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Exploring Fully Biodegradable PEG-Dendrimers as Vectors for Dual siRNA Delivery

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Abstract

In the last century, humans were able to double their life expectancy due to a tremendous evolution in the biomedical field. However, our society is now facing other diseases that require new therapeutic strategies. Gene therapy is a therapeutic approach that recently comes of age and carries the promise to treat and/or prevent several diseases at the most fundamental level of the human body: our genes. Among the gene therapy strategies, ribonucleic acid (RNA) interference (RNAi) is one of the most promising for the short-term downregulation of an overexpressing gene. Different nucleic acids (NAs), including small interfering RNAs (siRNAs), can integrate the RNAi machinery leading to gene silencing. However, the delivery of these small NAs faces several extra- and intracellular barriers and challenges. Therefore, it is imperative the development of an efficient, biocompatible and clinically suitable delivery carrier, *i.e.*, vector, which can be viral or non-viral. Viral vectors demonstrated high transfection efficiencies, but safety concerns soon led to the necessity for non-viral vectors.

Among the non-viral vectors, cationic dendritic structures emerged as promising NA vectors due to their ability to efficiently compact NAs into electrostatic complexes called "dendriplexes". However, the use of dendritic structures as NA vectors still faces some challenges, regarding nonbiodegradability of the most used dendrimers under physiological conditions, leading to bioaccumulation, charge-related cytotoxicity and reduced transfection efficiency. To fill this gap, our group recently developed a new family of fully biodegradable poly(ethylene glycol) (PEG)-dendritic block (fbB) copolymers to serve as vectors in nanomedicine.

The present dissertation explores fbB copolymers as vectors for single and dual siRNA delivery, targeting two different genes. The fbB copolymers showed excellent siRNA binding strength and siRNA complexation efficiency. Dendriplexes presented very small sizes, on the nanometre scale, narrow size distribution, globular morphology and slightly positive zeta potential. Furthermore, dendriplexes demonstrated a suitable compromise between the stability in the presence of serum and under endosomal pH and sustained siRNA release in the presence of an anionic polymeric competitor and at physiological pH. The fbB-based dendriplexes provided excellent siRNA protection against endonuclease degradation and led to good gene silencing effects.

The excellent physicochemical properties of the fbB-based siRNA dendriplexes, as well as their good biological performance, demonstrated the remarkable potential of these dendritic nanosystems to act as vectors for gene therapy applications.

Resumo

No último século, os seres humanos dobraram a sua esperança média de vida à nascença devido a uma tremenda evolução na área biomédica. No entanto, a nossa sociedade enfrenta agora outras patologias que requerem novas estratégias terapêuticas. A terapia genética é uma nova abordagem terapêutica que promete tratar e/ou prevenir várias doenças ao nível mais fundamental do corpo humano: os nossos genes. Entre as estratégias de terapia genética, o mecanismo de ácido ribonucleico (ARN) de interferência (ARNi) é uma das mais promissoras para a regulação negativa a curto prazo de um gene sobreexpresso. Vários tipos de ácidos nucleicos (ANs), incluindo pequenos ARNs interferentes (siRNA, do inglês *small interfering RNA*), conseguem integrar a maquinaria do ARNi induzindo o silenciamento genético. No entanto, a entrega desses pequenos ANs enfrenta várias barreiras e desafios extra- e intracelulares. Portanto, é imperativo o desenvolvimento de um transportador, isto é, vetor (viral ou não viral), eficiente, biocompatível e clinicamente adequado. Os vetores virais demonstraram elevadas eficiências de transfeção, mas preocupações com a segurança da sua utilização rapidamente levantaram a necessidade do desenvolvimento de vetores não virais.

Entre os vetores não virais, as estruturas dendríticas catiónicas emergiram como promissores vetores de AN devido à sua capacidade de compactar eficientemente os ANs em complexos eletrostáticos denominados "dendriplexos". No entanto, a utilização de estruturas dendríticas como vetores de AN ainda enfrenta alguns desafios, no que diz respeito à não biodegradabilidade dos dendrímeros mais utilizados sob condições fisiológicas, à citotoxicidade causada pelas cargas positivas e pela bioacumulação desses materiais sintéticos, e à reduzida eficiência de transfeção. Para colmatar esta lacuna, o nosso grupo desenvolveu recentemente uma nova família de copolímeros de bloco polietilenoglicol (PEG)-dendríticos totalmente biodegradáveis (fbB) para atuarem como vetores em nanomedicina.

A presente dissertação explora os copolímeros fbB como vetores para entrega de um ou dois siRNAs, visando dois genes diferentes. Os copolímeros fbB mostraram ligar-se fortemente aos siRNAs, resultando numa elevada eficiência de complexação. Os respetivos dendriplexos apresentaram uma estreita distribuição de tamanhos, na escala nanométrica, morfologia globular e potencial zeta ligeiramente positivo. Além disso, os dendriplexos demonstraram um compromisso adequado entre a estabilidade na presença de soro e sob pH endossomal e a libertação sustentada de siRNA na presença de um concorrente polimérico aniónico e sob pH fisiológico. Os dendriplexos baseados no fbB proporcionaram excelente proteção dos siRNA contra a degradação de endonucleases e levaram a bons níveis de silenciamento genético.

As excelentes propriedades físico-químicas dos dendriplexos de siRNA baseados no copolímero fbB, bem como o seu bom desempenho biológico, demonstraram o grande potencial destes nanossistemas dendríticos para atuar como vetores em aplicações de terapia genética.

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To the esteemed reader, who I am delighted to receive in this document.

Luís Francisco

"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change."

Charles Darwin

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List of Acronyms

AAV	Adeno-Associated Virus
AD	Alzheimer's Disease
ADA	Adenosine Deaminase
Ago	Argonaute
APP	Amyloid Precursor Protein
ASO	Antisense Oligonucleotide
BACE 1	ß-Site APP Cleaving Enzyme 1
Bis-HMPA	2,2-Bis(Hydroxymethyl)Propionic Acid
Cas9	CRISPR-Associated System 9
CNS	Central Nervous System
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
CuAAC	Copper(I)-Catalysed Huisgen 1,3-Dipolar Cycloaddition
DDAB	Dimethyldioctadecylammonium Bromide
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DLS	Dynamic Light Scattering
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DOPE	1,2-Dioleoylphosphatidylethanolamine
DOSPA	2,3-Dioleyloxy-N-[2-(Sperminecarboxamido)Ethyl]-N,N-Dimethyl-1-Propanaminium
DOTAP	1,2-Dioleoyl-3-(Trimethylammonium) Propane
DOTMA	N[1-(2,3-Dioleyloxy) Propyl]-N,N,N-Trimethylammonium Chloride
dsRNA	Double-Stranded RNA
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
eGFP	Enhanced Green Fluorescent Protein
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GABA	γ-Aminobutyric Acid

GAD	Glutamic Acid Decarboxylase
GATG	Gallic Acid–Triethylene Glycol
GATGE	GATG Ester
GDEPT	Gene-Directed Enzyme Prodrug Therapy
HCV	Hepatitis C Virus
HEPES	2-[4-(2-Hydroxyethyl) Piperazin-1-yl] Ethanesulfonic Acid
HIV	Human Immunodeficiency Virus
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
HSV	Herpes Simplex Virus
LDBC	Linear-Dendritic Block Copolymer
Luc	Luciferase
miRNA	Micro RNA
MPS I	Mucopolysaccharidosis I
MPS	Mononuclear Phagocyte System
mRNA	Messenger RNA
NA	Nucleic Acid
NF	Nuclease-Free
P/S	Penicillin-Streptomycin
PACT	Protein Activator of PKR
PAGE	Polyacrylamide Gel Electrophoresis
PAMAM	Poly(Amidoamine)
P-Bodies	Processing Bodies
PBS	Phosphate-Buffered Saline
PBzE	Poly(Benzyl Ether)
PD	Parkinson’s Disease
PDI	Polydispersity Index
pDNA	Plasmid DNA
PEG	Poly(Ethylene Glycol)
PEI	Poly(Ethylenimine)
PHPMA	poly(N-(2-Hydroxypropyl) Methacrylamide)
PLGA	Poly(D/L-Lactic-co-Glycolic Acid)
PLL	Poly(L-Lysine)
PLLA	Poly(L-Lactic Acid)
PMA	Poly(Methacrylate)
PMMA	Poly(Methyl Methacrylate)
PNIPAM	Poly(N-Isopropylacrylamide)
Pol	Polymerase
PPI	Poly(Propylene Imine)
pre-miRNA	Precusor mi-RNA
pri-miRNA	Primary miRNA

PTEN	Phosphatase and Tensin Homolog Protein
PTFE	Polytetrafluorethylene
PTGS	Post-Transcriptional Gene Silencing
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
RLC	RISC-loading complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RT	Room Temperature
SCID	Severe Combined Immune Deficiency
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
TAA	1,3,5-Triazaadamantane
TALEN	Transcription Activator–like Effector Nucleases
TBE	Tris/Borate/EDTA
TEM	Transmission Electron Microscopy
TRBP	TAR RNA-Binding Protein
U2OS	Human bone osteosarcoma cell line
UV-Vis	Ultraviolet-Visible
XPO5	Exportin-5
ZFN	Zinc Fingers Nucleases

Chapter 1

Introduction

In 1869, Miescher first identified what would be later called deoxyribonucleic acid (DNA) [1]. Only three-quarters of a century passed until Avery, Macleod, and McCarty demonstrate that DNA encodes genetic information [2]. Less than one decade later, in 1953, Watson and Crick revealed the double helix structure of DNA [3]. Half of a century later, the thirteen years of the Human Genome Project would finish revealing more 20.000 genes that compose the human genome, defining what we are [4]. These discoveries marked the beginning of our understanding of genetic information, the molecular pathways behind its regulation and their importance in a plethora of diseases. At the same time, new knowledge led to the development of new drugs and therapeutics for several gene-related diseases.

During the 1960s and 1970s, a new therapeutic concept arose — Gene Therapy — the replacement of defective DNA for exogenous "good" DNA [5–7]. Despite these innovative suggestions, the first human gene transfer only occurred in 1989, on tumour-infiltrating lymphocytes [8]. The next year, 1990, marked an utterly new era in the gene therapy field. Two girls, Ashanti De-Silva, and Cynthia Cutshall, four and nine years old, presenting severe combined immune deficiency (SCID) due to mutations in the adenosine deaminase (ADA) gene, enrolled in a clinical trial of a retroviral-mediated ADA gene transfer directed to T-lymphocytes. This chapter constituted the first successful gene therapy in humans [9].

To date, almost 3 000 gene therapy approaches reached clinical trials, and some of them already enter the market, reflecting in a global industry valued at around 500 million euros in 2018 [10]. In Portugal, gene therapy received considerable media coverage due to the case of Matilde, a baby girl that (along with Natália) recently received the Zolgensma, a gene therapy product for the treatment of spinal muscular atrophy type I [11].

Gene therapy is a promising strategy based on the delivery of exogenous nucleic acids (NAs) to defective cells or tissues in order to counteract or substitute an abnormally functioning gene, thus inducing a restorative therapeutic effect. The delivery of the therapeutic NAs can be mediated through a vector, *i.e.*, a carrier that protects the NAs and promote their internalisation into cells. This broad field includes several therapeutic approaches, namely (i) gene augmentation, (ii) gene silencing, (iii) suicide genes, and (iv) immuno-gene therapy [12]. Initially, the main focus of gene therapy research was directed to gene augmentation approaches. Gene augmentation or gene upregulation aims to restore the expression of a downregulated gene or to compensate for the production of a non-functional protein, for example, due to insertion or

missense mutation [13]. In this strategy, a plasmid DNA (pDNA) is usually delivered to the cell nucleus to induce the expression of a fully functional protein, thus compensating its absence.

Conversely, suicide gene therapy and immunogene therapy emerged as promising therapeutic approaches, primarily focusing on cancer therapy. On the one hand, suicide gene therapy or gene-directed enzyme prodrug therapy (GDEPT) consists of the delivery of a gene, known as suicide gene, that converts inactive prodrugs into toxic metabolites, thus inducing cell apoptosis [14]. On the other hand, immunogene therapy consists of a synergy between the concepts of both immunotherapy and gene therapy, resulting in a NA-based approach that leads to a safe, target-specific immune response. This strategy consists of the immune system stimulation through the (i) induction of antigene expression, the (ii) delivery of NAs encoding immunostimulatory cytokines, and/or (iii) the inhibition of the expression of immunosuppressive genes [15].

Contrary to gene augmentation, gene silencing or gene downregulation aims to restore the levels of an upregulated gene or to compensate an abnormally functioning protein, for example, due to nonsense or repeat mutation [13]. The final of the XX century marked a shift of the gene therapy research towards the gene silencing approach, due to the discovery of a naturally occurring mechanism of gene silencing by double-stranded ribonucleic acids (dsRNAs) — the RNA interference (RNAi) pathway —, which congratulated Andrew Z. Fire and Craig C. Mello with the Nobel Prize in Physiology or Medicine in 2006 [16]. Apart from RNAi, other methods to achieve gene silencing include the use of single-stranded antisense oligonucleotides (ASOs), or genome editing using Zinc Fingers Nucleases (ZFN), Transcription Activator–like Effector Nucleases (TALEN), or the clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated system 9 (Cas9) (CRISPR/Cas9) RNA guided systems [17]. Nevertheless, RNAi represents a rational path for the treatment of a plethora of inherited and acquired diseases, including cancer, and it is, by far, the most studied gene silencing approach for short-term gene downregulation.

1.1. RNA Interference

RNAi is a highly conserved natural cellular process of endogenous gene expression regulation and innate defence against invading viruses and jumping genes (transposons), that induces messenger RNA (mRNA) degradation, leading to post-transcriptional gene silencing (PTGS) [18]. RNAi is mediated by small (21 to 25 nucleotides), non-coding, dsRNAs, including micro RNA (miRNA), short hairpin RNA (shRNA) and small interfering RNA (siRNA), that target gene promoters or mRNA transcripts [19–21]. The RNAi pathway using exogenous dsRNAs has been used in a wide range of areas, from pest control (insects and virus) to medicine.

The RNAi mechanism is represented in Figure 1.1. In the cell nucleus, RNA polymerase (Pol) II or III catalyses the transcription of DNA into primary miRNA (pri-miRNA), a double-stranded stem-loop structure. Afterwards, the Microprocessor complex comprising Drosha, a ribonuclease (RNase) III, and the dsRNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8), cleaves the primary miRNA, forming the precursor miRNA (pre-miRNA), a short-hairpin 70 to 100 nucleotides duplex, commonly containing mismatches. Exportin-5 (XPO5) recognises the pre-miRNA 2-nt 3'-overhang and mediates the nuclear transport of the NA to the cytoplasm. Once in the cytoplasm, the pre-miRNA binds with Dicer, a specialised RNase III-like enzyme, and the cofactors TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT) [22]. Dicer cleaves the terminal loop of the pre-miRNA, thus forming a mature double-stranded miRNA with 18 to 25 nucleotides, and induces the formation of the RNA-induced silencing

complex (RISC)-loading complex (RLC) in association with an Argonaute (Ago) family endonuclease (Ago1-Ago4). Afterwards, the mature miRNA is loaded into Ago, unwound, and thermodynamic properties determine the sense strand (passenger), which is discarded, and the antisense strand (guide), which guides the mature RISC complex to the target mRNA, binding through partial complementary Watson-Crick base pairing (within the 3'-untranslated region). RISC association with the mRNA regulates gene expression through translational repression, mRNA degradation and/or mRNA cleavage, leading to gene silencing. On the one hand, perfect

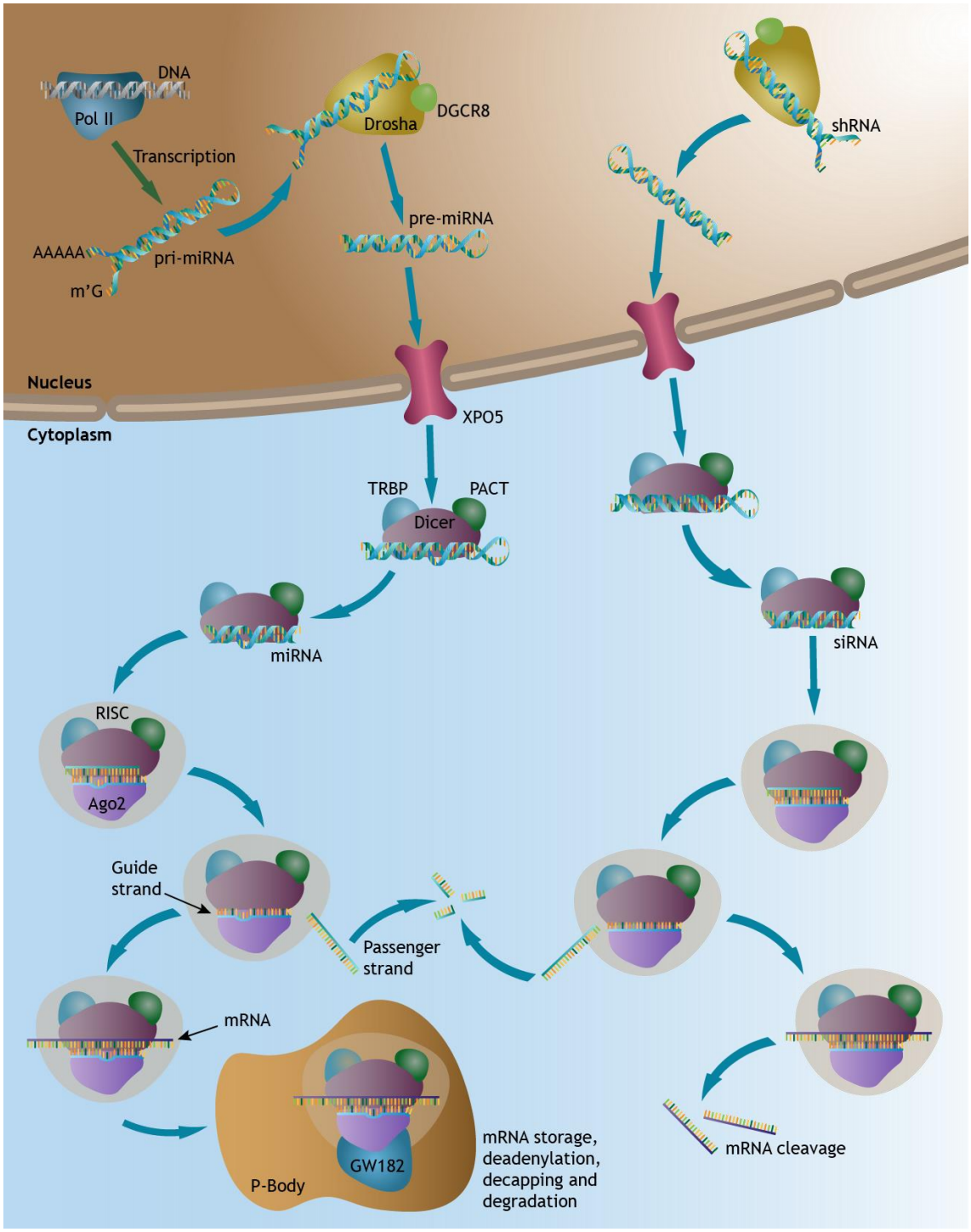

Figure 1.1 RNA interference: miRNA vs siRNA. The miRNA imperfectly binds to the target resulting in mRNA translational repression while siRNA perfectly binds to the target leading to mRNA cleavage.

or nearly perfect complementarity between miRNA and mRNA leads to site-specific cleavage by exonuclease action. On the other hand, reduced complementarity leads to mRNA sequestration in cytoplasmatic RNA processing bodies (P-bodies) and/or GW-bodies. GW-bodies are formed upon the recruitment of GW182 proteins due to the interaction with Ago proteins and are responsible for mRNA storage, deadenylation, decapping and degradation.

