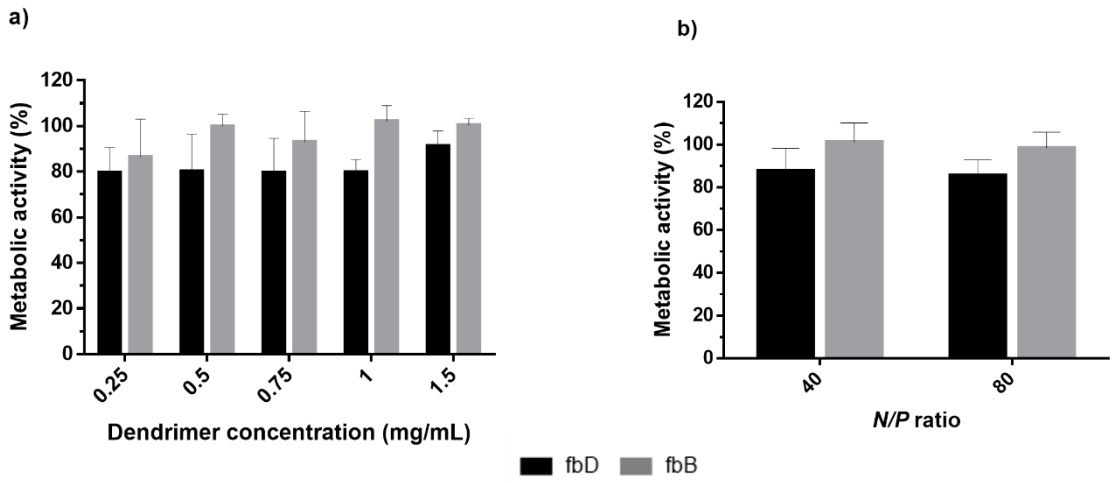

Figure 4.13 Relative metabolic activity (resazurin-based assay) using untreated cells as a reference determined after 24 h incubation of U2OS cells with **a)** PEG-dendritic structures diamine-terminated (fbD) and benzylamine-terminated (fbB) at different concentrations between 0.25 and 1.5 mg mL^{-1}; **b)** Dendriplexes at *N/P* 40 and 80 (equivalent to a dendritic concentration of *ca*. 0.25 and 0.5 mg mL^{-1}, respectively. One-way ANOVA testes were used for statistical analysis. No significant differences were obtained.

4.4.2. Cellular association and uptake

The dendrimers ability to associate/internalize the cell membrane was carefully investigated by flow cytometry and confocal fluorescence microscopy. U2OS cells were incubated at 37 ºC for 24 hours with dendriplexes carrying Cy5-labeled *siDNA*. Cy5 is a cyanine fluorescence marker. Then, through flow cytometry and confocal microscopy, the cells were analysed.

As showed in Figure 4.14a, cells treated with dendriplexes showed a shift to higher fluorescence intensity (FL) compared with untreated cells (black trace, Fig. 4.14a). This result is related to the dendriplex cell association/internalization. For both dendrimers, increasing *N/P* ratio increases FL, which can be explained with higher protection of the nucleic acid in dendriplex.

For all dendriplexes tested, fluorescence was detected in 100% or near 100% of the cells (Table S2.).

Although fbB dendrimers showed a very good ability to associate/internalizate siRNA, an excellent association/internalization was obtained by fbD dendriplexes with FL values very close to, or even higher than those of Lipofectamine® 2000 (L2k, which is a gold standard agent for *in vitro* transfection), above *N/P* 10 (Figure 4.14a). These results are indicating that both dendrimers presented advantages for cellular uptake thanks to the groups with which they were functionalized as previously commented (Section 4.2).

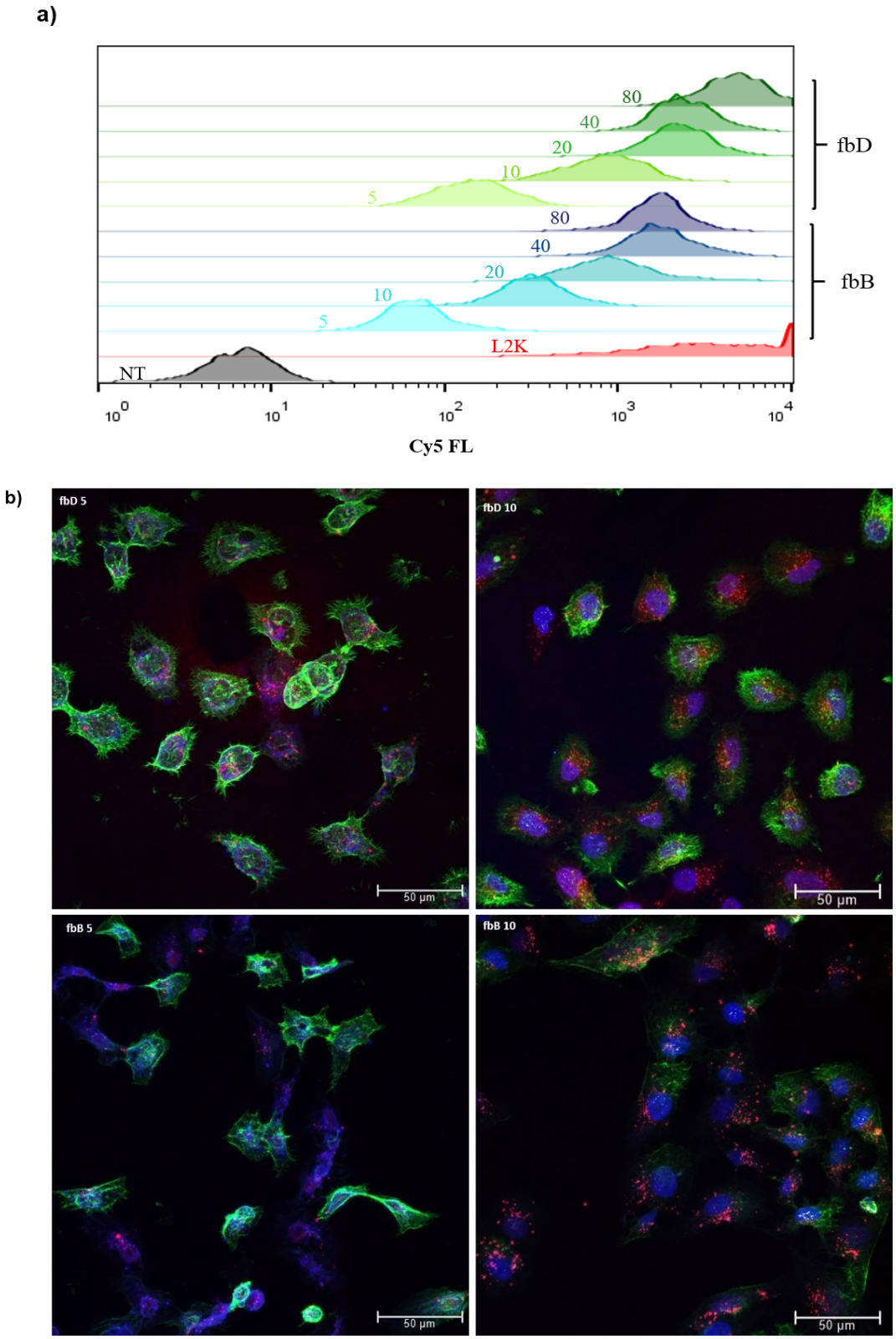

Figure 4.14 Cellular association of dendriplexes. Dendriplexes of Cy5 labeled *siDNA* (Cy5-*siDNA*) were incubated for 24 hours with U2OS cells at a final Cy5-*siDNA* concentration of 0.6 µM. Lipofectamine® 2000 (L2K) was used as a control following the manufacturer's instructions. Untreated cells were used as control. **a)** Flow cytometry characterization at different *N/P* ratios. The highlighted area is the population of cells with high relative FL. **b)** Confocal microscopy images (z-stacks) for fbD and fbB at *N/P* 5 and 10. Nuclei stained with DAPI (in blue). Actin filaments stained with Alexa Fluor 488 Phalloidin in green. Cy5-*siDNA* dendriplexes (in red). Scale bar: 50 µm.