The siRNA-mediated RNAi pathway shares several similarities with the miRNA pathway but presents some singularities. First, when siRNA interacts and activates the RISC complex, only Ago2 binds with the siRNA, cleaving the sense strand. The antisense strand remains associated with the RISC, guiding the complex to the target mRNA for cleavage by Ago2. However, the most significant difference between the miRNA and siRNA mechanisms resides on specificity. The miRNA pathway is not specific to a single mRNA since mRNAs complementary to the miRNA seed region (bases 2–8 from the 5′ end) of guide strands can be affected by RNAi. Conversely, the siRNA guide strand is virtually entirely complementary to a single target mRNA, thus inducing specifically targeted mRNA cleavage and, consequently, gene silencing.

The siRNA RNAi pathway can also be induced by shRNA. This dsRNA can be transcribed in the cell nucleus from a plasmid vector by RNA Pol III. Afterwards, similarly to miRNA, it is further processed by Drosha and transferred by XPO5 to the cytoplasm, where it is cleaved by Dicer into a siRNA. This strategy presents the advantage of being expressed in the cell nucleus, leading to long-term expression. However, the shRNA pathway inherently presents additional barriers to a possible delivery system, namely nuclear trafficking and entry. Moreover, a siRNA-based gene silencing system presents the advantage of avoiding the Microprocessor complex processing and the activation of interferon pathways associated with dsRNAs with over than 30 nucleotides [18, 19].

1.2. Nucleic Acid Delivery Barriers and Challenges

RNAi mediated by siRNA is a promising approach for the treatment and/or prevention of a wide range of diseases. However, this is a sophisticated approach that involves numerous extra- and intracellular barriers and challenges, as schematised in Figure 1.2. These factors may lead to foreign genetic material destruction or clearance, low transfection levels and reduced therapeutic effect in cells. Thus, several NA delivery systems employ a vector, *i.e.*, a carrier, to protect and promote the efficient NA delivery to cells. The following sections describe the main barriers and challenges faced by a gene therapy system, as well as the key NA vector engineering strategies and design parameters to overcome them.

1.2.1. Extracellular Barriers and Challenges

The extracellular barriers and challenges faced by a NA delivery system depend on the route of administration, whether topical, mucosal, oral or intravenous. Non-viral vectors usually present a positive surface charge to promote the interaction with NAs. In the bloodstream, highly positively charged particles, lead to unspecific serum and blood protein adsorption, leading to aggregation or the formation of a protein corona and further clearance. Moreover, recognition by the mononuclear phagocyte system (MPS) leads to the rapid clearance of genetic material in circulation due to opsonisation by the complement system and subsequent phagocytosis [23, 24]. Stabilisation of the NA-vector system with poly(ethylene glycol) (PEG), poly(N-(2-

hydroxypropyl)methacrylamide) (PHPMA), sugars, proteins, among others lead to increased blood circulation time but can prevent cellular uptake [25].

Furthermore, RNAi-based systems can lead to the activation of the innate immune system. Synthetic NAs are recognised by multiple transmembrane and cytosolic receptors (*e.g.* toll-like receptor and retinoic-acid-inducible gene I-like receptor) eliciting the response of both immune and non-immune cells. Consequently, this induces the formation of interferon and pro-inflammatory cytokines. Additionally, in the MPS organs (lungs, spleen, and liver), macrophages (Kupffer cells), that present complement receptors, phagocyte and destroy the foreign genetic material.

In the case of nonbiodegradable compounds, where the above-described process is insignificant, other factors should be considered, namely the vector molecular weight and particle size. Different MPS organs sequester materials larger than the renal threshold (size of 200 nm or molecular weight of 5000 $g \cdot mol^{-1}$), leading to bioaccumulation and toxicity [26, 27]. Thus, the criteria for a successful NA delivery vector design for systemic administration must include biodegradability, molecular weight and size of the vector, and the stability and surface charge of the system.

1.2.2. Intracellular Barriers and Challenges

After ensuring the stability of the NA delivery system in circulation, the genetic material of interest must be adequately delivered to the target cells in order to produce the desired therapeutic effect. However, RNAi systems face several intracellular barriers and challenges, including, cell membrane crossing, endosomal escape and cytosol trafficking to access the RNAi machinery.

The cell membrane poses the first barrier to cell NA delivery. It consists of an anionic lipophilic bilayer with embedded proteins that tightly controls and regulates the passage of different molecules. The negative charge density of naked small RNAs together with their considerably high molecular weight, hamper their ability to interact with and cross the cell membrane. Conversely, cationic molecules interact with the cell membrane through electrostatic interactions but can lead to cytotoxicity due to cell membrane disruption [28]. Therefore, the NA delivery system should present an overall slightly positive surface charge to be able to interact with and traffic through the cell membrane while avoiding charge related toxicity [23].

Apart from the system charge density, the main factors impairing nanoparticle cellular internalisation include size, shape, hydrophobicity, cargo (NA) properties, and cell type (which define cell receptors and, consequently, the internalisation pathways). Also, the cellular internalisation rate and pathways are highly dependent on the particle size and shape. Most NA delivery systems are internalised by phagocytosis (usually related with opsonisation), macropinocytosis, caveolae-mediated endocytosis (50 nm to 100 nm), clathrin-mediated endocytosis (50 nm to 150 nm), and other receptor-mediated clathrin- and caveolin-independent endocytosis [29–32]. Moreover, different studies suggest that spherical particles in the range of 30 nm to 50 nm are internalized at a maximum rate [33–36].

Generally, cellular internalisation through endocytosis leads to endosomal entrapment. Endosome presents a regulatory function, monitoring material entering the cell and either recycling it to the cell membrane or directing it to lysosomal degradation. Thus, if the NA delivery system cannot efficiently promote endosomal escape, it is further secreted to the extracellular environment or destroyed in the lysosome [37]. Different mechanisms have been proposed to achieve endosomal escape, including the increase of osmotic pressure and membrane

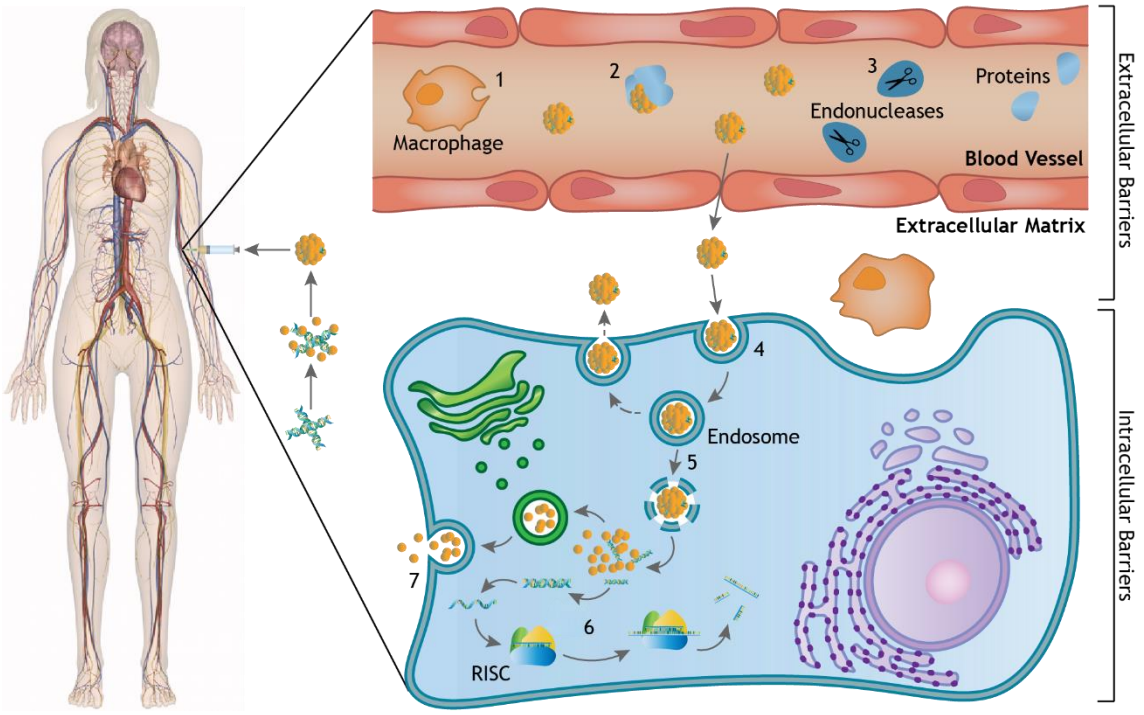

Figure 1.2 Nucleic acid delivery barriers and challenges. 1 – avoid recognition by the MPS; 2 – avoid unspecific protein binding; 3 - protection against endonuclease degradation; 4 – promote cellular internalisation; 5 – favour endosomal escape; 6 – NA release and access to the cytoplasmatic and RNAi machinery; 7 – avoid vector extra- and intracellular bioaccumulation.

destabilisation. Aminated polymers and peptides can mediate the increase of osmotic pressure through a "proton sponge effect" [38, 39]. Under the acidic environment of the endosomal compartment, amines become protonated, causing an influx of water and endosome buffering, leading to swelling and, consequently, bursting. Thus, the endosomal content is released to the cytosol. Also, gene vectors can be modified with hydrophobic moieties to tease the membrane destabilisation by interacting with the anionic lipid endosomal membrane. This mechanism is based on pore formation, causing membrane leakage [23, 24].

Once in the cytosol, the therapeutic NA needs to be released from the vector and access the cytoplasmic and RNAi machinery to allow an efficient gene silencing process. A major challenge in NA delivery vector system design is the extra- and intracellular vector bioaccumulation and ineffective clearance after accomplishing the biological function.

Furthermore, RNAi-based systems can lead to off-target effects (either by the NAs or the vectors) [40, 41]. Some studies reported non-specific effects of small RNAs on the gene regulation by endogenous miRNAs, due to matching between the seed region of the siRNA and the 3'UTR of the off-target gene [41, 42]. The improvement of the siRNA sequences using appropriate algorithms (*e.g.* BLAST), some "rules" for siRNA design and the introduction of chemical modifications (*e.g.* phosphorothioate and boranophosphate modifications) can reduce the mentioned "off-target" effects [28].

1.2.3. Endonuclease Degradation

Both endogenous and exogenous miRNAs, either derived from viruses or other foreign organisms present in the human diet (*e.g.* plants) and microbiome (*e.g.* bacteria), are present in several body fluids, including blood plasma and serum, urine, saliva, tears, and breastmilk, among others [43, 44]. Initially, the presence of endogenous miRNAs in body fluids was

attributed to cell lysis, but recent studies support the selective export of some circulating RNAs, possibly involved in cell-cell communication [45, 46]. Moreover, recent studies support that circulating RNAs complex with apoptotic bodies or lipid vesicles (exosomes and microvesicles) or bound high-density lipoproteins or RNA-binding proteins (including Ago2) [47]. These vehicles can stabilise and protect circulating miRNAs from RNase activity and thus, the naturally occurring miRNAs present outstanding stability in circulation (up to several hours) [48].

The synthetic counterparts, however, are unstable and rapidly degraded by ubiquitous RNase activity, presenting half-lives of less than a few minutes (2 min to 30 min) [49, 50]. These NA can be degraded either by extracellular (*e.g.* RNase I), lysosomal or cytosolic RNases. Thus, the protection of siRNA against endonucleases present in the extra- and intracellular milieu represents an additional challenge to these systems [51].

1.3. Delivery of Multiple Therapeutic Nucleic Acids

The vast majority of the described RNAi systems consist of the single delivery of a small NA to silence a specific gene. Nevertheless, in the literature, one can find several reports on the synergistic effect of the co-delivery of multiple substances, including the co-delivery of miRNAs or siRNAs with drugs, such as docetaxel, doxorubicin, etoposide, genistein or paclitaxel [52–56]. Moreover, multiple gene silencing mediated either by a single multiple targeting NA or by multiple single targeting NAs has also been reported [57–59]. However, the few reports on the design of dual and triple NA delivery systems, depicted in Table 1.1, are very recent (the majority from the last five years), indicating that this approach is still in its infancy and requires additional research. Furthermore, most of the studies reported so far focus on the multiple NA delivery and associated effects and use transfection agents or vectors inappropriate for further *in vivo* gene therapy [60].

In general, these strategies led to the efficient silencing of multiple genes, at least comparable to that of single delivery approaches. Moreover, some authors reported a synergistic effect mediated by the presence of other NAs ("helper" NAs), not only in dual siRNA delivery systems but also in siRNA/mRNA combinatory approaches [61]. Also, the simultaneous delivery of inter-dependent therapeutic NAs can result in improved global therapeutic effect [62]. Conversely, there are also combinatory approaches that lead to antagonistic effects [59, 60].

Despite the synergistic effect of combined approaches, some gene silencing applications do not require the downregulation of multiple genes. Recently, Ball *et al.* suggested the substitution of the "helper" NA by a mimicking negatively charged polymer [61]. Thus, the application of single targeting systems also benefits from the synergistic effect of the mimicking polymer.

1.4. Viral Vectors

The first successful *ex vivo* retroviral-mediated gene therapy clinical trial in 1990 brought enthusiasm and attention to the high efficiency of gene therapy mediated by viral vectors [9]. At that time, due to viruses' natural ability to transfect cells, viral vector-mediated gene therapy represented a limitless therapeutic approach for the treatment of several diseases, owing to high transgene efficiency and long-term transgene expression. In fact, viral vectors have been used in approximately 70% of the gene therapy clinical trials so far [63].

Table 1.1 Co-delivery of multiple therapeutic NAs. si- stands for a siRNA, m- stands for an mRNA and anti-miR stands for an anti-miRNA. [a] authors' notation, [b] for *in vitro* applications, [c] for *in vivo* applications. TGFß1 – Transforming Growth Factor ß 1, TGFßR2 - Transforming Growth Factor ß Receptor 2, CTGF - Connective Tissue Growth Factor, E1A - Adenovirus Early Region 1A, IVa2 – Adenovirus Packaging Protein 1, Pol – DNA Polymerase, Luc – Luciferase, FVII – Factor VII, MDR1 - Multi-Drug Resistance Protein 1, BCL2 - B-Cell Lymphoma 2 Apoptosis Regulator, p53 – Tumour protein 53, TNF – Tumour Necrosis Factor, ADAMTS5 - A Disintegrin And Metalloproteinase With Thrombospondin Motifs 5, PLGA - Poly(Lactic-co-Glycolic Acid), DHDEAC - N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium chloride, TNBC – Triple-Negative Breast Cancer.