So that, when being functionalized with diamine groups, it will double the density of positive charges (higher cationic multivalency) on the surface without needing to increase the generation/size of the system, what resulted in a higher positive zeta potential of the corresponding fbD dendriplexes (Section 4.3.3 and Figure 4.1d). On the other hand, when functionalized with benzylamine groups it is gained a hydrophobic facet that facilitates interaction with the cell membrane and promotes uptake.[124] However, the better association/internalization results for fbD dendriplexes seems to indicate that the higher positive zeta potential of fbD dendriplexes is mainly managing the higher association/internalization of this system, specially a the lower *N/P* ratios (*N/P* 5 and 10) studied where the ZP values for both systems (fbD and fbB) are more different.

Through the visualization of U2OS cells with the same contact time with dendriplexes through confocal microscopy, it was possible to confirm the cellular uptake (Figure 4.14b). This technique was employed to confirm the internalization of dendriplexes as well as their distribution inside the cell. Images revealed the dotted-like Cy5 fluorescence pattern. This pattern is not common in wild type U2OS cells so it is indicative of endosomal uptake of the dendriplexes (Figure S8). The images also showed a wide distribution of Cy5-*siDNA* in the cell cytoplasm and spherical agglomerates suggesting that *siDNA* is inside of endocytic vesicles.

4.4.3. Transfection efficiency

The dendriplexes ability to mediate gene silencing has been tested on U2OS cells stably expressing the fusion protein eGFP-Luciferase (U2OS/eGFPLuc cells). Cells were incubated at 37 ºC for 24 h with anti-eGFP siRNA dendriplexes. Transfection efficiency was assessed by measuring the decrease of fluorescence intensity, which was expressed as a percentage of FL obtained for untreated cells.

For both dendrimers, there was a great decrease in fluorescence intensity compared to untreated cells. Dendriplexes with the benzylamine group dendrimer silenced 50% of GFP expression, whereas diamine-terminated dendrimer had a greater silencing effect (around 70%) that could be related to the fact that this dendritic macromolecule is able to internalize higher amount of siRNA, as shown in the results of Section 4.4.2. The silencing effect was not dependent on *N/P* ratios, with this great silencing effect occurring even at the lowest ratio tested (*N/P* 20). As it can be seen in Figure 4.15, the FL percentage values are very close and equal to those obtained by the gold standard control, L2k, for fbB and fbD, respectively.

As previously commented (Sections 1.2.2.3 and 4.1), the hybrid-biodegradable PEG-dendrimers previously reported by our team[111] had showed the ability to interact with the siRNA,

compact it, protect it and transport it into cells, but, despite the dendritic shell degradability contribute to the siRNA release, low transfection efficiencies were obtained due to a poor endosomal escape.[111]

Here, the excellent transfection efficiencies obtained appear to indicate that the vector, after internalization, and having been in the endosomal pathway, was able to escape from the vesicle to the cytoplasm. Therefore, it seems confirmed the premise that the higher/fully biodegradability of the dendrimer lead to the formation of many charged fragments that favors the endosomal vesicle breakdown, allowing the siRNA release to the cytoplasm and fulfil its biological function.

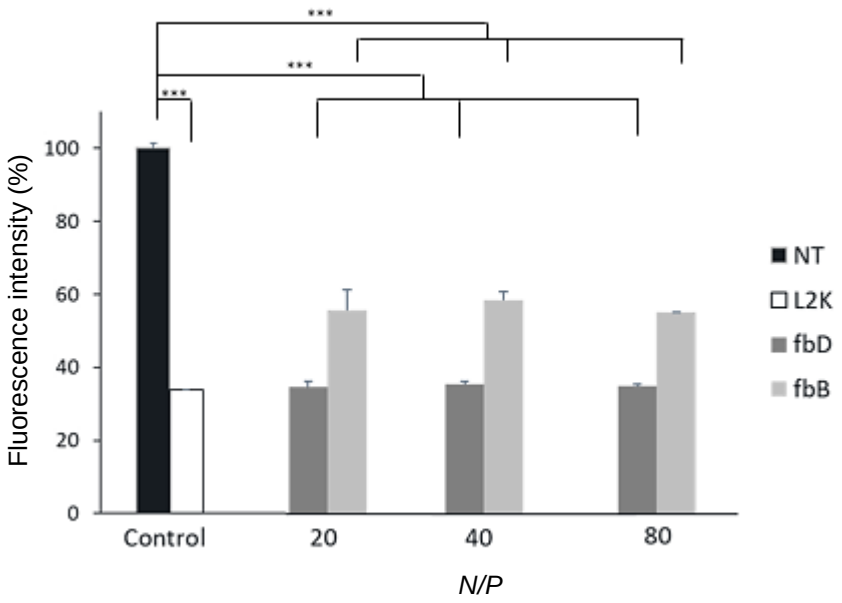

Figure 4.15 Percentage of fluorescence intensity upon 72 h post-transfection of anti-eGFP siRNA dendriplexes and different *N/P*, and L2k. (*n* =1) Final concentration of siRNA of 0.6 µM. One-way ANOVA tests were used for statistical analysis. Significant differences: ****p* < 0.001.

Chapter 5

Concluding Remarks

Gene therapy requires a thorough design of delivery systems that can safely and efficiently deliver nucleic acids to cells. Dendrimers are very promising candidates as non-viral vectors for nucleic acids delivery due to their unique characteristics as globular, well-defined, and branched structure, tunable nanosizes, low polydispersity and the presence of several functional groups that allow the surface multifunctionalization according to the desired properties.

Although most of the dendrimers currently developed have very good *in* vitro and *in vivo* results in several research labs around the world, not so many have gone into clinical trials, especially due to their cytotoxicity. The toxic effect on cells is mainly caused by bioaccumulation and, specifically with cationic dendrimers, such as ours, can be also caused by the high density of positive charges of the molecule. Thus, several teams worldwide are focused on developing dendritic structures that degrade into small fragments under physiological conditions to be easily excreted from the organism. However, the biodegradable nature of a molecule makes it susceptible to undesirable and/or premature degradation during synthesis, purification, functionalization or even during subsequent application steps, thus justifying the reduced number of biomedical studies using biodegradable dendrimers.

Here, the synthesis of novel fully biodegradable, biocompatible, non-toxic, and water-soluble azide-terminated PEG-GATGE dendritic block copolymers has been presented. Their repeating unit is composed of gallic acid (GA) core and branches of triethylene glycol butanoate, incorporating ester bonds (TGE). The functionalization of these dendritic systems succeeded by "click" chemistry with alkynated and deprotected amine groups allowed an efficient complexation of siRNA (close to or above 90% of complexed siRNA), being potential vectors for this kind of nucleic acid.

The size, morphology, and charge of the nanoparticles strongly influence the cell uptake and transfection efficiency. Thus, during the development of a vector for gene therapy, it is necessary to consider these physicochemical properties. In this work, the development of PEG-GATGE based dendriplexes with characteristics suitable for this purpose was shown. The dendriplexes showed sizes between 74 and 97 nm, spherical structures and positive surface charges.

These characteristics led to the successful nanoparticles uptake for every *N/P* ratio tested. Furthermore, no toxicity was found in U2OS cells after incubation with these particles.

The nature of the amine terminal moieties (diamine and benzylamine) of the dendrimer has shown to influence the internalization and transfection. Although both dendrimers (fbD and

fbB) were able to form dendriplex populations with very similar sizes, the cellular uptake and the GFP silencing effect resulting from siRNA transfection was higher for the diamine-terminated dendrimer (fbD).

Interestingly, the fully biodegradability contributed to a great increasing of the transfection efficiency when compared to hybrid-biodegradable dendritic structure previously reported by us.[111]

Combining the PEG-dendrimer amazing characteristics with the easy and efficient decoration periphery of these family of dendritic structures by "click" chemistry with other groups or ligand, the novel PEG-dendritic structure developed can be applied as vectors in many other ways, such as diagnosis, vaccines and drug delivery. Therefore, the biodegradable dendritic systems developed in this work is a great promise for biomedical applications.

Chapter 6

Future perspectives

In the near future, purification yields of the G2 and G3 will be optimized and the biological performance of the fully biodegradable PEG-GATGE will be further assessed, including siRNA protection from the endonuclease degradation and determination of lethal dose-50 (LD50) on cells, and evaluate the internalization mediation through imaging flow cytometry. Furthermore, additional transfection replicates will be performed. Moreover, degradability studies for both fully biodegradable PEG-dendrimers and dendriplexes will be carried out.

All biological performance will be reassessed *in vitro* with a neuronal cell line and then *in vivo*.

Later, dendrimers will be synthesized with targeting moiety and a complete study about the behaviour of dendriplexes *in vitro*, *in vivo* studies will be carried

References

1. Mccarty, M., Avery, O. & MacLeod, C. Studies on the chemical nature of the substance inducing transformation of Pneumococcal types - Induction of transformation by a desoxirribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* **79,** 137–58 (1944).
2. Dufès, C., Uchegbu, I. F. & Schätzlein, A. G. Dendrimers in gene delivery. *Adv. Drug Deliv. Rev.* **57,** 2177–2202 (2005).
3. del Pozo-Rodríguez, A., Solinís, M. Á. & Rodríguez-Gascón, A. Applications of lipid nanoparticles in gene therapy. *Eur. J. Pharm. Biopharm.* **109,** 184–193 (2016).
4. Jayant, R. D. *et al.* Current status of non-viral gene therapy for CNS disorders. *Expert Opin. Drug Deliv.* 1744–7593 (2016). doi:10.1080/17425247.2016.1188802
5. Mou, Q., Ma, Y., Jin, X. & Zhu, X. Designing hyperbranched polymers for gene delivery. *Mol. Syst. Des. Eng.* **1,** 25–39 (2016).
6. Kaufmann, K. B., Büning, H., Galy, A., Schambach, A. & Grez, M. Gene therapy on the move. *EMBO Mol. Med.* **5,** 1642–1661 (2013).
7. Ibraheem, D., Elaissari, A. & Fessi, H. Gene therapy and DNA delivery systems. *Int. J. Pharm.* **459,** 70–83 (2014).
8. Hosseinkhani, H. *et al.* Biodegradable nanoparticles for gene therapy technology. *J. Nanoparticle Res.* **15,** 1794 (2013).
9. Yin, H. *et al.* Non-viral vectors for gene-based therapy. *Nat. Rev. Genet.* **15,** 541–555 (2014).
10. Jones, C. H., Chen, C. K., Ravikrishnan, A., Rane, S. & Pfeifer, B. A. Overcoming nonviral gene delivery barriers: Perspective and future. *Mol. Pharm.* **10,** 4082–4098 (2013).
11. Ruponen, M., Yla, S. & Urtti, A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans : physicochemical and transfection studies. *Biochim. Biophys. Acta* **1415,** 331–341 (1999).
12. Kumar, S. R., Markusic, D. M., Biswas, M., High, K. A. & Herzog, R. W. Clinical development of gene therapy: results and lessons from recent successes. *Mol. Ther. Methods Clin. Dev.* **3,** 16034 (2016).
13. Liang, W. & Lam, J. in *Molecular Regulation of Endocytosis* 429–456 (2012).
14. Mintzer, M. A. & Simanek, E. E. Nonviral vectors for gene delivery. *Chem. Rev.* **109,** 259–302 (2009).
15. Pêgo, A. P., Oliveira, H. & Moreno, P. M. Biomaterial-Based Vectors for Targeted Delivery of Nucleic Acids to the Nervous System. *Springer Sci.* **4,** 127–151 (2013).
16. Mulligan, R. C. The Basic Science of Gene Therapy. *Science (80-.).* **260,** 926–932 (1993).
17. Gomes, C. P. *et al.* Translating chitosan to clinical delivery of nucleic acid-based drugs. *MRS Bull.* **39,** 60–70 (2014).
18. Oliveira, A. V., Bitoque, D. B. & Silva, G. A. Combining hyaluronic acid with chitosan enhances gene delivery. *J. Nanomater.* **2014,** 9 (2014).
19. de Souza, A. G., Bastos, V. A. F., Silva, I. B. B., Marangoni, K. & Goulart, V. A. Different gene therapy strategies: A overview for prostate cancer. *Curr. Gene Ther.* **16,** 1–5 (2016).
20. Edelstein, M., Abedi, M. & Wixon, J. Gene therapy clinical trials worldwide to 2007 - an update. *J. Gene Med.* **9,** 833–842 (2007).
21. Pushpendra, S., Arvind, P. & Anil, B. in *From Nucleic Acids Sequences to Molecular Medicine, RNA Technologies* 19–46 (2012).
22. Horton, H. M. *et al.* A gene therapy for cancer using intramuscular injection of plasmid DNA encoding interferon alpha. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1553–8 (1999).
23. Kim, H. *et al.* Gene therapy using plasmid DNA-encoded anti-HER2 antibody for cancers that overexpress HER2. *Cancer Gene Ther.* **23,** 341–347 (2016).
24. Weide, B., Garbe, C., Rammensee, H. G. & Pascolo, S. Plasmid DNA- and messenger

RNA-based anti-cancer vaccination. *Immunol. Lett.* **115,** 33–42 (2008).
25. Fan, C.-H. *et al.* Noninvasive, Targeted, and Non-Viral Ultrasound-Mediated GDNF-Plasmid Delivery for Treatment of Parkinson's Disease. *Sci. Rep.* **6,** 19579 (2016).
26. Patil, S. D., Rhodes, D. G. & Burgess, D. J. DNA-based therapeutics and DNA delivery systems: a comprehensive review. *AAPS J.* **7,** E61–E77 (2005).
27. Lew, D. *et al.* Cancer Gene Therapy Using Plasmid D N A : Study of DNA Pharmacokinetic Following Injection in Mice. **564,** 553–564 (1995).
28. Bennett, C. F. & Swayze, E. E. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* **50,** 259–293 (2010).
29. Kole, R., Krainer, A. R. & Altman, S. RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat. Rev. Drug Discov.* **11,** 125–140 (2012).
30. Dias, N. & Stein, C. a. Antisense Oligonucleotides : Basic Concepts and Mechanisms. *Mol. Cancer Ther.* **1,** 347–355 (2002).
31. DeVos, S. L. & Miller, T. M. Antisense Oligonucleotides: Treating Neurodegeneration at the Level of RNA. *Neurotherapeutics* **10,** 486–497 (2013).
32. Moreno, P. M. D. & Pêgo, A. P. Therapeutic antisense oligonucleotides against cancer: hurdling to the clinic. *Front. Chem.* **2,** 87 (2014).
33. Evers, M. M., Toonen, L. J. A. & van Roon-Mom, W. M. C. Antisense oligonucleotides in therapy for neurodegenerative disorders. *Adv. Drug Deliv. Rev.* **87,** 90–103 (2015).
34. He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **5,** 522–531 (2004).
35. Kim, D. H. & Rossi, J. J. RNAi mechanisms and applications. *U.S. Food Drug Adm.* **44,** 613–616 (2008).
36. Sen, G. L. & Blau, H. M. A brief history of RNAi: the silence of the genes. *Faseb J* **20,** 1293–1299 (2006).
37. Cullen, B. R. RNAi the natural way. *Nat. Genet.* **37,** 1163–1165 (2005).
38. Lee, R. C. The C . elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to & II-14. **75,** 843–854 (1993).
39. Ardekani, A. M. & Naeini, M. M. The role of microRNAs in human diseases. *Avicenna J. Med. Biotechnol.* **2,** 161–179 (2010).
40. Friedländer, M. R. *et al.* Evidence for the biogenesis of more than 1,000 novel human microRNAs. *Genome Biol.* **15,** R57 (2014).
41. de Fougerolles, A., Vornlocher, H.-P., Maraganore, J. & Lieberman, J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* **6,** 443–453 (2007).
42. Zamore, P. D. & Aronin, N. siRNAs knock down hepatitis. *Nat. Med.* **9,** 266–7 (2003).
43. Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H. & Williams, B. R. G. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5,** 834–839 (2003).
44. Jackson, A. L. Position-specific chemical modification of siRNAs reduces 'off-target' transcript silencing. *Rna* **12,** 1197–1205 (2006).
45. Robbins, M. *et al.* 2′-O-methyl-modified RNAs Act as TLR7 Antagonists. *Mol. Ther.* **15,** 1663–1669 (2007).
46. Chiu, Y.-L. & Rana, T. M. siRNA function in RNAi: a chemical modification analysis. *RNA* **9,** 1034–1048 (2003).
47. Watts, J. K., Deleavey, G. F. & Damha, M. J. Chemically modified siRNA: tools and applications. *Drug Discov. Today* **13,** 842–855 (2008).
48. Akhtar, S. & Benter, I. F. Nonviral delivery of synthetic siRNAs in vivo. *J. Clin. Invest.* **117,** 3623–3632 (2007).
49. Leiro, V., Santos, S. D. & Pêgo, A. P. Delivering siRNA with Dendrimers : In Vivo Applications. *Curr. Gene Ther.* **17,** 105–119 (2017).
50. Fargnoli, A., Katz, M., Bridges, C. & Hajjar, R. Gene Therapy in Heart Failure. *Handb. Exp. Pharmacol.* 251–263 (2016).
51. Dash, P. R., Read, M. L., Barrett, L. B., Wolfert, M. A. & Seymour, L. W. Factors

affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. **6,** 643–650 (1999).
52. Thomas, C. E., Ehrhardt, A. & Kay, M. a. Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* **4,** 346–358 (2003).
53. Wiethoff, C. M. & Middaugh, C. R. Barriers to Nonviral Gene Delivery. **92,** 203–217 (2003).
54. Robbins, P. D. & Ghivizzani, S. C. Viral Vectors for Gene Therapy. *Pharmacol. Ther.* **80,** 35–47 (1998).
55. Yu-Wai-Man, P. Genetic manipulation for inherited neurodegenerative diseases: myth or reality? *Br. J. Ophthalmol.* **1,** 1–10 (2016).
56. Cavazzana-calvo, M. *et al.* Gene Therapy of Human Severe Combined Immunodeficiency (SCID)– X1 Disease. **288,** 669–673 (2000).
57. Hacein-Bey Abina, S. *et al.* A Serious Adverse Event after Successful Gene Therapy for X-Linked Severe Combined Immunodeficiency. *N. Engl. J. Med.* **348,** 255–256 (2003).
58. Verma, I. M. & Weitzman, M. D. Gene Therapy: Twenty-First Century Medicine. *Annu. Rev. Biochem.* **74,** 711–738 (2005).
59. Park, J. H., Park, J. & Choi, J. S. Basic Amino Acid-Conjugated Polyamidoamine Dendrimers with Enhanced Gene Transfection Efficiency. **22,** 500–508 (2014).
60. Rehman, Z., Zuhorn, I. S. & Hoekstra, D. How cationic lipids transfer nucleic acids into cells and across cellular membranes : Recent advances. *J. Control. Release* **166,** 46–56 (2013).
61. Luten, J., van Nostrum, C. F., De Smedt, S. C. & Hennink, W. E. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. *J. Control. Release* **126,** 97–110 (2008).
62. Luo, K., He, B., Wu, Y., Shen, Y. & Gu, Z. Functional and biodegradable dendritic macromolecules with controlled architectures as nontoxic and efficient nanoscale gene vectors. *Biotechnol. Adv.* **32,** 818–830 (2014).
63. Oupicky, D. Effect of Albumin and Polyanion on the Structure of DNA Complexes with Polycation Containing Hydrophilic Nonionic Block. *Bioconjug. Chem.* **10,** 764–772 (1999).
64. Alexis, F., Pridgen, E., Molnar, L. K. & Farokhzad, O. C. Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles. **5,** 505–515 (2008).
65. Petersen, H. *et al.* Polyethylenimine- graft -Poly (ethylene glycol) Copolymers : Influence of Copolymer Block Structure on DNA Complexation and Biological Activities as Gene Delivery System. *Bioconjug. Chem.* **13,** 845–854 (2002).
66. Hommej, C. P., Let, B. A. S. S., Lard, M. A. R. & Spenlehauer, G. Stealth Me . PEG-PLA Nanoparticles Avoid Uptake by the Mononuclear Phagocytes System. **84,** 493–498 (2000).
67. Akinc, A. & Battaglia, G. Exploiting Endocytosis for Nanomedicines. *Cold Sping Harb Perspect Biol* **5,** 1–24 (2013).
68. Mislick, K. A. & Baldeschwieler, J. D. Evidence for the role of proteoglycans in cation-mediated gene transfer. **93,** 12349–12354 (1996).
69. Kirchhausen, T., Owen, D. & Harrison, S. C. Molecular Structure, Function, and Dynamics of Clathrin-Mediated Membrane Traffic. *Cold Spring Harb Perspect Biol* **6,** 1–26 (2014).
70. Jovic, M., Sharma, M., Rahajeng, J. & Caplan, S. The early endosome: a busy sorting station for proteins at the crossroads. *Histol Histopathol* **25,** 99–112 (2010).
71. Grant, B. D. & Donaldson, J. G. Pathways and mechanisms of endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **10,** 597–608 (2011).
72. Benjaminsen, R. V, Mattebjerg, M. A., Henriksen, J. R., Moghimi, S. M. & Andresen, T. L. The Possible ‘ Proton Sponge ’ Effect of Polyethylenimine (PEI) Does Not Include Change in Lysosomal pH. **21,** 149–157 (2013).
73. Grumezescu, A. *Nanobiomaterials in drug delivery*. *Elsevier* (Elsevier Inc., 2016). at <http://dx.doi.org/10.1016/B978-0-323-42866-8.00001-0>
74. Kostiainen, M. A., Smith, D. K. & Ikkala, O. Optically triggered release of DNA from