Nucleic Acid	Vector	Outcome	Ref.
si-TGFß1, si-TGFßR2, si-CTGF	TransIT-TKO transfection reagent	The triple siRNA combinatory approach led to significant gene downregulation (>80%) while reducing off-target effects, relatively to single and dual siRNA delivery.	[59]
si-E1A_4, si-IVa2_2, Pol-si2[a]	Lipofectamine® RNAiMAX transfection reagent	The siRNA dual and triple mixtures did not result in increased downregulation compared to single siRNA transfections.	[60]
si-Luc/ m-mCherry[b], si-FVII/ m-Luc[c]	Lipioid (306Oi10)--based nanoparticles	Enhanced siRNA-mediated silencing but reduced mRNA-mediated protein expression of the co-delivery compared with the single delivery, *in vitro*. 2-fold siRNA-mediated gene silencing and 3-fold mRNA-mediated protein expression, *in vivo*.	[61]
si-MDR1, si-BCL2	PLGA nanoparticles	Efficient simultaneous suppression of both genes, associated with drug resistance, resulting in increased tumour responsiveness to drugs.	[62]
anti-miR-10b, anti-miR-21	PLGA-*b*-PEG polymer	Cumulative effect in TNBC tumour growth reduction in a mice xenograft model (up to 40%), even at very low doses (0.15 mg/kg).	[64]
si-p53, si-TNF	siPORT™ NeoFX™ transfection reagent	Enhanced effects observed in double gene knockdown in two TNBC cell lines (Hs578T and MDA-MB-231).	[65]
si-Caspase 3, si-ADAMTS5	DHDEAC:cholesterol (1:1) liposomes	A synergistic effect was observed in dual siRNA delivery comparing with single siRNA delivery, leading to a reduction of the intravertebral disc degeneration.	[66]

Apart from retroviruses, some of the most used viruses in gene therapy are lentivirus, adenovirus, adeno-associated virus (AAV) and herpes simplex virus (HSV). The properties of these vectors are described in Table 1.2. Although lentiviruses belong to the *Retroviridae* family, this genus presents some unique properties worthy of distinction. Additional viruses that have been used with less frequency in gene therapy include alphavirus, flavivirus, rhabdovirus and measles virus, among others [67].

The genome of viruses is usually modified to produce recombinant virus, which ability to replicate has been reduced or disabled while maintaining the ability to express the transgene of interest. When choosing and engineering a viral vector-based NA delivery system, there are several factors to consider, such as (i) the ability to integrate the host cell genome, (ii) the transgene capacity, and (iii) the propensity to trigger the immune response. Moreover, there is a broad spectrum concerning the virus genome, either single- (*e.g.* AAV) or double-stranded (*e.g.* adenovirus, HSV) DNA, or positive (*e.g. Retroviridae* viruses, flavivirus) or negative (*e.g.* rhabdovirus) RNA [63].

The ability to integrate the host cell genome depends on the type of viral vectors. Integrating vectors, namely retrovirus and lentivirus, can integrate the host genome and, thus, are the preferred choice when transfecting actively dividing cells. Lentivirus, however, can integrate

the genome of quiescent cells, such as neurons, and represent the choice viral vector for *ex vivo* NA transfer [68, 69]. Integrating viral vectors are associated with safety concerns, including immunogenicity and possible insertional mutagenesis due to random integration in the host-cell genome, which may lead to oncogene activation [68]. Generally, adenovirus, AAV and HSV, however, do not present the ability to integrate the host cell genome, remaining episomal in the host cell nucleus and, thus, are mainly used in quiescent cells.

Novel modifications to viral vectors, including capsid engineering (pseudotyping), modifications on the glycoproteins of enveloped viruses (*e.g. Retroviridae* family, HSV), or restriction of the expression of specific promoters and enhancers, make possible to alter the virus' tropism to target a specific cell or tissue and improve both transduction efficiency and specificity [67].

Table 1.2 Properties of viral vectors. +ssRNA – positive single-stranded RNA, ssDNA – single-stranded DNA.

Virus	Packaging Capacity	Genome	Advantages	Limitations
Retrovirus	8 kb	+ssRNA	Long-term expression (chromosomal integration)	Insertional mutagenesis Incapability to transfect non-dividing cells
Lentivirus	8 kb	+ssRNA	Low cytotoxicity Long-term expression (chromosomal integration)	Insertional mutagenesis
Adenovirus	<7.5 kb	dsDNA	High transfection efficiency Short-term transient expression	Strong immunogenicity
AAV	<4 kb (more in dual AAV vectors)	ssDNA	Low pathogenicity and toxicity Stable transgene expression	Immune response (repeated administration) Limited capacity
HSV	>30 kb	dsDNA	Latent infection Long-term expression (episome) Low toxicity Large packaging capacity	Immunogenicity

1.4.1. Viral Vectors in Research

Retroviruses, commonly derived from the Murine Leukaemia Virus, were the first Food and Drug Administration (FDA) approved vectors for clinical trials. Retroviruses have been used in gene therapy systems targeted to SCID, cardiovascular diseases, and cancer [69–72]. Oncolytic retrovirus-based systems induced tumour cell death and provided prolonged survival in animals with a pre-existing immune response to the vectors, supporting the potential for re-administration [73].

Lentiviral vectors have been employed in the gene therapy of monogenic metabolic disease, inducing organ pathology reversion and haematological phenotype correction, without the presence of genotoxicity indicators, and different types of cancer, such as pancreatic [69, 74]. Lentiviruses have also been exploited as RNAi vectors targeted to Central Nervous System (CNS) diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), either to deliver shRNA, miRNA or siRNAs [13, 74]. In two different PD models, lentivirus-based systems have shown to successfully knockdown the overexpression of α-synuclein or γ-aminobutyric acid (GABA)-producing enzyme glutamic acid decarboxylase 67 (GAD67) resulting in normalised neuronal activity [75, 76]. In AD model, lentiviruses vectors lead to an efficient silencing of ß-site amyloid

precursor protein (APP) cleaving enzyme 1 (BACE1), leading to a reduction of APP [77]. Consequently, ß-amyloid production decreased, resulting in reduced neurodegeneration. Furthermore, different lentiviral-based systems provided safe integration and efficient inhibition of infection by human immunodeficiency virus (HIV) [78, 79].

Adenovirus family have more than one hundred different serotypes reported. In gene therapy, serotypes 2 and 5 stand out since their presence in the population is rare, thus avoiding pre-existing immunity [68, 80]. Adenoviruses have been used mainly in cancer therapy, either using specific cancer-targeting ligands or chimeric types 5 and 3 or types 11 and 3 [63, 81–83]. These vectors resulted in specific tumour cells infection and effective oncolysis both in the infected and non-infected tumours ("bystander anti-tumour activity"). Moreover, adenovirus-based gene therapy is usually related to short-term transgene expression and, consequently, limited duration of therapeutic activity. Thus, these viruses have been used in combinatory systems with Sleeping Beauty transposase system or CRISPR-Cas9 system, leading to long-term therapeutic activity due to chromosomal integration or gene editing, respectively [63, 84]. The first approved gene therapy products, Gendicine and Oncorine (H101), were adenovirus-based gene therapies.

AAV demonstrated great potential for the gene therapy of a wide range of diseases, including the Rett Syndrome, Huntington's disease, cystic fibrosis, motor diseases (*e.g.* Duchenne and limb-girdle muscular dystrophies), various forms of haemophilia, and blindness [69, 85–89]. Remarkably, the use of dual AVV systems (using two AVV vectors) increased the packaging capability of AAV-based NA delivery systems. In AAV vectors, there is a serotype dependency on the cell tropism and transduction profile of the virus. While serotypes AAV1 and AAV5 preferentially transduce CNS cells, serotype AAV8 effectively transduce hepatic cells [67]. Some of the gene therapy products that reached the market were or are based on different AVV vectors, including Glybera (AVV1), Luzturna (AVV2) and the recently mediatic Zolgensma (AVV9).

HSV can be a particularly interesting NA delivery vector when long-term gene expression is required due to the ability to remain latent within the host-cells after infection. Thus, HSV-based vectors have been applied in a wide range of diseases, including painful diabetic neuropathy, as an inhibitor of the development and progression of painful neuropathy, and cancer by causing cytotoxicity due to overexpression, leading to tumour growth inhibition and extended life survival [90, 91]. The gene therapy Imlygic, targeted to the treatment of melanoma, was based on the HSV-1.

Despite the potential as of viral vector-mediated gene therapy for a plethora of diseases, the use of viral vectors soon raised some scepticism due to reports of associated strong immunogenicity and insertional mutagenesis. The concerns with the use of viral vectors become clear after the death of an 18-year old patient in 1999, due to an adenovirus-based gene therapy targeted to ornithine transcarbamylase, a non-life threating disease [72]. A few years later, a severe adverse event due to proviral integration occurred in a patient who had been treated with a retroviral-based gene therapy for the X-linked severe combined immunodeficiency 30 months earlier [92]. Moreover, the use of viral vectors in gene therapy applications is limited by low scale production and storage difficulties [93].

1.5. Non-Viral Vectors

To overcome the disadvantages of viral vector-mediated NA delivery, mainly due to safety concerns and continuous administration, researchers focused on the engineering of non-viral

chemical vectors. These vectors are mainly based on inorganic nanoparticles, exosomes, lipids, polymers and dendrimers, which interact with NAs primarily *via* electrostatic interactions. There are a plethora of possibilities among non-viral vectors, favouring the design of an efficient, biocompatible, biodegradable and targeted NA delivery system. However, the efficiency of non-viral vector-based systems is still below that of viral vector-based systems, and further research is required.

Generally, non-viral NA vectors present a cationic nature to promote the interaction with the negatively charged NAs, protecting them from the degradation of extra- and intracellular nucleases. Moreover, the positive charges of the cationic vector interact with the negatively charged glycoproteins and proteoglycans of the cell membrane promoting cellular uptake [94–96]. These systems are usually characterised by the ratio between the positively chargeable amine groups of the vector (N, from nitrogen) and the number of phosphate groups of the NA backbone (P), *i.e.*, the N/P ratio.

1.5.1. Inorganic Nanoparticles

Inorganic nanoparticles, composed of metals (*e.g.* gold, iron oxide or quantum dots), inorganic salts (*e.g.* calcium phosphate) or ceramics (*e.g.* silica) are surface-coated with cationic polymers (*e.g.* poly(ethylene imine) - PEI) or molecules (*e.g.* ß-cyclodextrin), thus allowing NA complexation [97–100]. The interest of these particles in gene therapy comes from the generally low cytotoxicity at low concentrations (due to their inert nature), low immunogenicity, low polydispersity, high surface-to-volume ratio and proper storage stability. Moreover, inorganic particles have very reduced sizes (10 nm to 100 nm) and have been used to transfect a wide variety of post-mitotic cells, both *in vitro* and *in vivo* [101–104].

The versatile properties of inorganic nanoparticles regarding the compound type, size, shape, charge and hydrophobicity, allow suitable tailoring to achieve biocompatibility, controlled release, stability under physiological conditions and targeting [97]. The excellent cost-effectiveness of functionalization allows the engineering of nanoparticles with unique properties, either electrical, magnetic, mechanical, or optical, for a specific NA delivery application [97, 104]. Super-paramagnetic properties of iron-oxide based nanoparticles have been used for remote NA delivery through magnetofection, and gold nanoparticles were used in the engineering of a pH-sensitive NA delivery system [102]. Moreover, nanoparticles are used to deliver different types of NAs, including pDNA, miRNA, siRNA and oligonucleotides for the gene therapy of several diseases, including cancer, cardiovascular and neurodegenerative diseases [100, 102, 105–107].

However, inorganic nanoparticles are usually cytotoxic at higher concentrations and their stability under biological fluids, such as blood plasma, should be carefully addressed since they are susceptible to physicochemical changes which can lead to aggregation [105].

1.5.2. Exosomes

Exosomes are membrane vesicles of endocytic origin that present small dimensions (30 nm to 120 nm). These vesicles are secreted by several types of cells and originate in multivesicular bodies, fuse with the plasma membrane and are secreted by cells into the extracellular environment. Exosomes play an essential role in many physiological processes, including immune response regulation, antigen presentation, protein secretion, cytokine transport and cell-to-cell communication [108–110].

These vesicles present high potential as NA vectors due to the similarity with cell membranes, and natural carrier properties, including in the transport of mRNAs and miRNAs. Furthermore, exosomes are highly abundant in biological fluids, having high stability in circulation, and present intrinsic targeting properties due to the presence of important surface proteins (vary depending on the cell origin of the exosome), enhancing their capacity to overcome some biological barriers. Also, the dimensions of these vesicles make them suitable for NA delivery to tumours or inflamed tissues due to selective and enhanced permeability and retention effect [108–111].

The use of exosomes as exogenous NA delivery vectors is still in its infancy [108, 111]. In fact, the first *in vivo* use of exosomes as an RNAi NA delivery system occurred less than ten years ago [112]. However, exosomes have already been exploited as NA delivery vectors for RNAi in a wide range of diseases, including cancer and neurological, inflammatory, ocular, liver, kidney and cardiovascular diseases [108–110]. Some examples of the applications of exosomes include the *in vivo* delivery of siRNA to mouse brain inducing the knockdown of a therapeutic target in AD by more than 60%, and the *in vitro* siRNA delivery to tumour cells resulting in significant target gene expression knockdown and cell death [112, 113].

The major disadvantage of exosomes as NA vectors is their hard protocols for isolation and purification (*e.g.*, ultracentrifugation or ultrafiltration), loading (*e.g.*, incubation, sonication or electroporation), targeting and delivery. Moreover, some safety concerns must be addressed, since these vesicles are involved in the pathogenesis of numerous diseases and their endogenous role is not entirely elucidated [108, 109, 111].

1.5.3. Lipids

Lipids are amphiphilic compounds with a positively charged hydrophilic head and a fatty acid hydrophobic tail, connected by a linker [68, 114, 115]. Different lipid-based nanoparticles have been employed in NA delivery systems, including nanoemulsions, liposomes and solid lipid nanoparticles. Cationic nanoemulsions are dispersions of an oil phase in an aqueous phase, stabilised by an emulsifying agent, generally a cationic surfactant, forming a single, continuous phase of droplets able to complex NAs [94, 116–118]. Conversely, because of their amphiphilic character, lipids form liposomes, with the ability to entrap NAs into nano-sized complexes called “lipoplexes” [68, 94, 114]. Solid lipid nanoparticles present similar composition to liposomes but are solid both at room and body temperatures. These nanoparticles are smaller than liposomes and present some structural differences [94, 119, 120].

Generally, the efficiency of lipid-based NA delivery systems depends on the size (20 nm to 1 μm), charge, nature of the lipid anchor and biodegradability of the linker bondage. The ideal lipoplex N/P ratio is approximately 1, and beyond a ratio of 3 lipoplexes become cytotoxic [68, 94, 95]. Conversely, in the case of solid lipid nanoparticles, the preferred N/P ratios are between 5 and 15 [119]. In the case of nanoemulsions, the nature and concentration of the stabiliser also have a preponderant role [116–118, 121].

The first synthesised lipid employed for NA delivery was N[1-(2,3-dioleyloxy)propyl]-N,N,N, trimethylammonium chloride (DOTMA), developed by Felgner *et al.* in 1987 [122]. Since then, several lipids have been used in lipid-based NA delivery systems, including lipoplexes based on 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), dimethyldioctadecylammonium bromide (DDAB), 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium (DOSPA), among others. Cationic lipids are usually mixed with helper neutral lipids, such as 1,2-dioleoylphosphatidylethanolamine (DOPE) or cholesterol, to increase the complex

stability, prolongate the blood circulation time, promote the endosomal escape, and promote the release of the NAs from the complex [95, 96, 114]. Two of the most commonly used transfection reagents, Lipofectamine® 2000 and Lipofectine®, are composed of a 3:1 w/w mixture of DOSPA and DOPE and a 1:1 w/w mixture of DOTMA and DOPE, respectively [123].

Cationic lipids have been used in NA delivery systems targeted to ocular diseases, infectious diseases, such as HIV and hepatitis C virus (HCV), mucopolysaccharidosis I (MPS I), lysosomal storage disorders, pulmonary disorders and cancer [68, 120, 121, 124, 125]. In particular, nanoemulsions administration is highly versatile and can be mediated through parenteral, oral, topical, nasal and ocular routes [117]. Solid lipid nanoparticles have been described as the choice of a delivery system for siRNA [126].

Despite the extensive use of lipid-based nanoparticles as NA vectors, they are commonly associated with high cytotoxicity and limited blood circulation time, limiting its utility not beyond vascular endothelial cells, due to degradation by MPS [68, 94]. Due to these facts, lipid-based gene therapy systems present, generally, reduced efficiency *in vivo* [115].

1.5.4. Polymers

Polymers with cationic character at physiological pH can entrap anionic NAs, forming compacted structures called "polyplexes". One of the first polymers used in gene therapy was poly(L-lysine) (PLL) [127]. Since then, a considerable number of other polymers have been used to engineer both *in vitro* and *in vivo* gene therapy systems, including chitosan, PEI, poly(amidoamine) (PAMAM), poly(methacrylate) (PMA), poly(D/L-lactic acid) (PLLA) and poly(D/L-lactic-co-glycolic acid) (PLGA), among others [95, 114, 115]. The molecular weight and length of the polymer is related to the net positive charge and, consequently, to polymer toxicity and the ability to entrap NAs [68, 95]. Moreover, labile bonds can be included into the polymer backbone to be susceptible to hydrolysis or to respond to an endogenous or exogenous stimulus, such as temperature, pH, hypoxia, redox gradient, ultrasound and light [114, 128, 129].

Polymers present a wide versatility regarding composition, molecular weight and side chains density, allowing the tuning of the physicochemical properties of the polyplex [114]. Nevertheless, cationic polymers present considerable charge-related toxicity, leading to poor transfection efficiency (especially *in vivo*), and polymer polydispersity. However, due to the plethora of synthetic chemical possibilities when designing a polymer-based NA delivery system, it is possible to mask their surface charge with several ligands and moieties [68, 115].

1.5.5. Dendrimers

Dendrimers are novel macromolecules that present unique structural characteristics: a nanosized, globular, highly branched and controlled structure, very low polydispersity, and a plethora of controllable functionalization possibilities due to the presence of a high number of terminal groups [95, 114]. Moreover, in their cationic form, dendrimers can efficiently condense NAs through electrostatic interactions into nanosized complexes called "dendriplexes". Dendrimers are the central focus of Chapter 2.