multivalent dendrons by degrading and charge-switching multivalency. *Angew. Chemie - Int. Ed.* **46,** 7600–7604 (2007).
75. Lee, D. *et al.* Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery. *Pharm. Res.* **24,** 157–167 (2007).
76. Russ, V., Günther, M., Halama, A., Ogris, M. & Wagner, E. Oligoethylenimine-grafted polypropylenimine dendrimers as degradable and biocompatible synthetic vectors for gene delivery. *J. Control. Release* **132,** 131–140 (2008).
77. Kostiainen, M. A., Kotimaa, J., Laukkanen, M. L. & Pavan, G. M. Optically degradable dendrons for temporary adhesion of proteins to DNA. *Chem. - A Eur. J.* **16,** 6912–6918 (2010).
78. Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J. & Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **24,** 1121–31 (2003).
79. Jevprasesphant, R. *et al.* The influence of surface modification on the cytotoxicity of PAMAM dendrimers. *Int. J. Pharm.* **252,** 263–266 (2003).
80. Duncan, R. & Izzo, L. Dendrimer biocompatibility and toxicity. *Adv. Drug Deliv. Rev.* **57,** 2215–2237 (2005).
81. Chaplot, S. P. & Rupenthal, I. D. Dendrimers for gene delivery - A potential approach for ocular therapy? *J. Pharm. Pharmacol.* **66,** 542–556 (2014).
82. Noriega-Luna, B. *et al.* Applications of dendrimers in drug delivery agents, diagnosis, therapy, and detection. *J. Nanomater.* **2014,** 1–19 (2014).
83. Kaur, D., Jain, K. & Kumar, N. A review on comparative study of PPI and PAMAM dendrimers. *J. Nanoparticle Res.* **18,** 1–14 (2016).
84. Buhleier, E., Wehner, W. & Vögtle, F. Cascade and Nonskid Chain like Syntheses of Molecular Cavity Topologies. *J. Chem. Inf. Model.* **53,** 155–158 (1978).
85. Tomalia, D. A. *et al.* A New Class of Polymers: Starburst-Dendritic Macromolecules. *Polymer Journal* **17,** 117–132 (1985).
86. Valdés Lizama, O., Vilos, C. & Durán-Lara, E. Techniques of Structural Characterization of Dendrimers. *Curr. Org. Chem.* **20,** 2591–2605 (2016).
87. Wu, L. P., Ficker, M., Christensen, J. B., Trohopoulos, P. N. & Moghimi, S. M. Dendrimers in Medicine: Therapeutic Concepts and Pharmaceutical Challenges. *Bioconjug. Chem.* **26,** 1198–1211 (2015).
88. Massadeh, S., Al-Aamery, M., Bawazeer, S., AlAhmad, O. & AlSubai, R. Nano-materials for Gene Therapy : An Efficient Way in Overcoming Challenges of Gene Delivery. *J. Biosens. Bioelectron.* **7,** 1–12 (2015).
89. Leiro, V., Santos, S. D., Lopes, C. D. F. & Pêgo, A. P. Dendrimers as Powerful Building Blocks in Central Nervous System Disease : Headed for Successful Nanomedicine. *Adv. Funct. Mater.* **1700313,** 1–20 (2017).
90. Svenson, S. & Tomalia, D. A. Dendrimers in biomedical applications - Reflections on the field. *Adv. Drug Deliv. Rev.* **57,** 2106–2129 (2005).
91. Lee, C. C. *et al.* Designing dendrimers for biological Designing dendrimers for biological applications. *Nat. Biotechnol.* **23,** 1517–1526 (2006).
92. Aulenta, F., Hayes, W. & Rannard, S. Dendrimers : a new class of nanoscopic containers and delivery devices. **39,** 1741–1771 (2003).
93. Supattapone, S., Nguyen, H. B., Cohen, F. E., Prusiner, S. B. & Scott, M. R. Elimination of prions by branched polyamines and implications for therapeutics. **518,** 0–5 (1999).
94. Augustus, E. N., Allen, E. T., Nimibofa, A. & Donbebe, W. A Review of Synthesis , Characterization and Applications of Functionalized Dendrimers. **7,** 8–14 (2017).
95. Labieniec-Watala, M. & Watala, C. PAMAM dendrimers: Destined for success or doomed to fail? Plain and modified PAMAM dendrimers in the context of biomedical applications. *J. Pharm. Sci.* **104,** 2–14 (2015).
96. Márquez-Miranda, V. *et al.* Effect of terminal groups of dendrimers in the complexation with antisense oligonucleotides and cell uptake. *Nanoscale Res. Lett.* **11,** 66 (2016).
97. Kesharwani, P. *et al.* PAMAM dendrimers as promising nanocarriers for RNAi therapeutics. *Mater. Today* **18,** 565–572 (2015).

98. Biswas, S. & Torchilin, V. P. Dendrimers for siRNA delivery. *Pharmaceuticals* **6,** 161–183 (2013).
99. Shao, N. *et al.* Comparison of generation 3 polyamidoamine dendrimer and generation 4 polypropylenimine dendrimer on drug loading, complex structure, release behavior, and cytotoxicity. *Int. J. Nanomedicine* **6,** 3361–3372 (2011).
100. Dhanikula, R. S., Argaw, A., Bouchard, J. & Hildgen, P. Methotrexate Loaded Polyether-Copolyester Dendrimers for the Treatment of Gliomas : Enhanced Efficacy and Intratumoral Transport Capability. **5,** 105–116 (2008).
101. Wasiak, T. *et al.* Phosphorus Dendrimers Affect Alzheimer's (Aβ1−28) Peptide and MAP-Tau Protein Aggregation. *Mol. Pharm.* **9,** 458–469 (2012).
102. Klajnert-maculewicz, B., Fernandez-villamarin, M. & Fernandez-megia, E. Dendrimers reduce toxicity of A β 1-28 peptide during aggregation and accelerate fibril formation accelerate fibril formation. *Nanomedicine Nanotechnology, Biol. Med.* **8,** 1372–1378 (2012).
103. Zeng, Y. *et al.* Effects of PAMAM dendrimers with various surface functional groups and multiple generations on cytotoxicity and neuronal differentiation using human neural progenitor cells. *J. Toxicol. Sci.* **41,** 351–370 (2016).
104. Fant, K. *et al.* Effects of PEGylation and Acetylation of PAMAM Dendrimers on DNA Binding , Cytotoxicity and in Vitro Transfection Efficiency. *Mol. Pharm.* **7,** 1734–1746 (2010).
105. Vanderkerken, S. *et al.* Structure – activity relationships of poly (L -lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. **83,** 169–182 (2002).
106. Kim, T. *et al.* PAMAM-PEG-PAMAM : Novel Triblock Copolymer as a Biocompatible and Efficient Gene Delivery Carrier. 2487–2492 (2004).
107. Wang, W., Xiong, W., Wan, J., Sun, X. & Xu, H. The decrease of PAMAM dendrimer-induced cytotoxicity by PEGylation via attenuation of oxidative stress. *Nanotechnology* **20,** 1–7 (2009).
108. Garcia, J. P. Exploring New PEG-dendritic Block Copolymers as siRNA Delivery Vectors. (Faculdade de Engenharia da Universidade do Porto, 2015).
109. Lee, J. S., Huh, J., Ahn, C. H., Lee, M. & Park, T. G. Synthesis of novel biodegradable cationic dendrimers. *Macromol. Rapid Commun.* **27,** 1608–1614 (2006).
110. Ma, X. *et al.* Facile synthesis of polyester dendrimers as drug delivery carriers. *Macromolecules* **46,** 37–42 (2013).
111. Leiro, V. *et al.* Biodegradable PEG-dendritic block copolymers: synthesis and biofunctionality assessment as vectors of siRNA. *J. Mater. Chem. B* **5,** 4901–4917 (2017).
112. Barnard, A. *et al.* Degradable Self-Assembling Dendrons for Gene Delivery : Experimental and Theoretical Insights into the Barriers to Cellular Uptake. 20288–20300 (2011).
113. Mart, P., Movellan, J., Gonz, R., Sierra, T. & Serrano, L. New Ionic bis-MPA and PAMAM Dendrimers : A Study of Their Biocompatibility and DNA- Complexation. *Macromol. Biosci.* **15,** 657–667 (2015).
114. Leiro, V., Paula, A., Garcia, P. & Tomás, H. The Present and the Future of Degradable Dendrimers and Derivatives in Theranostics. *Bioconjug. Chem.* **26,** 1182–1197 (2015).
115. Twibanire, J. d A. K. & Grindley, T. B. Polyester dendrimers: Smart carriers for drug delivery. *Polymers (Basel).* **6,** 179–213 (2014).
116. Poll, D. G. Van Der *et al.* Design, Synthesis and Biological Evaluation of a Robust Biodegradable Dendrimers. *Bioconjug. Chem.* **21,** 764–773 (2010).
117. Guillaudeu, S. J. *et al.* PEGylated Dendrimers with Core Functionality for Biological Applications. *Bioconjug. Chem.* **19,** 461–469 (2008).
118. Tornøe, C. W., Christensen, C. & Meldal, M. Peptidotriazoles on Solid Phase: [1, 2, 3]-Triazoles by Regiospecific Copper (I)-Catalyzed 1, 3-Dipolar Cycloadditions of Terminal Alkynes to Azides. **67,** 3057–3064 (2002).
119. Rostovtsev, V. V, Green, L. G., Fokin, V. V & Sharpless, K. B. A Stepwise Huisgen

Cycloaddition Process: Copper (I) -Catalyzed Regioselective Ligation of Azides and Terminal Alkynes. *Angew. Chemie - Int. Ed.* **41,** 2596–2599 (2002).
120. Amaral, S. P., Fernandez-Villamarin, M., Correa, J., Riguera, R. & Fernandez-Megia, E. Efficient multigram synthesis of the repeating unit of gallic acid-triethylene glycol dendrimers. *Org. Lett.* **13,** 4522–4525 (2011).
121. Fernandez-Megia, E., Correa, J. & Riguera, R. 'Clickable' PEG - Dendritic block copolymers. *Biomacromolecules* **7,** 3104–3111 (2006).
122. Jean, M. Low molecular weight chitosan nanoparticulate system at low N:P ratio for nontoxic polynucleotide delivery. *Int. J. Nanomedicine* **7,** 1399–1414 (2012).
123. Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomedicine* **7,** 5577–5591 (2012).
124. Yoo, H. & Juliano, R. L. Enhanced delivery of antisense oligonucleotides with fluorophore-conjugated PAMAM dendrimers. **28,** 4225–4231 (2000).
125. Touchefeu, Y., Harrington, K. J., Galmiche, J. P. & Vassaux, G. Gene therapy, recent developments and future prospects in gastrointestinal oncology. *Aliment. Pharmacol. Ther.* **32,** 953–968 (2010).

Supplementary information

Table S1. Sequence of nucleic acids used

Nucleic acid type	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

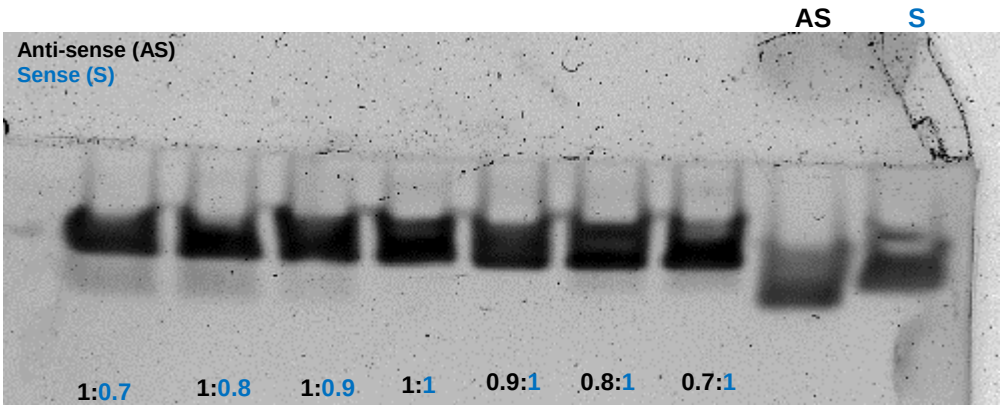

Figure S1. *siDNA* sense (S) and anti-sense (AS) sequences were annealed at different AS:S molar ratios. The nucleic acid was stained with SYBRGold® and gel visualized in a GelDoc XR imaging system. Results showed the conditions in which there are no free sequences for maximized annealing efficiency.

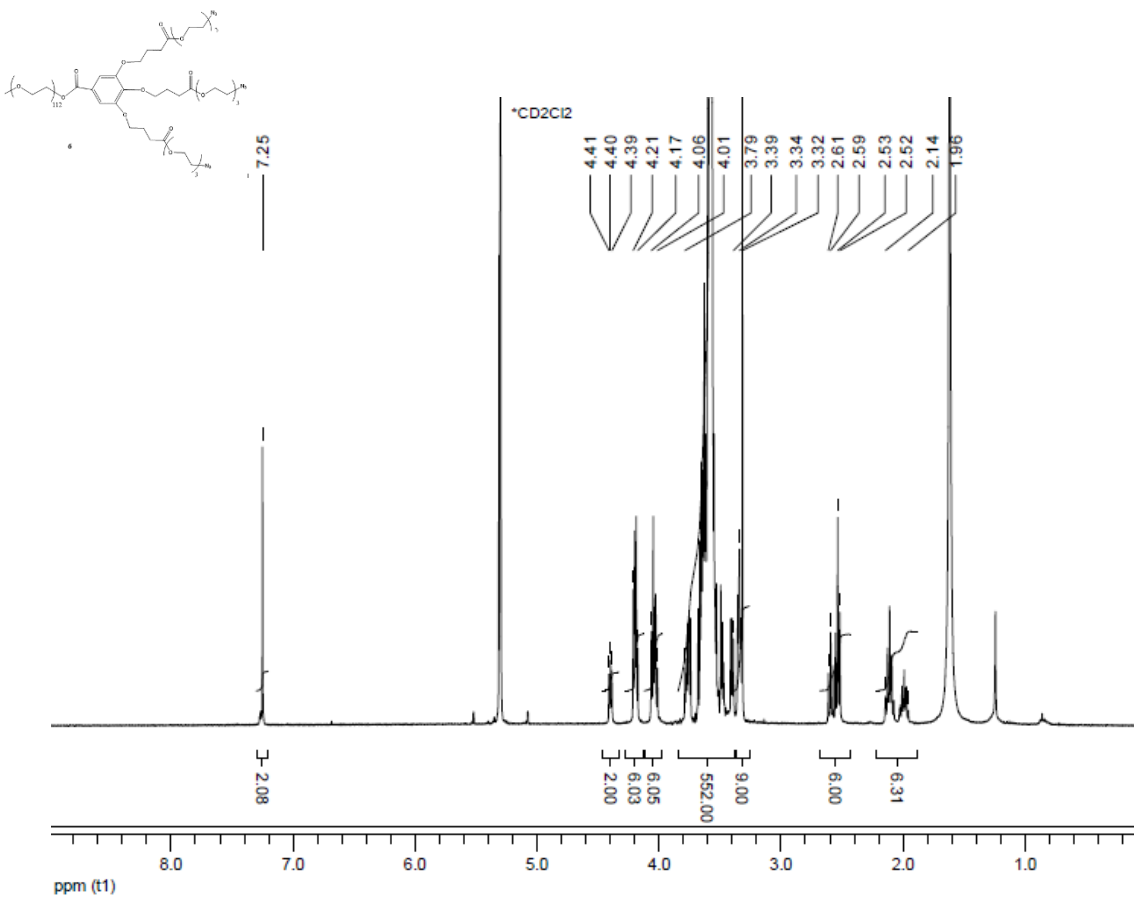

Figure S2. ^{1}H NMR Spectra of **6**. Solvent peak labelled as * in spectra.