1.6. Summary

Gene therapy is a promising field that aims to treat or to prevent a plethora of diseases using therapeutic NAs. Depending on the strategy and NA, this therapy has the potential to

upregulate or downregulate a specific gene or even to promote cell death or the response of the immune system, thus inducing a therapeutic effect. Among the gene therapy approaches, RNAi mediated by small RNAs, including miRNAs and siRNAs, represents a promising strategy for short-term gene silencing. Moreover, different reports support the synergistic effect of multiple NA delivery for both single and multiple gene silencing. However, a NA therapy system faces numerous extra- and intracellular barriers and challenges. Thus, the engineering of a proper NA delivery vehicle is imperative for an effective gene therapy approach. The NA vectors are divided into two major categories, viral and non-viral.

Due to their excellent transfection efficiency, viral vectors are highly used in clinical trials. However, these vectors are related with considerable toxicity and immunogenicity issues. Among them, AAV vectors stand out due to their improved safety profile and excellent transfection efficiency. However, AAV-based NA delivery systems present limited transgene capacity. Other viral vectors, such as AV present higher transgene capacity and targeting efficiency but are highly associated with immunogenicity. Thus, considering the side-effects of gene therapy mediated by viral vectors, new NA delivery strategies have been investigated.

Non-viral vectors were expected to overcome the problems of viral counterparts. These vectors can be engineered from naturally occurring or synthetic compounds. Different vectors have shown to efficiently complex and protect NAs from enzymatic degradation and to lead to high targeting and moderate transfection efficiencies. Among the non-viral vectors, lipids and polymers have been the most used vectors due to their ability to complex NAs, versatility and considerable efficiency. However, the overall efficiency of these systems is still below that of gene therapy mediated by viral vectors. Thus, more research is still required in this field. More recently, dendrimers have emerged as new attractive NA vectors due to their key structural features, such as highly branched and controllable nanostructure, forming very low polydisperse dendriplexes with NAs. Dendrimers are further discussed in Chapter 2, particularly in the context of NA vectors for gene therapy applications.

Chapter 2

Dendrimers

In 1978, Buhleier, Wehner, and Vögtle reported the synthesis of "cascade" molecules, introducing the concept of repetitive growth with branching [130]. In 1985, Tomalia's group described the first successful synthesis of a new class of highly branched macromolecules: dendrimers. The term derives from the Greek words "dendron", meaning "tree", and "meros", meaning "part". In the following years, Tomalia, Newkome, and Fréchet groups published a series of new reports on these compounds [131–133].

Dendrimers are nanosized macromolecules with a well-defined, radially symmetrical and densely branched structure, exhibiting high molecular weight and a very reduced polydispersity [28, 134]. These molecules are composed of three main components (Figure 2.1):

(i) a central core with two or more functional groups,

(ii) interior layers – generations – of branched repeating units or monomers,

(iii) functional end-groups on the surface.

Dendrimers are defined according to their number of generations (G), *i.e.*, the number of layers between each cascade point (subdivision point of each branch). As the number of generations increases, the number of terminal functional groups increases exponentially, the dendrimer diameter tends to increase linearly, and its appearance becomes more globular [135, 136]. Furthermore, the terminal groups can be functionalized with different ligands, contrast agents, drugs, among other moieties. Thus, dendrimers arouse great enthusiasm in several areas, especially in nanomedicine, for instance, in diagnostic imaging, drug delivery, and NA delivery [28, 137–139].

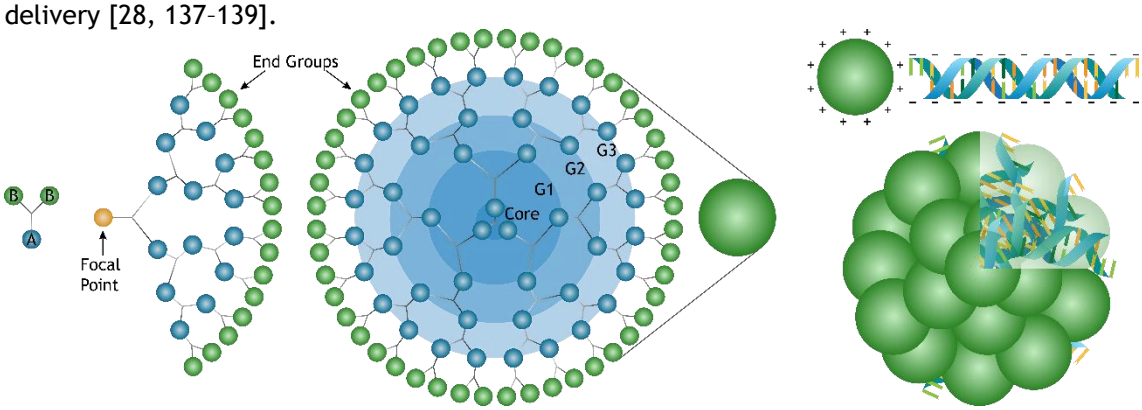

Figure 2.1 Monomers, dendrons, dendrimers and dendriplexes. Scheme of an AB_2 monomer, a G4 dendron, a G4 dendrimer and a dendriplex.

The following sections present a review of the main strategies to synthesise dendrimers, as well as the most commonly used dendrimers as NA vectors. Furthermore, several approaches to overcome the disadvantages of these dendrimers are discussed, including surface modifications, PEGylation (as in the case of PEG-dendritic block copolymers) and, more recently, the development of biodegradable dendrimers.

2.1. Synthesis

During the last three decades, dendrimer synthesis evolved significantly, as reviewed elsewhere [140]. The synthesis of dendrimers occurs either using a divergent or convergent approach (Figure 2.2). The first developed dendrimers were synthesised *via* step-by-step iterative coupling methods and involved costly and multiple protection and deprotection reactions. Later, orthogonal coupling strategies, boosted by the emergence of "click" chemistry reactions, allowed to reduce the number of reactions, leading to improved efficiency of dendrimers synthesis.

In 1985, Tomalia, Newkome and Vögtle introduced the divergent growth strategy, also known as the inside-out approach. In this method, the synthesis of the dendrimers initiates from a multifunctional core and a monomer with an activated group and multiple protected groups. The active functionalities of the monomer react with the active functionalities of the core, thus forming a dendritic layer [141]. Afterwards, the peripheral groups of the monomer are activated or deprotected, thus originating a G1 dendrimer with reactive groups. The process is repeated, extending the dendrimer outwards, to add new layers, *i.e.*, to increase the dendrimer generations, or the end-groups are further post-functionalized.

In 1990, Hawker and Fréchet described the synthesis of dendrimers through a convergent modality [133]. This approach consists of the synthesis of perfectly branched dendrons presenting a focal point that is activated and coupled to a multifunctional core. Afterwards, the activation of the functional end-groups originates a reactive dendrimer [141, 142]. Likewise, the construction of dendrons can also occur *via* the convergent growth or the previously described divergent method.

In some cases, the conventional dendrimers syntheses present some disadvantages, namely, numerous time-consuming protection and deprotection reactions (at least two reactions per dendrimer generation), an excess of reagents and monomers (inefficient at higher generations), and the risk of defect introduction (especially at higher generations). Thus, researches have been focusing on the development of faster and more time- and cost-effective strategies to synthesise high molecular weight dendrimers with a large number of functional end-groups while reducing the number of reaction steps and the consumption of reagents and monomers.

During the turning of the millennium, several accelerated synthesis strategies were proposed, including

(i) the use of hypermonomers, containing several functional groups (four or more),
(ii) double stage convergent growth (implies the use of a hypercore),
(iii) double exponential growth, using a monomer with orthogonal functionalities at the focal point and the peripheral groups, and
(iv) orthogonal strategies using chemoselective reactions.

Orthogonal strategies benefited from the introduction of the "click" chemistry concept, by Kolb, Finn and Sharpless, in 2001 [140, 143]. "Click" chemistry refers to a group of highly selective, efficient and mild condition chemical reactions that forms stable products with reduced

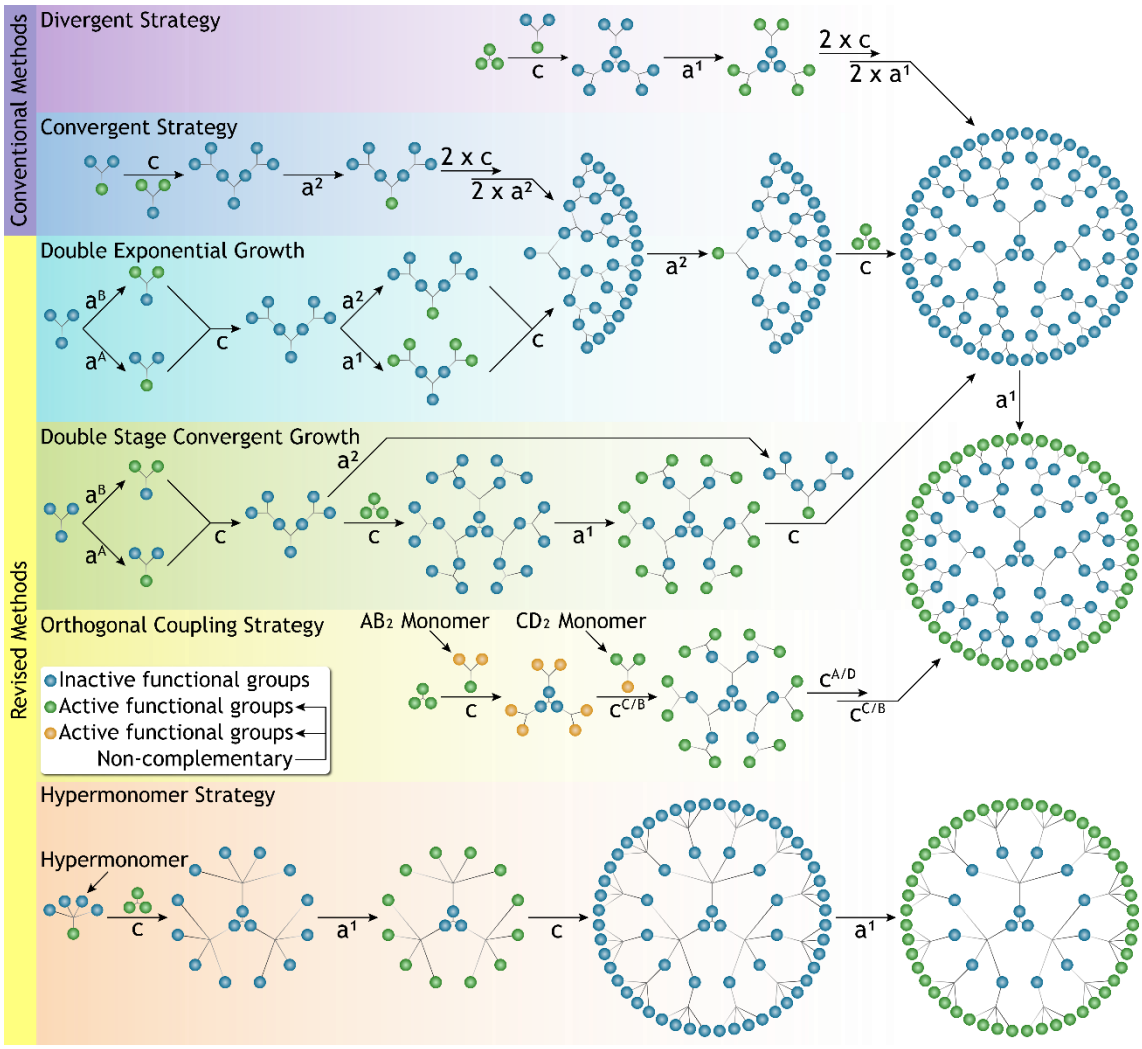

Figure 2.2 Dendrimers synthesis. Comparison between conventional approaches and accelerated approaches. c – coupling, a^1 – activation of the peripheral groups, a^2 – activation of the focal point, $c^{C/B}$ – coupling of the C functionalities of the CD_2 monomer to the B functionalities of the AB_2 monomer, $c^{A/D}$ – coupling of the A functionalities of the AB_2 monomer to the D functionalities of the CD_2 monomer.

or inexistent byproducts, as is the case of the copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC). These reactions present remarkable advantages, including high yields (100% or close) and simple purification procedures [144]. In a remarkable proof-of-concept, Antoni *et al.* reported the orthogonal synthesis of a G6 dendrimer based on thiol-ene and CuAAC reactions, in a single day, including purifications [145].

2.2. Dendrimers for Small Interfering RNA Delivery

In their cationic form, dendrimers have proven to be highly efficient NA carriers due to their ability to condense NAs into complexes called “dendriplexes”. These compact structures are formed mainly *via* electrostatic interactions between the positively charged cationic dendrimers and the negatively charged NAs. Some studies also pointed to the non-negligible contribution of hydrophobic interactions and hydrogen bond formation [146]. Dendriplexes allow proper protection of NAs from degradation and enhanced cellular uptake and transfection efficiency [134, 138]. Thus, cationic dendrimers present boundless potential as non-viral vectors for NA delivery applications.

In 1993, Haensler and Szoka first reported the use of cationic dendrimers in NA delivery [147]. Since then, a plethora of cationic dendrimers has been described in the literature. Some examples of dendrimer families that have been used as NA vectors include PAMAM, poly(propylene imine) (PPI), PLL, 2,2-bis(hydroxymethyl)propionic acid (bis-HMPA), poly(benzyl ether) (PBzE), polymelamine(triazine), carbosilane or gallic acid–triethylene glycol (GATG) dendrimers. As a result of their commercial availability, the most studied dendrimers in therapeutic applications are PAMAM, PPI and PLL dendrimers (Figure 2.3). The synthesis of both PAMAM and PPI dendrimers occurs through a divergent route based on the repeating unit amidoamine or propylamine, respectively, taking advantage of the thiol-Michael addition [131][148]. PPI dendrimers are smaller than PAMAM dendrimers of the same generation, present a higher density of surface groups, but are more hydrophobic. Both dendrimers have been widely used in drug and NA delivery applications due to their water solubility, non-immunogenicity, and relatively proper biocompatibility [40, 149–153]. PAMAM dendrimers are the base of some commercially available reagents for *in vitro* NA transfection, such as PolyFect® and SuperFect®, which have shown to mediate efficient siRNA delivery [28]. PLL dendrimers are composed of the naturally occurring monomer L-lysine and thus, present good biocompatibility and water solubility [154, 155]. It is worthy of note that, despite the biodegradability of linear PLL polymers, the ability of proteases to degrade PLL dendrimers *in vivo* has not yet been elucidated. Moreover, despite the ability of PLL dendrimers to complex NAs, these interactions are relatively weak, leading to inadequate NA condensation, protection, and delivery [156, 157]. Also, PLL based dendrimers usually lead to reduced transfection efficiency due to deficient endosomal escape after endocytosis, attributed to the lack of buffering effect of the dendrimers. To mitigate these disadvantages, PLL dendrimers have been modified with weak-basic amphiphilic peptides or oleic acid [155]. These modifications lead to increased transfection efficiency, both *in vitro* and *in vivo*, while maintaining the suitable biocompatibility and low cytotoxic nature of these dendrimers [156–158].

2.2.1. Dendrimers Toxicity

The cytotoxic profile of dendrimers mainly depends on the nature and number of surface terminal groups, as well as on the size of the dendrimer, which impacts the in vivo biodistribution and pharmacokinetics. One should notice that both dendrimer size and number of functional groups are related to the dendrimer generation [134]. Moreover, the main disadvantage of the use of cationic dendrimers resides on their increased charge-related cytotoxicity compared with neutral or anionic counterparts [28]. Like other cationic systems, these dendrimers lead to adverse side effects due to electrostatic interactions with the anionic biological membranes. These interactions lead to cell membrane disruption, either due to membrane thinning or pore formation, leakage of intracellular components and subsequent necrosis [159, 160].