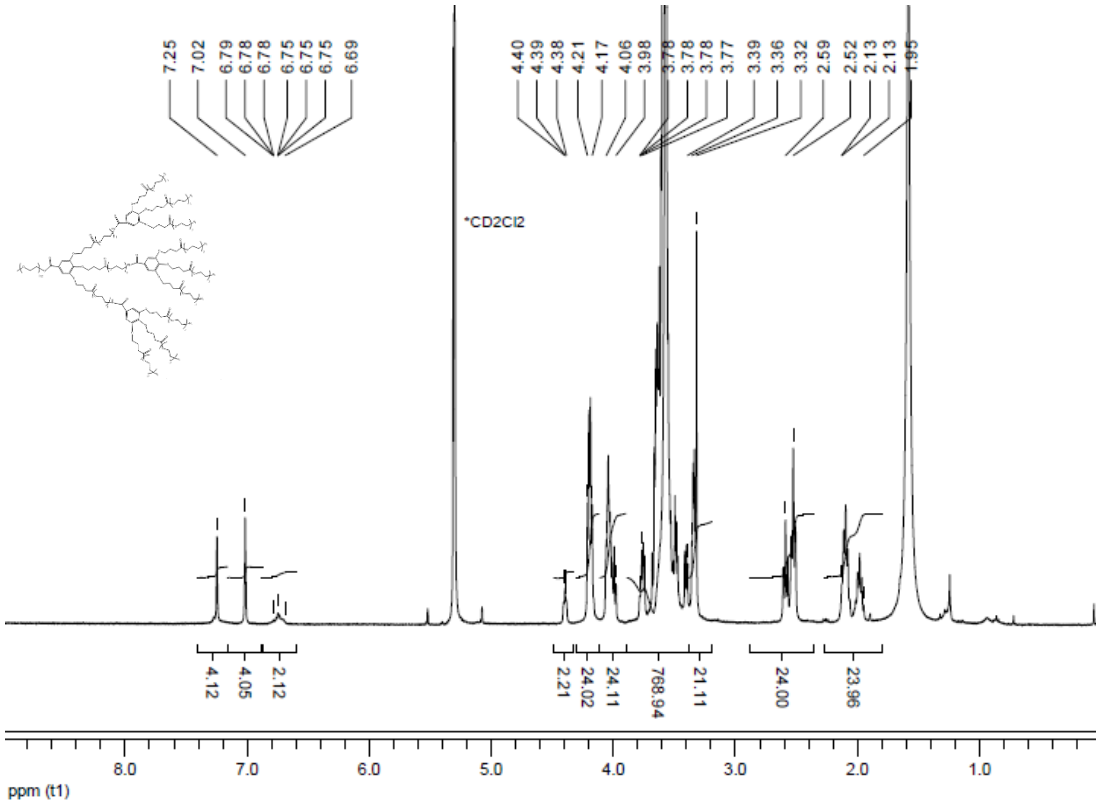

Figure S3. ^{1}H NMR Spectra of **7**. Solvent peak labelled as * in spectra.

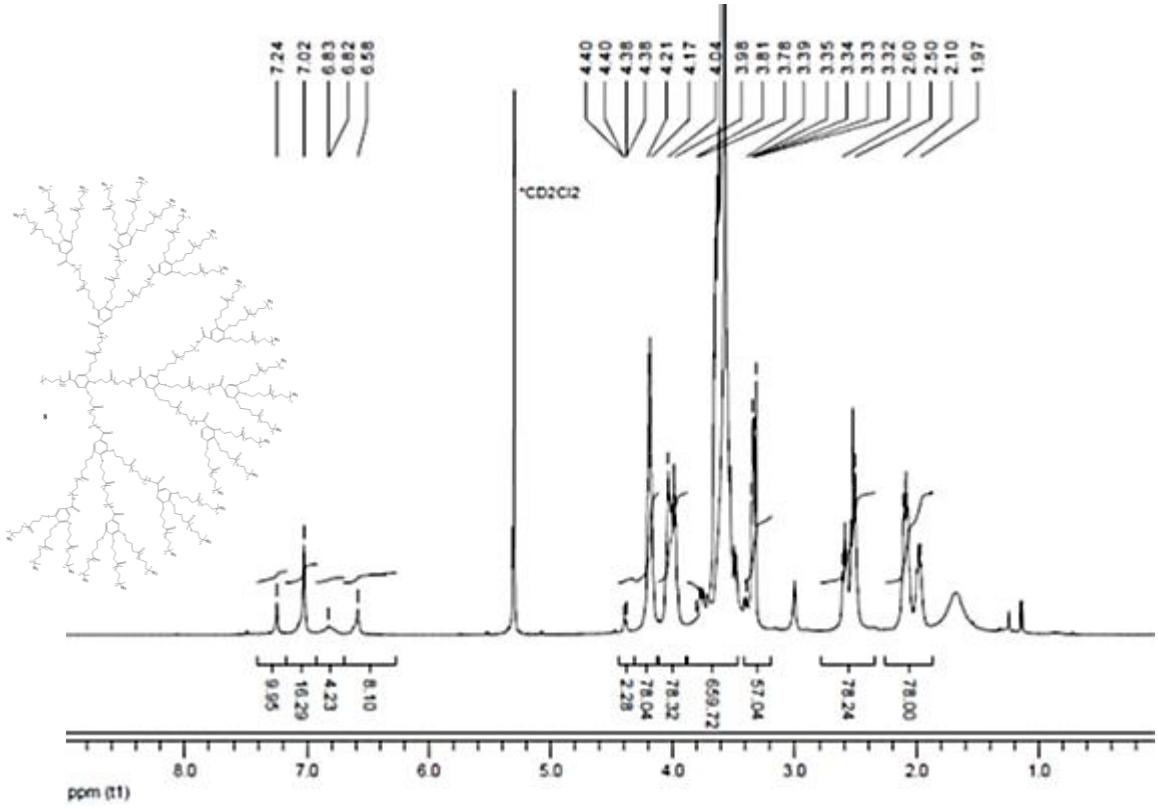

Figure S4. ^{1}H NMR Spectra of **8**. Solvent peak labelled as * in spectra.

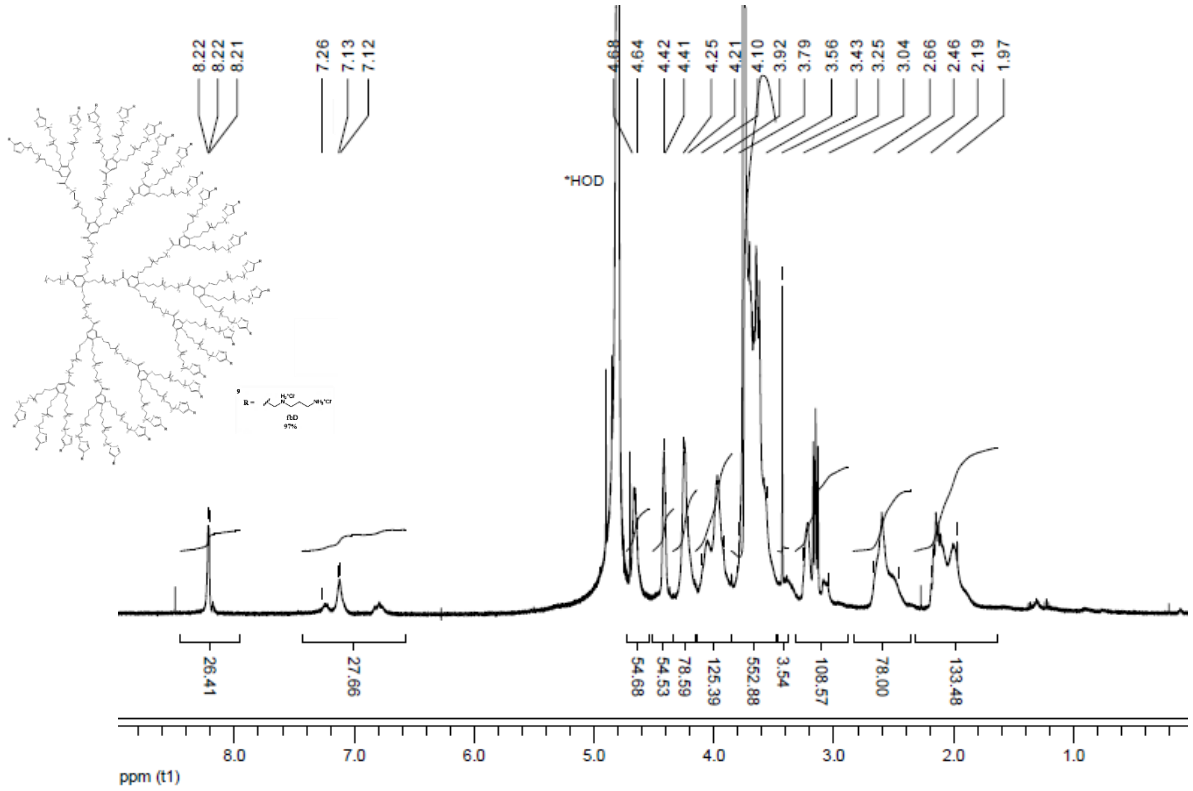

Figure S5. ^{1}H NMR Spectra of fbD. Solvent peak labelled as * in spectra.