Furthermore, there is strong evidence that these dendrimers lead to the activation of the innate immune response, mitochondrial dysfunction and altered expression of various endogenous genes, ultimately leading to apoptotic cell death [40]. Nyitrai *et al.* reported the impairment of mitochondrial oxidation mediated by PAMAM dendrimers, suggesting mitochondrial depolarization [161]. Moreover, Lee *et al.* demonstrated that PAMAM dendrimers lead to mitochondrial dysfunction inducing cell apoptosis [162]. There are some studies reporting alteration of the gene expression pattern, not only on PAMAM and PPI dendrimers but also on carbosilane

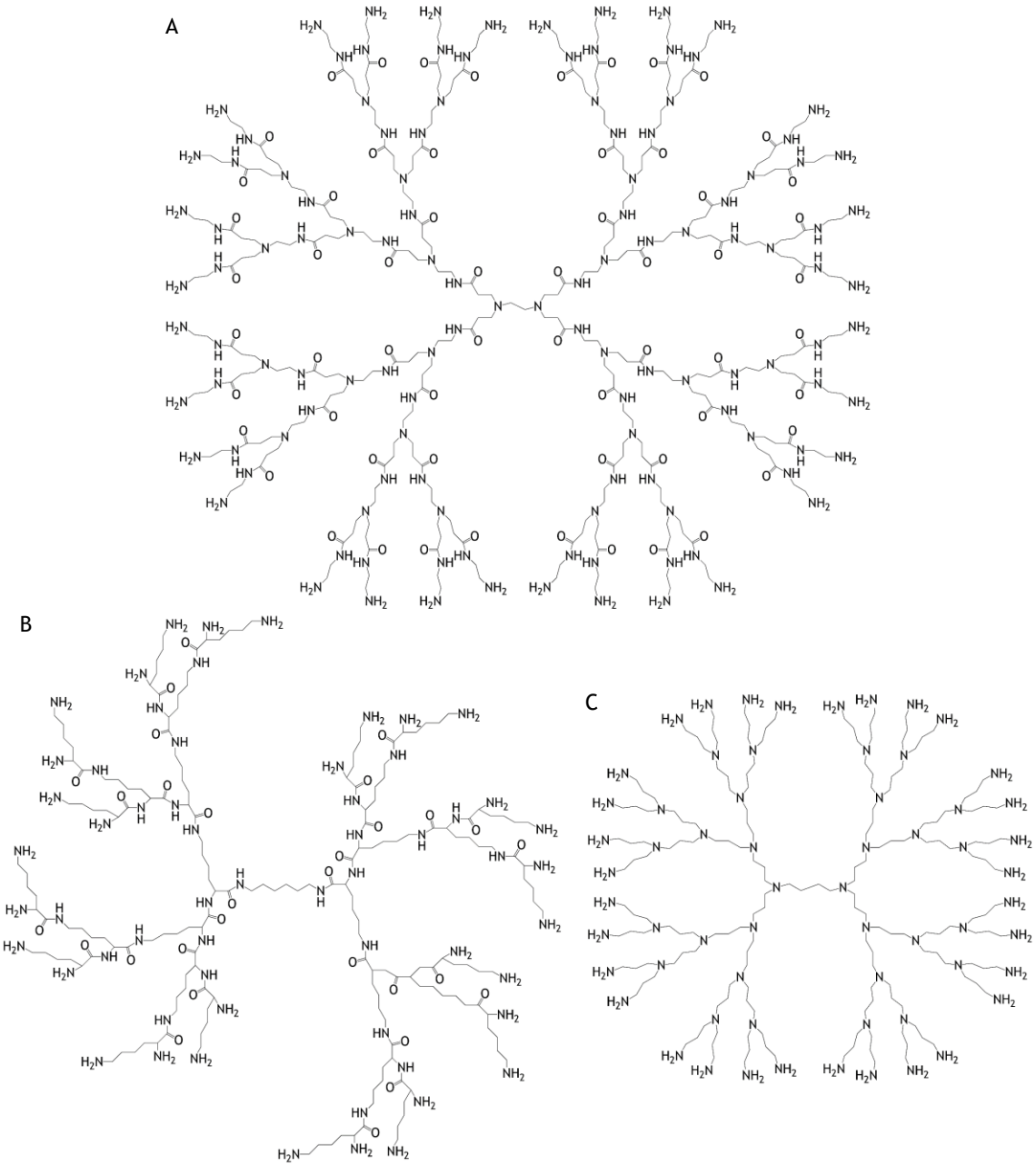

Figure 2.3 Commercially available dendrimers. PAMAM (A), PLL (B) and PPI (C) dendrimers.

dendrimers. Besides, changes in gene expression appear to depend on dendrimers generation, at least in PPI dendrimers [40]. Unspecific gene expression changes can either represent a therapeutic benefit or a challenge since it can severely impair different cellular pathways and mechanisms. If genes related to cell integrity and division are affected, this can lead to cell death by apoptosis or uncontrollable cell division.

Apart from genotoxicity, PAMAM dendrimers trigger the increased production of intracellular reactive oxygen species (ROS), highly correlated with the molar dose of the amino peripheral groups [163]. Moreover, several studies demonstrated that the production of ROS induced by PAMAM dendrimers leads to increased genotoxicity, cytokine production and autophagy flux, ultimately leading to either apoptotic or necrotic cell death [163, 164].

Besides, the *in vivo* cytotoxicity of dendrimers is influenced by the dendrimer dose and concentration, and by the administration route. Systemic administration of dendrimers may

result in haemolysis due to interaction with red blood cells, and alterations of the haemoglobin content and the number and morphology of both red and white blood cells [137].

Despite studies supporting relatively proper biocompatibility of both PAMAM and PPI dendrimers, an important limitation of these dendrimers resides on their nondegradable character under physiological conditions. Thus, the repeated use of these dendrimers can lead to cytotoxicity by bioaccumulation inside cells and tissues. In fact, both PAMAM and PPI dendrimers and the corresponding dendriplexes have shown to accumulate in several organs, such as pancreas, liver, spleen, kidneys, lungs, heart and brain [165].

Considering the adverse effects of cationic dendrimers, researchers joined efforts towards the development of new strategies to mitigate their non-specific cytotoxicity. Some advances regarding the engineering of dendrimers for NA delivery include

(i) surface modifications,
(ii) the well-known PEGylation, and
(iii) the development of biodegradable dendrimers.

2.2.2. Surface Engineering

Aiming to reduce dendrimers and dendriplexes associated toxicity while maintaining or potentiating internalisation and transfection efficacies, dendrimers surface have been modified with different moieties.

Mirroring the great number of fluorinated pharmaceutical molecules, dendrimers fluorination represents a promising surface engineering strategy to reduced dendrimers cytotoxicity, due to positive charge density reduction [166]. Moreover, perfluoroalkylation and fluorination improve serum stability, cellular uptake, endosomal escape, and intracellular NA dissociation, leading to high transfection efficiency [166].

Cyclic oligosaccharides, namely cyclodextrins, bind to cell membrane lipids and can improve dendrimer solubility, stability, and biocompatibility by masking the positive charges of dendrimers. Moreover, cyclodextrins enhance cell membrane affinity of functionalized dendrimers, and consequently, the transfection efficiency of the system [25, 167]. Other saccharides, including mannose and lactose, have also been used to modify dendrimers surface, resulting in enhanced toxicity profile [168].

Decoration of dendrimers with amino acids, such as aspartic acid, cysteine, glycine, histidine, phenylalanine and serine, showed to reduce dendrimer toxicity and haemolicity, to enhance dendrimer solubility and, ultimately, to result in higher transfection efficiencies [169, 170].

Apart from fluorous compounds, saccharides and amino-acids, dendrimers toxicity has also been reduced using other moieties. Janaszewska *et al.* reported PAMAM dendrimers modified with 4-carbomethoxypyrrolidone that demonstrated reduced toxicity and showed no influence in intracellular ROS levels [171]. In a different strategy, Kolhatkar reported the reduction of toxicity in acetylated PAMAM dendrimers by more than 10-fold [172].

Other strategies to reduce the cytotoxicity of dendrimers include the preparation of zwitterionic dendrimer or surface modifications with anti-biofouling polymers, which are characterised by their low cytotoxicity, low haemolicity and non-immunogenicity [172–175]. Modifications with these polymers result in reduced cytotoxicity, enhanced serum stability and reduced unspecific protein binding [175]. There are different possibilities to functionalize dendritic structures with polymers, including the graft of polymers to the dendrimer surface or the functionalization of the dendritic macromolecule at the focal point with linear polymers, thus

forming a linear-dendritic block copolymer (LDBC) [176]. These latter macromolecules are further discussed in the next section.

2.2.3. PEG-Dendritic Block Copolymer

As discussed in section 1.2.1, one foremost barrier to NA delivery through intravenous administration is the interaction of the system with serum proteins and the membranes of erythrocytes. These interactions lead to reduced half-life circulation time of the NA delivery system due to clearance by MPS and can further lead to vascular obliteration and embolism due to aggregates formation [177]. To improve the stability and circulation time of the NA delivery system, LDBCs have been synthesised from anti-biofouling polymers. LDBC can be based on AB diblock, ABA triblock, or star-shaped AnBn dendrimers, where A is the dendritic block and B is the linear chain [178]. The conjugation of two distinct macromolecules aims at a synergistic effect and allow increased properties tuning [179]. Several polymers have been used as the linear chain in LDBCs, including PEG, PEI, poly(methyl methacrylate) (PMMA) and poly(N-isopropylacrylamide) (PNIPAM).

PEG has been the polymer most commonly used in many biomedical applications. Due to the hydrophilic nature of PEG, PEG-dendritic block copolymers based on hydrophobic dendrons lead to amphiphilic macromolecules. Thus, PEGylation improves the solubility and biocompatibility of the copolymers and sterically stabilises the complexes, due to sequestration and masking of positive charges. Moreover, as a consequence of the shielding effect, the interaction of the complexes with the plasma components is suppressed, leading to increased circulation times [177]. However, in some cases, excessive PEGylation can interfere with the formation of compact condensates, leading to large dendriplexes with high polydispersity, and lower NA protection [180, 181]. Besides, steric hindrance due to the presence of PEG chains may hamper the interaction of PEG-DBC based dendriplexes with cell membranes. Reduced interaction with biological membranes results in inefficient internalisation and endosomal escape and, consequently, low transfection efficiency. The increased biocompatibility generally reported for PEG-DBCs when compared to non-PEGylated counterparts can advent from this low internalisation efficiency [177]. Thus, a crucial issue associated with the nanoparticle PEGylation arises – the PEG dilemma. Therefore, the lengths and abundance of PEG chains need to be appropriately balanced.

The solution to this problem consists of finding a balance between efficient transfection and appropriate biocompatibility. A hydrolysable linkage between the linear polymer and the focal point of the dendron is a promising approach to surpass the low endosomal escape [182]. On the one hand, the system can benefit from the PEG chain. On the other hand, under the acidic conditions of the endosome, the disruption of the PEG-dendritic block leads to charges unmasking, promoting the endosomal escape.

Moreover, dendrimers PEGylation also enhances the targeting possibilities of the dendrimer by incorporating specific targeting ligands, such as carbohydrate groups, proteins, aptamers and peptide ligands, at the end of the PEG chain [183, 184]. In this way, the suitable exposure of the targeting molecule to the nanoparticle surface is ensured in order to favour the interaction with the cell receptors [183].

2.2.4. Biodegradable Dendrimers

Despite the improved biocompatibility of the commonly used dendrimers promoted by the surface modifications and PEGylation previously mentioned, one cannot neglect the possibility of toxicity by bioaccumulation of these nondegradable materials [165]. Hence, recently, researchers have been focusing on the design and synthesis of biodegradable dendrimers, presenting labile bonds, that degrade in time into minor fragments under the physiological environment. Then, the small subproducts would be excreted or eliminated through metabolic pathways [134, 185].

Several factors affecting the degradation rate of dendrimers include [134]:

(i) type of cleavable chemical bond;
(ii) hydrophobicity of the repeating units – hydrophilic ones result in faster degradation rates of compounds;
(iii) size – larger dendrimers, presenting increased packaging, have slower degradation rates;
(iv) location of the cleavable linkage – structures presenting internal hydrolysable linkages have faster degradation rates.

Several classes of biodegradable dendrimers have been investigated, integrating different labile linkers, such as acetal/ketal, cis-aconityl, disulphide, ester, and hydrazine bonds [134, 185]. The major families of biodegradable dendrimers are polyacetal and polyester dendrimers. Other biodegradable dendrimer families have been described, including a 1,3,5-triazaadamantane (TAA) monomer-based dendrimer, reported by Balija *et al.* [186].

Polyacetal dendrimers present high sensitivity at acidic pH and non-acidic metabolites. Thus, they present exciting potential for drug and NA delivery to tissues where the pH is different from that of healthy tissues, such as in tumour or inflammatory tissues (where the pH is 0.5 to 1.0 pH below that of healthy tissues) [187]. Despite the potential of these monomers in the development of acid-responsive biodegradable dendrimers, the research around polyacetal/polyketal dendrimers is limited, probably due to harsh synthesis conditions and difficult degradation rate control [134, 185].

As above-mentioned, despite the number of different labile bonds, the dominant portion of the reported biodegradable dendrimers is based on ester bonds. This is due to their biocompatibility and a good compromise between biodegradability trait and relative ease of synthetic manipulation [185, 188]. Among the biodegradable polyester dendrimers, the most documented are those based on the bis-HMPA monomer, which is commercially available at relatively low cost. However, the use of bis-HMPA based dendrimers as NA vectors faces some barriers, especially regarding premature or undesired degradation. To partially overcome this problem, some authors suggested the combination of the biodegradable nature of bis-HMPA dendrimers and the robustness of other nonbiodegradable dendrimers, such as polyamide dendrimers [189]. These and other similar dendrimers, consisting of alternating ester bonds and other non-cleavable linkages are known as “alternating dendrimers”. These dendrimers present, in general, increased degradation time, thus being more appropriate in NA delivery applications. However, the synthesis of alternating dendrimers is laborious, and problems regarding bioaccumulation and ineffective endosomal escape and NA release continue to persist [134].

Although the vast majority of the reported biodegradable polyester dendrimers is based on the bis-HMPA monomer, some groups also reported the synthesis of polyester dendrimers based on alternative monomers [190]. However, the preparation of these ester-based dendritic nanocarriers is challenging due to undesired backbone degradation during synthesis,

purification, and subsequent functionalization and processing steps. Consequently, in the literature, only a few the number of biodegradable dendritic structures containing esters were reported for specific functions in biomedicine [134, 190, 191].

2.3. Summary

Dendrimers are nanosized macromolecules with a well-defined, radially symmetrical and densely branched structure, exhibiting high molecular weight and a very reduced polydispersity. These macromolecules are usually synthesised either using a divergent or convergent approach, *via* a step-by-step iterative coupling method. However, several optimised strategies have been proposed, including the use of hypermonomers and orthogonal growth, tacking advantage of "click" chemistry.

In the cationic form, dendrimers can complex NAs *via* electrostatic interactions into condensates called "dendriplexes". These complexes protect NAs from degradation and enhance their cellular uptake. Thus, dendrimers have great potential as non-viral vectors for gene therapy. However, the most used cationic dendrimers in biomedical applications lead to charge-related cytotoxicity and bioaccumulation due to their nonbiodegradable character.

Different strategies to improve dendrimers biocompatibility have been reported, including surface modifications or the conjugation with anti-biofouling polymers, such as PEG, and, ultimately, the development of biodegradable dendrimers. Despite the efforts towards the development of biodegradable dendrimers, only a few studies report their application in biomedical systems. Moreover, among them, just very few groups reported dendritic structures to act as NA delivery systems.

2.4. Scope, Aim and Objectives

Considering the gap regarding a proper NA delivery vector for gene therapy applications, our group synthesised and explored the biological function of a new family of hybrid biodegradable (hb) G2 PEG-GATG ester (GATGE) dendritic block copolymers. These hb LDBC are a type of "alternating dendrimers" consisting of a nonbiodegradable core and a biodegradable shell. These copolymers present azides as terminal groups allowing the straightforward functionalization, *via* CuAAC ("click" chemistry) with different ligands. In this case, and to explore them as siRNA delivery vectors, hb PEG-GATGE were functionalized with different amine groups, either diamine or benzylamine [182, 192]. The functionalization with diamine is intended to increase the positive multivalency of dendritic systems, thus boosting the strength of the electrostatic interactions with the NAs, without increasing the dendron generation. However, as mentioned in section 2.2, the contribution of hydrophobic interactions to assure the stability of the dendriplexes cannot be neglected. Thus, the functionalization with benzylamine is intended to strengthen the hydrophobicity of the system. The hb PEG-GATGE copolymers demonstrated efficient siRNA complexation and an outstanding ability to internalise the NAs. However, poor transfection efficiencies were obtained due to the partial degradability of the vector together with the presence of PEG.

To overcome these issues, new fully biodegradable (fb) G3 PEG-GATGE dendritic block copolymers were proposed, presenting 40 ester bonds as degradation points across the whole dendritic structure, including a new decisive point of degradation: a hydrolysable linkage

connecting the PEG and the dendritic part [192]. The fully degradable nature of these dendritic vectors was expected to favour the endosomal escape as well as the siRNA release from the dendriplexes, rendering higher transfection efficiencies. In fact, this new fb G3 PEG-GATGE, diamine- (fbD) and benzylamine-terminated (fbB, depicted in Figure 2.4), demonstrated excellent siRNA transfection efficiency in several cell lines [193, 194].