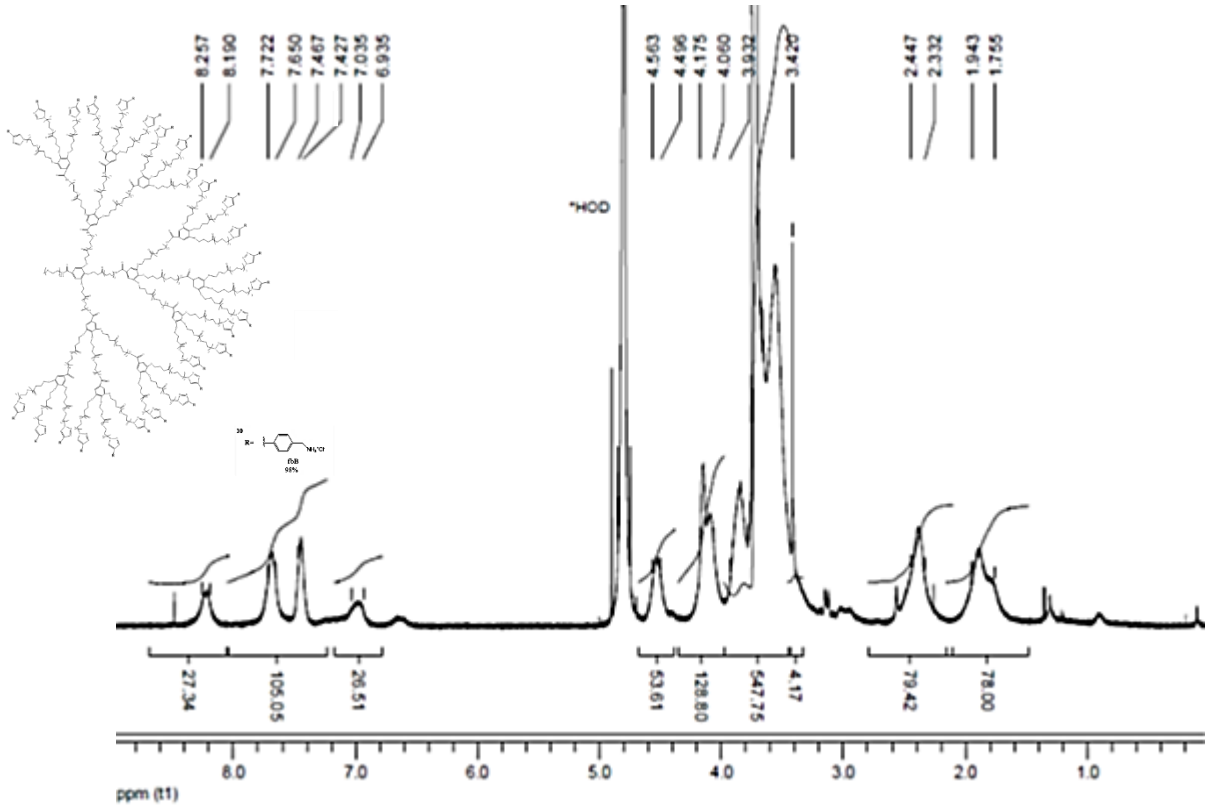

Figure S6. ^{1}H NMR Spectra of fbB. Solvent peak labelled as * in spectra.

Table S2. Percentage of cells with Cy5 fluorescence, 24 hours after incubation with dendriplexes or lipofectamine complexes.

Sample	*N/P*	% of positive cells
Untreated cells		1.1
L2K		93.2
fbD	**5**	99.1
	10	98.9
	20	98.7
	40	99.1
	80	97.8
fbB	**5**	98.4
	10	98.6
	20	96.8
	40	98.6
	80	97.2

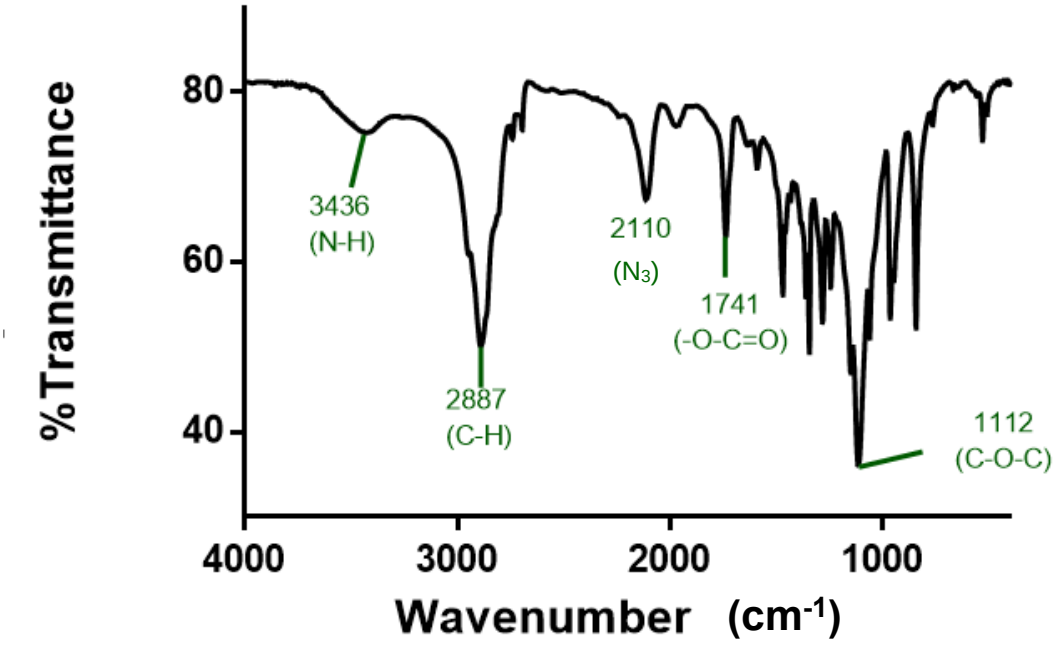

Figure S7. FTIR transmittance spectrum of fully biodegradable third-generation dendrimer (**8**) with functional groups and wavenumber identified in green. (KBr)

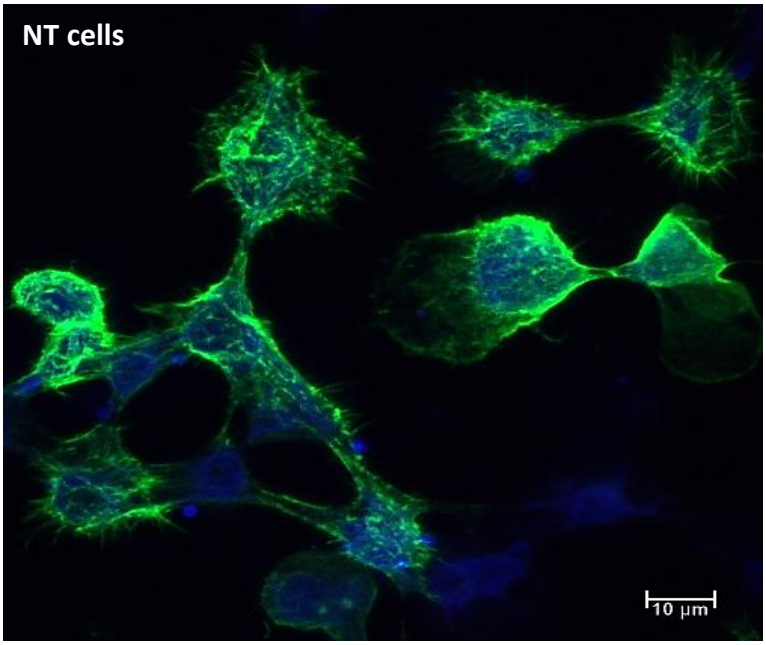

Figure S8. Confocal microscopy image of U2OS cells (wild type) without incubation with dendriplexes. Nuclei stained with DAPI (in blue). Actin filaments stained with Alexa Fluor 488 Phalloidin in green. Cy5-*siDNA* dendriplexes (in red). Scale bar: 10 µm.