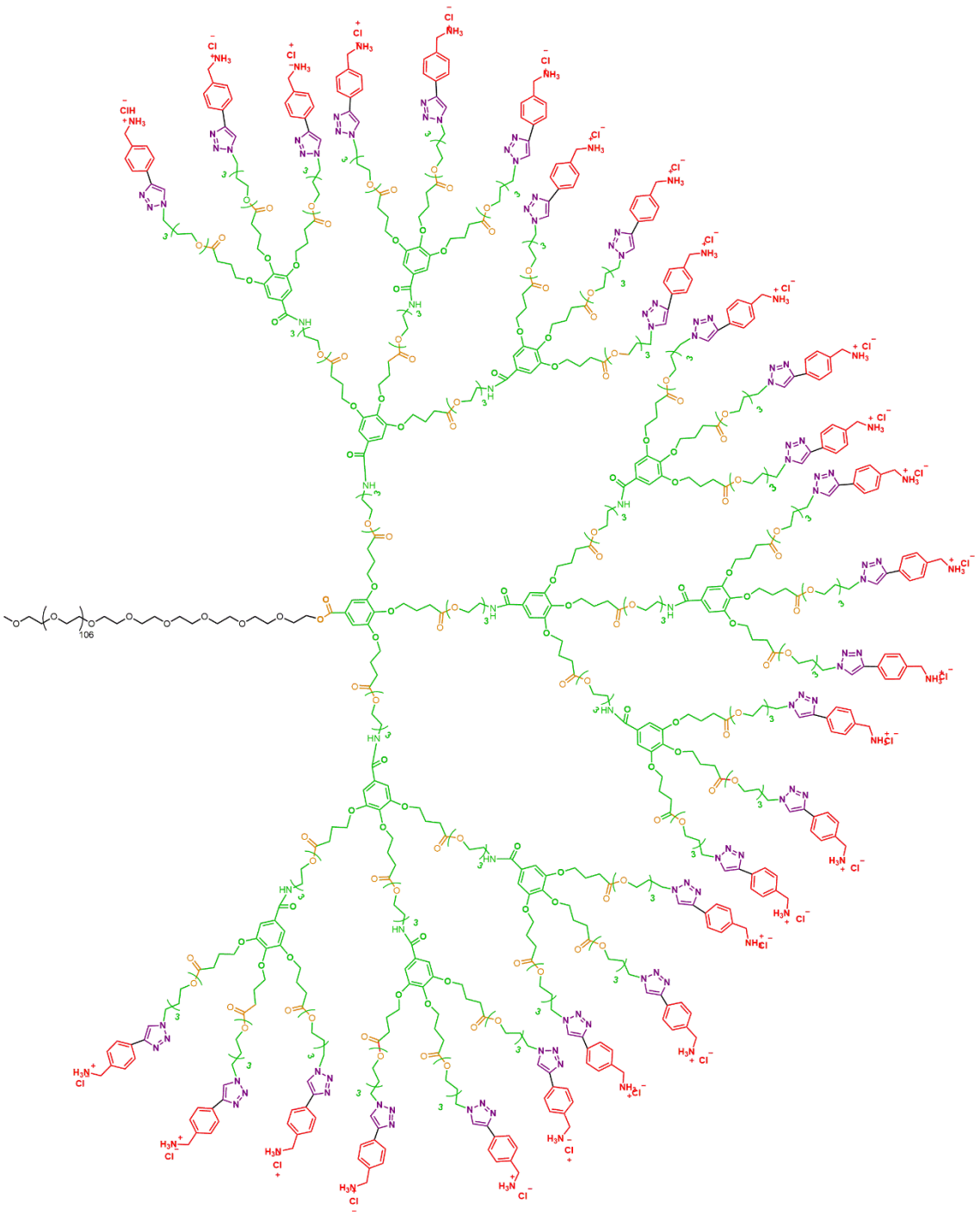

Figure 2.4 Benzylamine terminated fb G3 PEG-GATGE dendritic block copolymers (fbB).

Encouraged by the excellent biocompatibility and siRNA transfection efficiency results obtained with fbB and the recent findings regarding the co-delivery of multiple siRNAs, the main objective of the present work consists of the thorough assessment of these copolymers as

vectors for single and dual siRNA delivery. The efficiency of the fbB as NA vectors was tested using two siRNA sequences that target the mRNAs coding the Phosphatase and Tensin Homolog Protein (PTEN) and the enhanced Green Fluorescent Protein (eGFP), si-PTEN and si-eGFP, respectively. Dendriplexes formed with a single siRNA sequence were characterized regarding different physicochemical properties: (i) siRNA binding ability and complexation efficiency, (ii) size and morphology, (iii) zeta potential, (iv) stability in the presence of serum, (v) stability in the presence of an anionic competitor and (vi) stability at different pHs. These dendriplexes were also analysed regarding their ability to protect the siRNAs against endonuclease degradation and silencing effect.

To access the ability of the fbB to act as dual siRNA delivery vectors two different systems were prepared: (i) a 1:1 mixture of both single siRNA dendriplexes and (ii) dendriplexes from a 1:1 mixture of both siRNAs. These dendriplexes were characterised regarding complexation efficiency and size. Finally, silencing studies were performed, not only to evaluate the efficiency of fbB to act as dual siRNA delivery vectors but also to evaluate the presence or not of a synergistic effect related to the co-delivery of multiple siRNAs.

Chapter 3

Materials and Methods

As described in the in section 2.4, the research work developed during the present dissertation accounts with a complete physicochemical characterisation of dendriplexes with two different siRNA sequences, as well as the evaluation of the developed dendriplexes in the scope of an innovative gene therapy approach, targeting two different mRNAs.

3.1. Materials

The present dissertation evaluates the PEG-GATGE dendritic block copolymer (fbB), synthesised by our group (nanoBiomaterials for Targeted Therapies – nBTT, INEB/i3S), as NA vector. The dendritic copolymers consist of a 5000 kDa PEG chain connected at the focal point of a GATGE dendron functionalized with benzylamine *via* by "click" chemistry. The repeating unit consists of a gallic acid core and triethylene glycol butanoate arms, incorporating ester bonds at the dendritic arms.

The efficiency of the developed dendrimers as NA vectors was tested using two siRNA sequences, si-PTEN and si-eGFP, both with 21 bp. The sequences of both si-PTEN and si-eGFP were supplied by Integrated DNA Technologies and are present in Table 3.1.

The transfection efficiency mediated by the fbB copolymer was evaluated in human bone osteosarcoma cell line stably expressing the eGFP-Luciferase fusion protein (U2OS/eGFP-Luc), kindly gifted by Prof. Edvard Smith (Karolinska Institute, Sweden).

Table 3.1 siRNA sequences used in the present dissertation.

siRNA	Sequence 5' to 3'
si-PTEN	Sense: **CGA CUU AGA CUU GAC CUA U**AU
	Antisense: **AUA GGU CAA GUC UAA GUC G**AA
si-eGFP	Sense: **GGC UAC GUC CAG GAG CGC A**CC
	Antisense: **UGC GCU CCU GGA CGU AGC C**UU

The remaining reagents and materials used throughout the experimental procedures and the respective suppliers are presented in Table 3.2.

Table 3.2 Materials and reagents used in the present dissertation and respective suppliers. TBE - Tris/Borate/Ethylenediamine tetraacetic acid, HEPES - 2-[4-(2-Hydroxyethyl) Piperazin-1-yl] Ethanesulfonic Acid, DMSO - dimethyl sulfoxide.

Reagent/Material (Reference)	Supplier
0.22μm Polyethersulfone Filter (SFPES030022S)	Lubio Scientific
0.45μm Polytetrafluorethylene Filter (28145-493)	VWR
0.6mL RNase-Free Tubes (MCT-060-L-C)	Axygen®
10× TBE buffer (MB27701)	NZYTech
24/96-well Polystyrene Flat Bottom Plates (353072/353047)	Falcon®
30% Acrylamide/Bis Solution, 29:1 (1610156)	BioRad
40μl Micro Cuvette (ZEN0040)	Malvern
5mL Polystyrene Round Bottom Test Tubes (352054)	Falcon®
6× NZYDNA Loading Dye (MB13101)	NZYTech
96-well Polystyrene Black Flat Bottom Plates (655090)	Greiner Bio-One International
96-well Polystyrene Round Bottom Plates (650101)	Greiner Bio One
Ammonium Persulfate (APS, 21300.260)	VWR
D-(+)-Glucose (G7528)	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAX™ (10569010)	Gibco™
Fetal Bovine Serum (12657011)	Gibco™
Folded Capillary Zeta Cell (DTS1070)	Malvern Panalytical
Formvar/Carbon Film-Coated Mesh Nickel Grids (FCF-100-NI)	Electron Microscopy Sciences
Glacial Acetic Acid (131008)	PanReact AppliChem
Heparin (H3149)	Sigma-Aldrich
HEPES (H3375)	Sigma-Aldrich
Lipofectamine® 2000 (11668027)	Invitrogen™
Nuclease-Free Water (129114)	Qiagen
One-Step TB Green® PrimeScript™ RT-PCR Kit II (Perfect Real Time, RR066A)	Takara Bio
Opti-MEM® (31985070)	Gibco™
Penicillin-Streptomycin (P4333)	Sigma-Aldrich
RNase I (AM2294)	Ambion™
Sodium Acetate (106268)	Merck
Sodium Dodecyl Sulphate (817034)	Merck
SYBR® Gold Nucleic Acid Stain (10000× in DMSO, S11494)	Molecular Probes, Invitrogen™
Tetramethylethylenediamine (A1148)	PanReact
Trypsin (T-0646)	Sigma-Aldrich

3.2. Dendriplexes Preparation

Dendriplexes between fbB and siRNA were prepared at different N/P ratio. Firstly, the dendritic copolymer was dissolved in nuclease-free (NF) water at a concentration of 6 $g \cdot L^{-1}$ using a vortex for 30 s followed by thermomixer for 10 min at 1000 rpm and room temperature (RT). This solution was subsequently filtered using a 0.45 μm polytetrafluorethylene (PTFE) filter to

remove possible aggregates. Finally, dendriplexes were prepared by adding siRNA (0.6 μM, otherwise indicated) to the corresponding volume of dendritic copolymer solution (N/P ratios = 5, 10, 20, 40, and 80), in 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) + 5% w/V Glucose. Afterwards, the samples were vortexed for 10 s and incubated for 30 min at RT before subsequent experiments to allow the complex stabilisation.

The required volume of dendritic copolymer, V_{fbB}, for a given N/P ratio and final dendriplex solution volume, V_{total}, is given by Eq. 1,

$$\frac{N}{P} = \frac{N_{fbB} \times \frac{c_{fbB}(g \cdot L^{-1}) \times V_{fbB}}{M_{fbB}}}{2 \cdot bp_{siRNA} \times c_{siRNA}(mol \cdot L^{-1}) \times V_{total}} \Leftrightarrow$$

$$\Leftrightarrow V_{fbB} = \frac{N}{P} \times \frac{2 \cdot bp_{siRNA} \times c_{siRNA}(mol \cdot L^{-1}) \times V_{total}}{N_{fbB} \times \frac{c_{fbB}(g \cdot L^{-1})}{M_{fbB}}} \quad \text{Eq. 1}$$

where N_{fbB} is the number of primary amines per fbB molecule, c_{fbB} is the concentration of filtered fbB solution (5.6 g·L^{-1} as obtained by spectrophotometry for different fbB batches), M_{fbB} is the molecular weight of fbB (20814.5 g·mol^{-1}), b_{psiRNA} is the average number of bp of the siRNA sequence, and c_{siRNA} is the molar concentration of the siRNA solution (usually 20 μM).

3.3. Physicochemical Characterization

3.3.1. Spectrophotometry

Due to the intrinsic ultraviolet-visible (UV-Vis) absorption of PEG-GATGE, fbB mass loss upon filtration was quantified through absorbance reading (λ = 250 nm), in a spectrophotometer (LAMBDA 35 UV-Vis Spectrophotometer, PerkinElmer, USA) using quartz cells. The calibration curve was obtained using different concentrations of unfiltered dendrimer solution (0.0025 g·L^{-1} to 0.05 g·L^{-1}). The filtered solution was diluted 200× prior to analysis. The presented data are expressed as a mean ± standard deviation (SD) of three independent sample measurements of three different fbB batches.

3.3.2. Polyacrylamide Gel Electrophoresis Shift Assay

The interaction strength between fbB dendrimer and siRNA was assessed through polyacrylamide gel electrophoresis (PAGE) shift assay. Polyacrylamide gels were prepared in tris/borate/ethylenediamine tetraacetic acid (EDTA) (TBE) buffer, with 4% stacking and 15% resolving gel (polymerisation catalysed by adding ammonium persulfate at 0.1% V/V and tetramethylethylenediamine at 0.08% V/V). Dendriplex solutions were prepared at N/P ratios of 5, 10, 20, 40 and 80, as previously described. Afterwards, loading dye (1×) was added to the solutions, and the samples were subjected to electrophoresis at 90 V for 30 min. A solution with the same amount of siRNA presented in the loaded samples was used as control. The gels were stained with SYBRGold® 10000× NA stain, diluted in TBE buffer (1:10000), for 10 min. After staining, the gels were visualised using GelDoc™ XR+ Imager (Bio-Rad Laboratories, Inc., USA) and analysed using Image Lab 6.0.1 (Bio-Rad Laboratories, Inc., USA). Dendriplexes complexation, *i.e.*, the binding between the dendritic copolymer and the siRNA, is indicated by limited migration (retention) of the NAs in the electrophoretic field.

3.3.3. SYBR Gold® Intercalation Assay

The efficiency of the fbB to condense and complex siRNA was studied by quantifying the uncomplexed siRNA through SYBR Gold® intercalation assay. Dendriplexes nanoparticle solutions were prepared as previously described. Subsequently, the samples were incubated in 96-well plates in TBE buffer with 1:100 SYBR Gold® 10000× and a final volume of 200 µL, for 10 min to allow the intercalation of the free siRNA. After incubation, a microplate reader (Synergy™ Mx, BioTek Instruments, Inc., USA) was used to measure the fluorescence (λ_{exc} = 485 nm, λ_{em} = 540 nm).

The results correspond to the percentage of complexed siRNA (100% corresponding to fully condensed and complexed NA) of duplicates. The presented data are expressed as a mean ± SD of three independent sample measurements.

3.3.4. Size and Zeta Potential

Size and polydispersity index (PDI) of the dendriplexes prepared at N/P ratios of 5, 10, 20, 40 and 80 were measured at 633 nm by a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., UK), following the manufacturer specifications. The experiments were conducted at RT with a detection angle of 173 ° in ZEN0040 micro cuvettes, using solutions with a final volume of 80 µL without dilution. The results correspond to mean hydrodynamic diameters, determined by cumulative analysis (Z-average mean). The hydrodynamic diameter, D_h ($D_h = 2R_h$), is determined based on the Stokes-Einstein equation, given by Eq. 2

$$R_h = \frac{k_B T}{6\pi\eta D} \qquad \text{Eq. 2}$$

where R_h is the hydrodynamic radius, k_B is the Boltzman constant ($k_B = 1.381 \cdot 10^{-23}$ J·K^{-1}), T is the thermodynamic temperature (in K), η is the dynamic viscosity of the solvent (in kg·m^{-1}s^{-1}) and D is the translational diffusion coefficient (in m^2·s^{-1}).

Nanoparticles surface charge was measured on the same equipment, through Laser Doppler Electrophoresis, using a capillary cell. Dendriplexes solutions were prepared with a final volume of 250 µL and diluted to 750 µL in type III water, prior to measurement. The determination of the Zeta Potential, ζ, was conducted based on the Henrys's equation, depicted in equation Eq. 3,

$$\zeta = \frac{U_E 3\eta}{2\varepsilon f(ka)} \qquad \text{Eq. 3}$$

where U_E is the electrophoretic mobility, f(ka) is Henry's function, ε is the dielectric constant, and η is the viscosity. The equation was simplified considering the Smoluchowski approximation, in which f(ka) = 1.5.

The data were analysed using the Zetasizer Software (v7.12, Malvern Instruments Ltd., UK). The presented data are expressed as a mean ± SD of three independent sample measurements.

3.3.5. Transmission Electron Microscopy

Morphology of the dendriplexes was evaluated by negative staining transmission electron microscopy (TEM). Dendriplexes solutions were prepared as previously described at N/P ratios

of 5 and 80. Afterwards, 10 µL of samples were mounted on formvar/carbon film-coated mesh nickel grids and left standing for 2 min. Subsequently, 2 µL of 1% uranyl acetate was added on to the grids and left standing for 10 s. The liquid in excess was removed with filter paper, and the grid was left dry prior to imaging. The visualisation was carried out on a JEM 1400 TEM (JEOL Ltd., Japan) operated at 80 kV. Images were digitally recorded using a CCD digital camera Orious 1100 W (Japan).

3.3.6. Dendriplex Stability in Biological Fluids

The dendriplexes stability was analysed in physiological media. Dendriplexes solutions were prepared using the si-eGFP as previously described at N/P ratios between 10 and 80. The dendriplexes were incubated at 37 °C with 1× phosphate-buffered saline (PBS) + 10% V/V fetal bovine serum (FBS), for 1 h and 4 h. Afterwards, dendriplexes stability was assessed through the hydrodynamic diameter profile, determined through DLS (as previously described). The size profile of PBS with FBS (without dendriplexes) was used as blank.

3.3.7. Heparin Dissociation Assay

Dendriplexes were prepared as previously described at N/P 80 and incubated at 37 °C with heparin solutions 1:1 V/V at increasing concentrations (final heparin concentrations of 0.0, 2.5, 5.0, 7.5, 10.0, 25.0 and 50.0 $mg \cdot L^{-1}$) for 2 h. Afterwards, the extent of dissociated siRNA from the dendriplexes was evaluated through PAGE shift assay (as previously described).

3.3.8. Dendriplex Degradation Studies

The degradation profile of the dendriplexes was studied both at endosomal and physiological pH (pH 5.0 and pH 7.4, respectively). The dendriplexes solutions were prepared as previously described at N/P 80 and incubated with acetate buffer solution (60 mM NaOAc, pH 4.7) or HEPES/Glucose buffer (40 mM HEPES + 10% w/V Glucose, pH 7.4) at 1:1 V/V at 37 °C, for 1 h, 24 h and 48 h. After incubation, the released siRNA was evaluated through (i) PAGE shift assay (as previously described) after incubation with heparin (7.5 $mg \cdot L^{-1}$) for 2 h at 37 °C to destabilise the complexes, and (ii) SYBR Gold® complexation assay (as previously described).

3.3.9. Endonuclease Protection Assay

Endonuclease protection assay was conducted to evaluate the ability of the fbB to protect the siRNA from enzymatic degradation. Firstly, it was conducted an assay to evaluate the concentration of RNase I required to degrade de studied siRNAs within 5 min. Solutions with different concentrations of RNase I were prepared in NF-water, followed by the addition of the NA sequences (0.3 µM). The samples were incubated at 37 °C for 5 min, 15 min, 30 min or 60 min and the endonuclease was inactivated with sodium dodecyl sulphate (SDS) at a final concentration of 0.1% w/V, following the manufacturer instructions. The siRNA degradation by RNase I was assessed by PAGE shift assay (as previously described). The minimum endonuclease concentration required to completely degrade the siRNA within 5 min was considered for the endonuclease protection assay.

To conduct the endonuclease protection assay, dendriplexes were prepared at N/P 80 as previously described and incubated with the determined concentration of RNase I at 37 °C for 5 min, 15 min, 30 min or 60 min (final siRNA concentration of 0.3 µM). The solutions were

incubated with 0.1% w/V SDS for 30 min to inactivate the RNase and dissociate the complexes. The siRNA degradation was evaluated through PAGE shift assay (as previously described).

3.4. Cells

3.4.1. Cell Culture

U2OS/eGFP-Luc cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% V/V heat-inactivated FBS (56 °C for 30 min) and 1% V/V Penicillin-Streptomycin (P/S) and incubated at 37 °C, 5% CO_2 (CCL-170A-8-SS, Esco, Singapore).

3.4.2. Silencing Studies

U2OS/eGFP-Luc cells were seeded at a density of $2.5 \cdot 10^4$ cells per cm^2, in 500 µL (24-well plates) or 150 µL (96-well plates) P/S and FBS supplemented DMEM and incubated at 37°C, 5% CO_2 for 24 h to reach 70% to 90% confluence prior to transfection. At transfection time, the medium was replaced with non-supplemented DMEM (rinsed twice with 1× PBS). Four different sets of dendriplexes were prepared at N/P ratios of 5, 10, 20, 40 and 80: (i) from si-PTEN, (ii) from si-eGFP, (iii) a 1:1 mixture of si-PTEN and si-eGFP dendriplexes (Mix of Ddp), and (iv) preparing a 1:1 mixture of both siRNAs and adding the fbB to form the dendriplexes (Mix of siRNA).

Cells were transfected using 25 µL of dendriplexes solution in a final volume of 150 µL (96-well plate) or 50 µL in 300 µL (24-well plate) in the case of single siRNA dendriplexes and 50 µL and 100 µL in the case of mix of dendriplexes and mix of siRNAs (each siRNA at 0.1 $pmol \cdot \mu L^{-1}$). Cells were then incubated at 37°C, 5% CO_2 for 24 h. Afterwards, the medium was replaced with fresh supplemented DMEM and incubated again at the same conditions. 96 h post-transfection, cells were prepared for silencing analysis through quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (96-well plate, for PTEN mRNA quantification) or fluorescence-activated cell sorting (FACS)/flowcytometry (24-well plate, for eGFP quantification), as described in sections 3.4.3 and 3.4.4. Non-treated cells were used for calibration, Scramble-transfected cells were used as the negative control, and cells whose transfection was mediated by Lipofectamine® 2000 were used as the positive control. The Scramble dendriplexes were prepared at N/P 80 as described for si-PTEN and si-eGFP dendriplexes. Lipofectamine® 2000 lipoplexes were prepared according to manufacturer instructions in Opti-MEM®. Similarly to dendriplexes, four different polyplex solutions were prepared: (i) from si-PTEN, (ii) from si-eGFP, (iii) a mixture of (i) and (ii), and (iv) from a 1:1 mixture of siRNAs. The final Lipofectamine® 2000 concentration was 0.15% V/V in si-PTEN or si-eGFP only transfected cells – (i) and (ii) – and 0.30% V/V in mixture of polyplexes and mixture of siRNAs – (iii) and (iv). The siRNAs concentrations were the same as in the case of dendriplexes.

3.4.3. Quantitative Reverse Transcriptase Polymerase Chain Reaction

96 h post-transfection, the medium was discarded, and cells were rinsed twice with 1× PBS (100 µL). Afterwards, 30 µL of cell lysis buffer (10 mM Tris-HCl, pH 7.4, 0.25% IGEPAL CA-630 and 150 mM NaCl) was added per well, followed by incubation on ice for 10 min. The lysate was transferred to RNase-free microtubes and centrifuged at 700 g for 5 min at 4 °C. The supernatant was transferred for new microtubes and stored at -80 °C or used directly in qRT-PCR plate.

One-step qRT-PCR was prepared in 384-well plate using the One-Step TB Green™ PrimeScript™ RT-PCR Kit II. For reverse transcriptase and qRT-PCR, 1 µL of each lysate was added to 0.4 µL of PrimeScript™ 1 step Enzyme Mix 2 in 1× One-Step TB Green® RT-PCR Buffer 4 (final volume of 10 µL). The primers were present at 300 nM, and the respective sequences are shown in Table 3.3. The qRT-PCR was run on a CFX 384™ Real-Time PRC System C100 Touch™ Thermal Cycler (Bio-Rad) and the cycling conditions were: (i) reverse transcription – 42 °C (5 min), (ii) hot start – 95 °C (10 s), (iii) PCR amplification (40 cycles) – 95 °C (10 s, denaturation), 55 °C (30 s, annealing) and 72 °C (30 s, extension). All samples were run in the qPCR plates as triplicates for each gene. The data were processed in CFX Maestro™ Software 1.1 (v4.1.2433.1219, Bio-Rad Laboratories, Inc., USA), and mRNA expression levels were computed by the relative quantification method based on the exponential transformation of ΔCt values (2-ΔΔCt). PTEN mRNA expression was normalised against the endogenous control hypoxanthine phosphoribosyltransferase 1 (HPRT1) and calibrated to untransfected cells. Scramble-transfected cells were used as negative control and cells which transfection was mediated by Lipofectamine® 2000 were used as the positive control. The presented data are expressed as the mean of the three technical replicates.

Table 3.3 PCR primers for PTEN and HPRT1 mRNAs.

Primer	Primers sequence 5' to 3'
PTEN	Forward: GGA GTA TCT TGT GCT CAC CC
	Reverse: TGG ATC AGA GTC AGT GGT GT
HPRT1	Forward: GTA ATG ATC AGT CAA CGG GGG AC
	Reverse: CCA GCA AGC TTG CAA CCT TAA CCA

3.4.4. Flow Cytometry

96 h post-transfection the medium was discarded, and the plate wells were rinsed twice with 1× PBS (200 µL). The cells were trypsinised, centrifuged and resuspended in 1× PBS with 2% V/V FBS. The median fluorescence intensity was analysed by flow cytometry in a BD Accuri™ C6 Plus Cytometer (BD Biosciences, USA). Scramble-transfected cells were used as the negative control, and cells whose transfection was mediated by Lipofectamine® 2000 were used as the positive control. Data were analysed using the FlowJo software (FlowJo X 10.0.7r2, FlowJo, LLC) and calibrated to untransfected cells. The present data correspond to the mean of two technical replicates.

3.4.5. Statistical Analysis

Significant differences were examined using one-way ANOVA. Tukey's multiple comparison test was further employed after ANOVA for all samples since the homogeneity of variances was observed in all cases. A p-value of <0.05 was considered statistically significant in all studies. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, USA) and the Python module StatsModels (v0.10.1).

Chapter 4

Results and Discussion

4.1. Dendriplexes Preparation

Dendriplexes between fully biodegradable G3 benzylamine terminated PEG-GATGE (fbB) and two different siRNA sequences, si-PTEN and si-eGFP, were prepared in HEPES-Glucose buffer (20 mM + 5% w/w, pH 7.4), at different N/P ratios ranging from 5 to 80. HEPES buffer is ideal for use in biomedical application since it is widely used to maintain physiological pH despite carbon dioxide concentration changes, such as the case of cell culture. Moreover, the presence of glucose promotes dendriplexes stability and compaction due to the formation of hydrogen bonds. Thus, the preparation of dendriplexes in HEPES-Glu buffer is optimal for direct application in further *in vitro* and even *in vivo* tests.

Before dendriplexes preparation, the fbB was easily solubilised in NF-water at 6 $g{\cdot}L^{-1}$ by vortexing for 30 s and agitating at 1000 rpm for 10 min. Afterwards, the copolymer solution was filtered in a 0.45 µm PTFE filter. The fbB concentration after filtration was determined by spectroscopy, taking advantage of the intrinsic UV-Vis absorption of the copolymer. The average concentration of fbB, quantified in three different batches was 5.6 $g{\cdot}L^{-1}$ ± 0.1 $g{\cdot}L^{-1}$. This slight mass loss, corresponding to only 7% of the total mass, is due to the filtration of small aggregates that do not solubilise. Filtration is an essential step in the preparation of dendriplexes since the presence of aggregates result in high polydisperse complexes and could lead to further cytotoxicity. Moreover, the low mass losses obtained are a relevant aspect when considering the efficiency of the experimental procedure of the production of a therapeutic agent.

4.2. Physicochemical Characterization of Dendriplexes

The condensation and complexation of siRNA with non-viral vectors is intrinsically challenging due to the small size, rigidity and reduced charge density of siRNA. Thus, the interactions with cationic vectors tend to be weaker, when comparing with bigger NA, such as pDNA. Deficient interactions between the vector and the NAs can lead to incomplete NA encapsulation and the formation of large and widely dispersed complexes. Consequently, the

vectors are not able to protect NA from degradation, leading to poor transfection efficiencies. In order to evaluate the ability of our fbB to complex, protect and act as vectors of different siRNA (si-PTEN and si-eGFP) the corresponding dendriplexes were exhaustively assessed and compared regarding their physicochemical properties.

4.2.1. Small Interfering RNA Binding Ability

A suitable NA vector must be able to efficiently complex the NAs. The formation of dendriplexes between cationic dendritic molecules and NAs is similar to other cationic molecules, namely lipids and polymers, and is mostly driven by electrostatic interactions, as previously mentioned. However, the hydrophobic interactions promoted by the dendritic backbone as well as by the benzylamine end-groups cannot be neglected and aim to increase the complexation efficiency and the stability of the system.

In order to assess the ability of the copolymer to condense and complex the siRNAs as a function of both N/P ratio, and siRNA sequence, two studies were performed: (i) PAGE shift assay and (ii) SYBR Gold® complexation assay. PAGE shift assay and SYBR Gold® complexation assay are complementary studies. On the one hand, PAGE shift assay is a qualitative technique to study the interaction strength between the copolymers and the siRNAs. Weak interactions between the siRNA and the vector lead to deficient complexation, resulting in free NA, which migrates throughout the gel when an electrophoretic field is applied. The use of polyacrylamide gels in detriment of the commonly used agarose gels is due to its increased resolving power for small NA sequences and is considered to give more reproducible results. Even so, the gel band intensity does not precisely correlate with the amount of uncomplexed siRNA and, thus, cannot be used as a quantitative method. On the other hand, SYBR Gold® is a highly sensitive fluorescence dye, that presents a substantial fluorescence enhancement (approximately 1000-fold) when bounding to accessible (free) NA. Thus, SYBR Gold® complexation assay is ideal for complexation efficiency quantification.

As presented in Figure 4.1 (A), the amount of free NA migrating throughout the gel decreases with increasing N/P ratios (increasing amounts of the dendritic copolymer). The reduction in free NA was due to the increased number of interactions between the dendrimer and the siRNA molecule, hence favouring the complexation process. At N/P ratio equal or greater than 40, the interaction strength between the dendritic copolymers and the siRNAs was visibly stronger, as a large amount of siRNA remains well-condensed and complexed in the dendriplexes and thus, was unable to migrate in the presence of an electric field.

As shown in Figure 4.1 (B), the complexation efficiency of the dendriplexes increased as the N/P ratio increased, for both siRNA sequences. Moreover, excellent percentages of complexed siRNA were observed, even at the lowest N/P ratio studied (N/P 5: 64% siRNA complexed). For N/P ratios equal or greater than 10, the dendritic copolymers provided outstanding siRNA complexation, between 75% and 95%, independently of the siRNA sequence. Interestingly, dual siRNA delivery systems provided increased complexation efficiency, with more than 80% of complexed siRNA at the lowest N/P ratio (n = 1). One should notice that complexed siRNA molecules can be partially exposed to the dendriplex surface allowing SYBR Gold® binding, especially in less stable complexes.

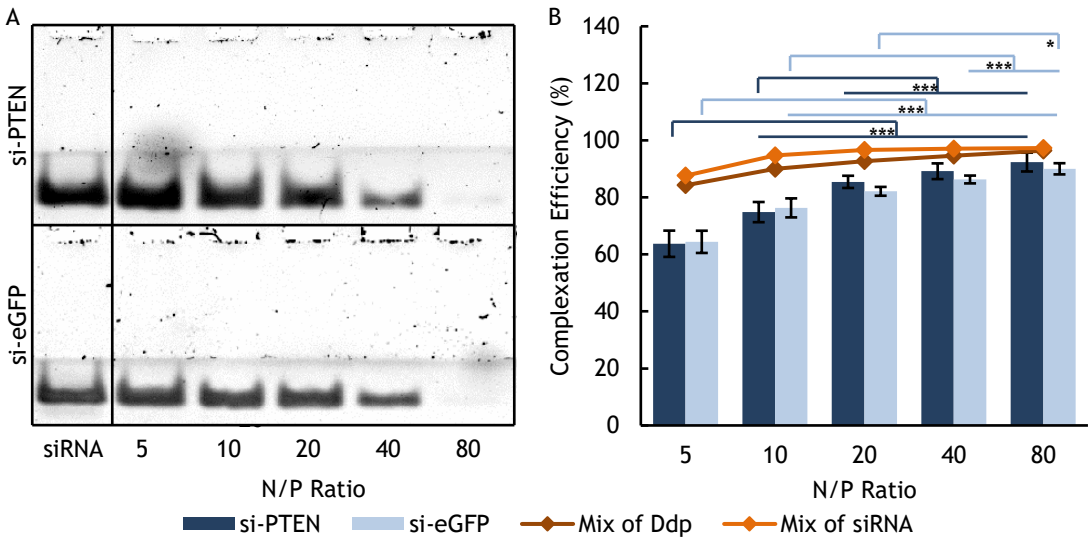

Figure 4.1 siRNA complexation by fbB. A – PAGE of both fbB/si-PTEN and fbB/si-eGFP dendriplexes at different N/P ratios. B - SYBR Gold® exclusion assay of the same dendriplexes as well as those used for dual siRNA delivery (Mix of Ddp and Mix of siRNA). Data represent the percentage of inaccessible siRNA (n = 3 ± SD for si-PTEN and si-eGFP dendriplexes and n = 1 for Mix of Ddp and Mix of siRNA dendriplexes). One-way ANOVA tests were used for statistical analysis. Significant differences: * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

4.2.2. Size and Morphology

As discussed in section 1.2.2, the size and shape of nanoparticles play an essential role in the internalisation pathway and efficiency. Thus, dendriplexes size and morphology were studied through DLS and TEM, respectively. Since dendriplexes will be administered in solution in the corresponding *in vivo* and/or clinical application, hydrodynamic diameter obtained through DLS is more representative of the complexes size. DLS measurements consider non-covalent interactions between the solvent and the particles and include any molecule adsorbed or attached to the particle surface. Conversely, size determination using TEM is valid in the dry state, which is usually a more compact state, thus resulting in slightly lower dimensions.

The size distribution profile was similar for dendriplexes formed with both siRNA sequences and was found to be independent of N/P ratio. DLS measurements pointed to a narrow particle size distribution within the nanometer scale, with an average hydrodynamic diameter of 39.0 nm ± 0.5 nm and PDI (measures the homogeneity of a population) of 0.25 ± 0.02, as shown in Figure 4.2 (A) and (B), respectively. Moreover, these results correlate well with those obtained in the dual siRNA delivery systems, were the average hydrodynamic diameter was 40.0 nm ± 2.0 nm with a PDI of 0.24 ± 0.03 (n = 1). As previously discussed, the complexation of siRNA is challenging, resulting in considerably big and poorly defined nanocomplexes. In fact, the vast majority of the reported siRNA dendriplexes present sizes between 100 nm and 300 nm [195-198]. For instance, the siRNA dendriplexes formed with the previously reported hb G2 PEG-GATGE presented a hydrodynamic diameter of approximately 175 nm [182]. However, our G3 fbB form siRNA dendriplexes with a size of 3 to 10-fold smaller than the dendriplexes formed with the previous vector. This size reduction is due to increased multivalency of the G3 copolymer (27 protonated amines, comparing with 9 in G2), allowing efficient siRNA complexation using less amount of dendritic material, thus resulting in smaller dendriplexes.

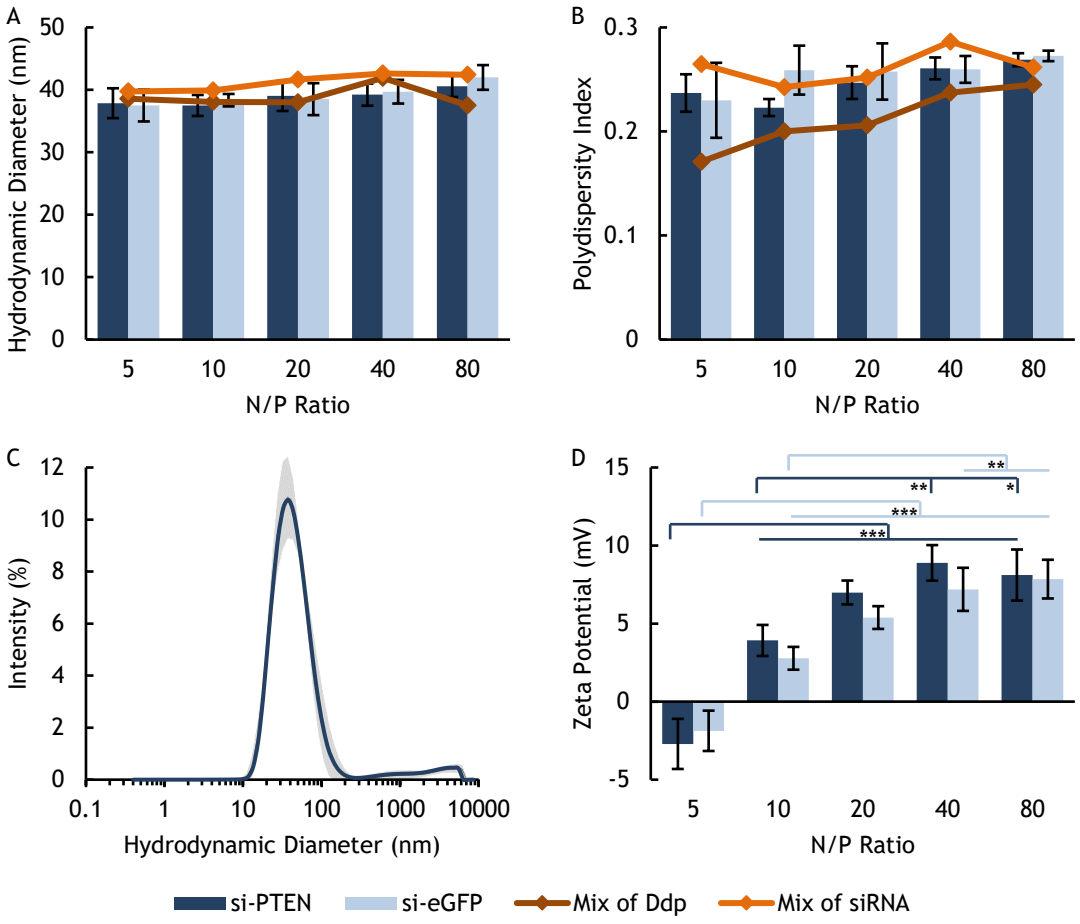

Figure 4.2 Size and surface charge of the dendriplexes. A - Average hydrodynamic diameter of the fbB/siRNA dendriplexes measured by DLS at different N/P ratios. B – PDI of the size distribution of the dendriplexes. C - Hydrodynamic diameter distribution of the fbB/siRNA dendriplexes obtained through DLS (average of all dendriplexes, n = 3); shadow represents SD. D – Zeta potential of the single siRNA dendriplexes at different N/P ratios. Data are expressed as n = 3 ± SD for si-PTEN and si-eGFP dendriplexes and n = 1 for Mix of Ddp and Mix of siRNA dendriplexes. One-way ANOVA tests were used for statistical analysis. Significant differences: * – $p<0.05$, ** – $p<0.01$, *** – $p<0.001$.

TEM experiments showed that the dendriplexes present a spherical morphology (Figure 4.3). Moreover, size measurements based on TEM images correlated with those obtained by DLS. As previously explained, slight differences are related to the sample state: in solution when analysed through DLS and in the dry state when analysed by TEM.

Considering the results obtained through DLS and TEM, the very reduced hydrodynamic diameter and the globular morphology of fbB/siRNA dendriplexes are ideal for cellular internalisation. On the one hand, several studies point to an optimal size range for maximum internalisation rate of nanoparticles between 30 nm and 50 nm, range in which the dendriplexes reported in this work are inserted [33–35]. On the other hand, different studies demonstrated that spherical particles have a higher uptake rate than ellipsoids or rod-shaped nanoparticles [35, 36]. Moreover, considering the size distribution profile obtained, the internalisation pathway of these complexes probably involves both clathrin- and caveolae-mediated mechanisms, as well as macropinocytosis [32, 34].

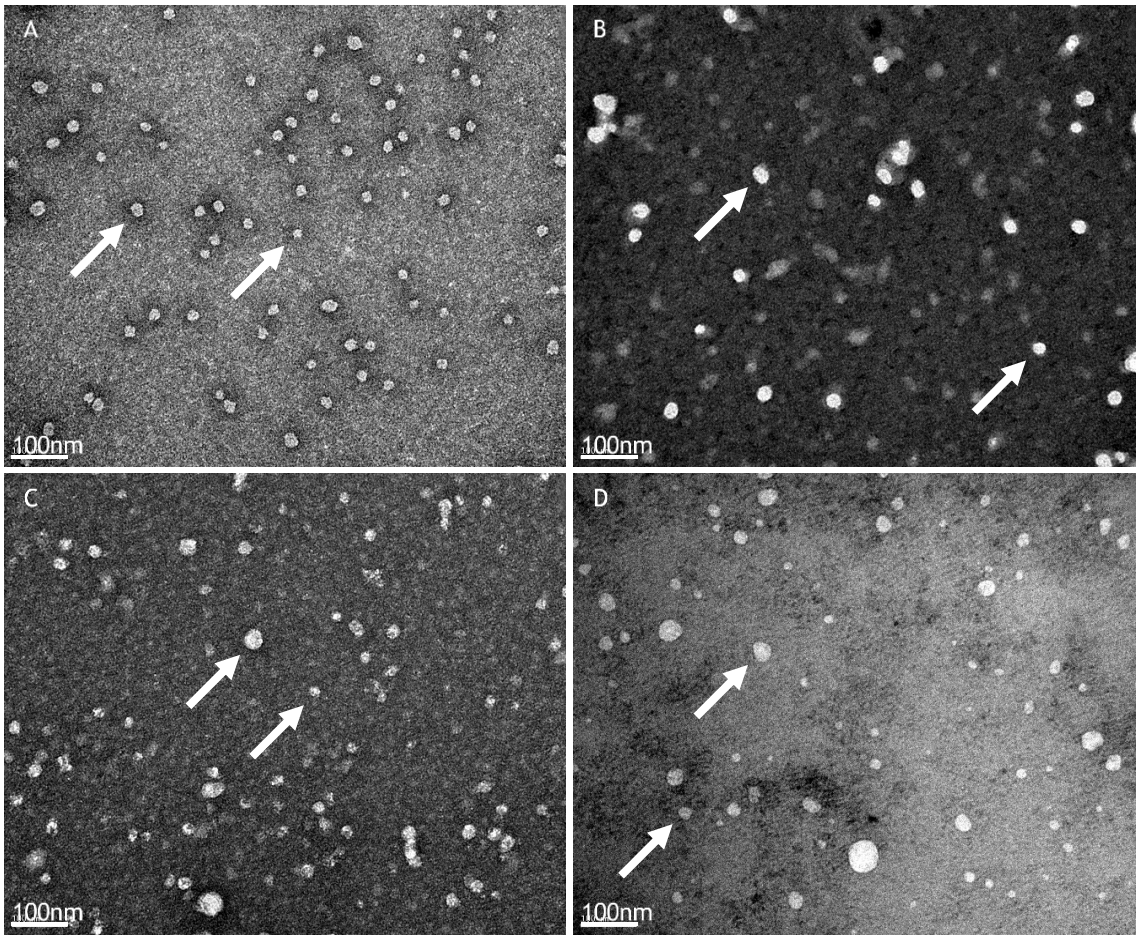

Figure 4.3 TEM images of the dendriplexes. A – Dendriplexes formed with si-PTEN at N/P 5. B – Dendriplexes formed with si-PTEN at N/P 80, C – Dendriplexes formed with si-eGFP at N/P 5. D – Dendriplexes formed with si-eGFP at N/P 80.

The stability of the dendriplexes was further studied simulating plasma conditions. For this study fbB/si-eGFP dendriplexes were prepared at N/P ratios ranging between 10 and 80 and incubated during 1 h and 4 h in 1× PBS containing FBS at 10% V/V. The results depicted in Figure 4.4 suggest that increased concentrations of the biodegradable copolymer resulted in increased stability of the complexes. Dendriplexes prepared at N/P 10 were less stable, as observed by the considerable decrease of the 40 nm peak (characteristic of the dendriplexes size distribution) relatively to the 10 nm peak (blank, *i.e.*, serum), even after 1 h of incubation. Conversely, at N/P 80, the hydrodynamic diameter distribution profile of the dendriplexes remains stable, even up to 4 h of incubation in serum. These results agree with those obtained in the PAGE shift assay, as increased N/P ratios provide higher siRNA binding strength and, therefore, higher stability. Moreover, the results suggest negligible unspecific protein aggregation. This effect can be attributed to the presence of PEG chain, that masks the surface charge of the dendriplexes [177].

4.2.3. Zeta Potential

The net surface charge of a delivery system is a major factor determining cellular uptake efficiency. Laser Doppler Electrophoresis was used to investigate the surface charge of both single siRNA dendriplexes as shown in Figure 4.2 (D). All developed dendriplexes showed a pos-

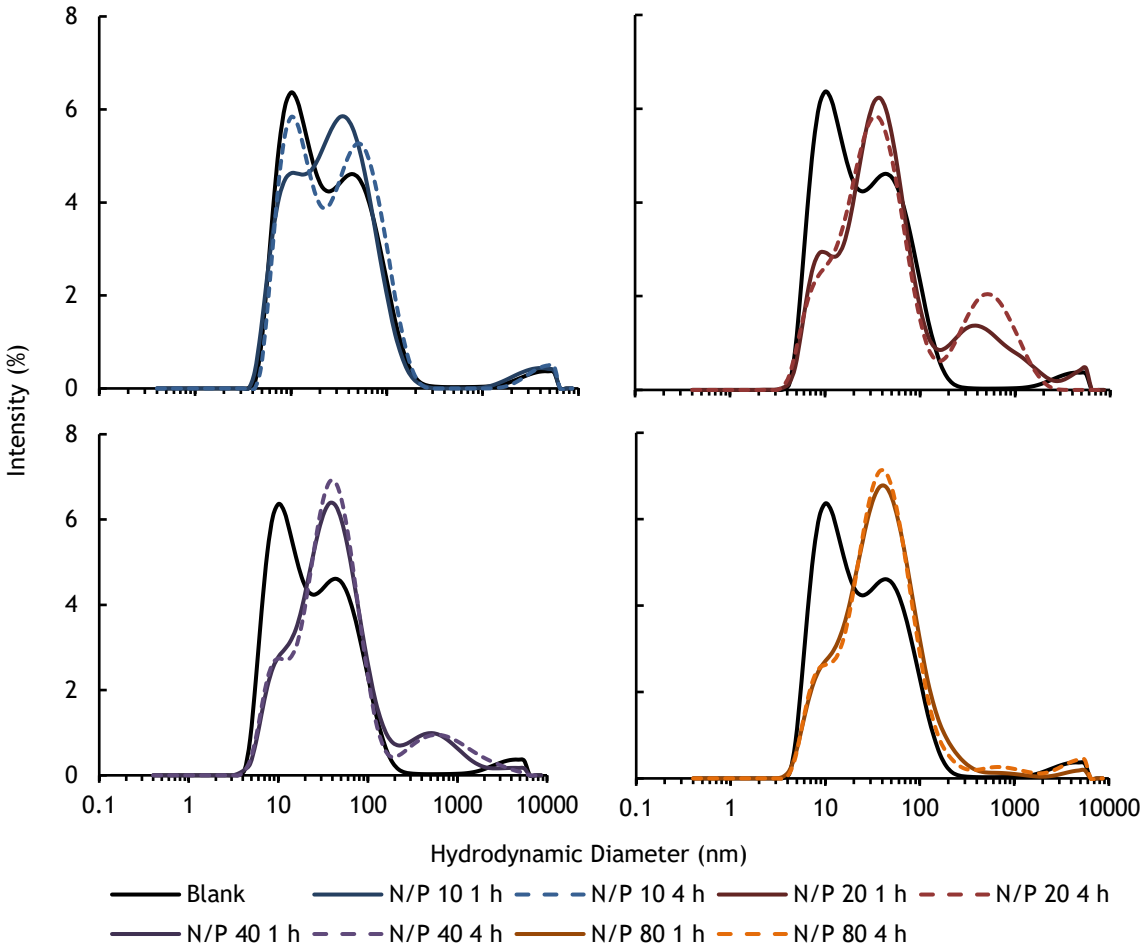

Figure 4.4 Dendriplexes degradation under physiological fluids. Hydrodynamic diameter distribution of the dendriplexes after incubation in 1× PBS + 10% V/V FBS for 1h and 4 h, at N/P ratios from 10 to 80.

itive zeta potential, except those prepared at N/P 5. This slightly negative zeta potential observed at N/P 5 (-1.9 mV ± 0.6 mV) can be attributed to a low amount of fbB, which results poorly stable complexes in a conformation that exposes the uncondensed siRNA molecules to the surface.

Higher N/P ratios result in an increased amount of fbB available for siRNA complexation and, therefore, the zeta potential of the condensates was positive. After complete siRNA complexation, the surface charge of the nanoparticles reached a plateau of +7.4 mV ± 1.2 mV at N/P 20 (no statistically significant differences in N/P ranges from 20 to 80). The neutralisation of the charge dependency on N/P ratio can be attributed to the PEGylation, that masks the charges of the complexes. Moreover, the slightly positive surface charge of dendriplexes resulted from a counterbalance between the cationic dendron and the PEG-chain.

Furthermore, despite statistically insignificant, slight differences between both sequences were observed. In general, the zeta potential of fbB/si-PTEN dendriplexes was slightly superior to that of fbB/si-eGFP dendriplexes. These small differences can be related to different packaging of the NAs inside the dendriplexes due to the differences in the siRNA sequences.

4.2.4. Stability of Dendriplexes in the Presence of Heparin

Thinking of an intravenous administration, the success of a NA delivery system depends on the ability to penetrate the extracellular matrix (ECM) and reach the target cells. During this pathway, NA delivery systems can be destabilised due to unspecific interactions of the cationic vectors with negatively charged ECM components, such as glycosaminoglycans, leading to premature NA release [177]. On the other hand, the transfection efficiency depends on the capacity of dendriplexes to release the siRNA once inside the cell [182]. To study the ability of fbB dendritic copolymers to surpass the ECM, dendriplexes were incubated with heparin, a polyanion commonly used to study the destabilisation and release of NAs from dendriplexes. The released siRNA after 2 h of incubation of dendriplexes (N/P 80) with different heparin concentrations at 37 °C was assessed through PAGE, as showed in Figure 4.5.

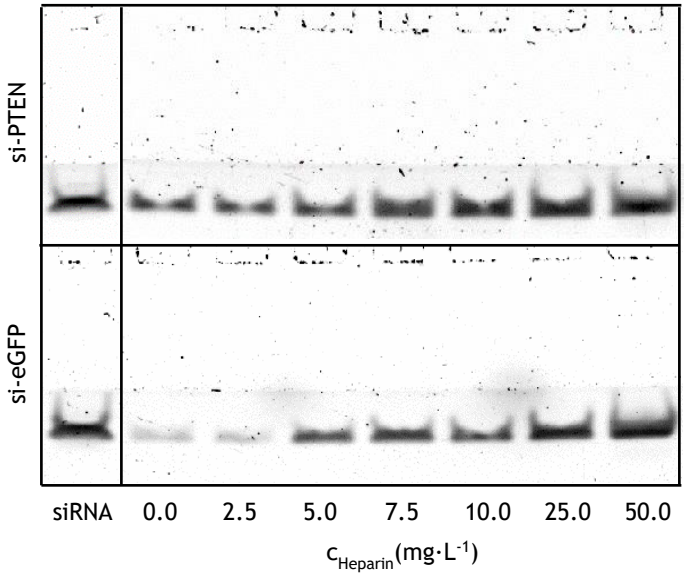

Figure 4.5 Heparin dissociation assay of dendriplexes at N/P80. The siRNA lane had the same amount of free siRNA used for the preparation of the dendriplexes.

As expected, the released siRNA increased as a function of the increased heparin concentration. Moreover, in both cases, the incubation of the dendriplexes at 37 °C for 2 h led to partial siRNA release from the dendriplexes, due to the biodegradability of the vectors (first lane, $c_{Heparin}$ = 0.0 mg·L^{-1}). The complete destabilisation of the complexes occurred at a heparin concentration approximately 3-fold (25 mg·L^{-1}) that of siRNA (8.25 mg·L^{-1}). Considering the obtained results, fbB showed a balance between the stability in the presence of an anionic competitor and the ability to release the NA. A concentration of 7.5 mg·L^{-1} of heparin led to partial destabilisation of dendriplexes in both sequences and was used in further biodegradability studies (section 4.2.5).

4.2.5. Dendriplexes Degradation Studies

Dendriplexes were evaluated regarding their capacity to release siRNA as a function of pH (endosomal/acidic pH and physiological pH, 5.0 and 7.4, respectively) and time (1 h, 24 h and 48 h). The released siRNA and, therefore, the effect of the dendriplexes degradation in the release of siRNA was evaluated through (i) PAGE shift assay and (ii) SYBR Gold® complexation assay. In the PAGE experiments, before the shift assay, dendriplexes were incubated with 7.5

mg·L^{-1} heparin for 2 h (determined through heparin dissociation assay, as previously described in section 4.2.4).

In both assays, there were no significant differences between both sequences, either regarding interaction strength or amount of complexed siRNA. These results are in agreement with the results discussed in section 4.2.1, where similar interaction strength and complexation efficiency was found for both sequences. Conversely, it was found a dependency of both binding strength and amount of complexed siRNA on the pH. As shown in Figure 4.6 (A), for dendriplexes prepared with both sequences, the interaction strength remained stable up to 48 h at acidic pH since the amount of released siRNA was similar at all studied time points. These results agreed with those of the complexation efficiency presented in Figure 4.6 (B). While at pH 5.0, the complexation efficiency remained above 85% after 48 h, at pH 7.4 the complexed siRNA was reduced to 40% during this time. These results suggest that dendrimers degradation leads to instability of the dendriplexes, especially at pH 7.4, thus leading to siRNA release. Moreover, these studies confirm the biodegradable nature of the proposed NA vectors under physiological pH, as well as appropriate stability under endosomal pH.

Considering all stability studies, dendriplexes presented a good compromise between the stability in the presence of serum and under endosomal pH and sustained siRNA release due to their biodegradable character at physiological pH and in the presence of a polymeric anionic competitor.

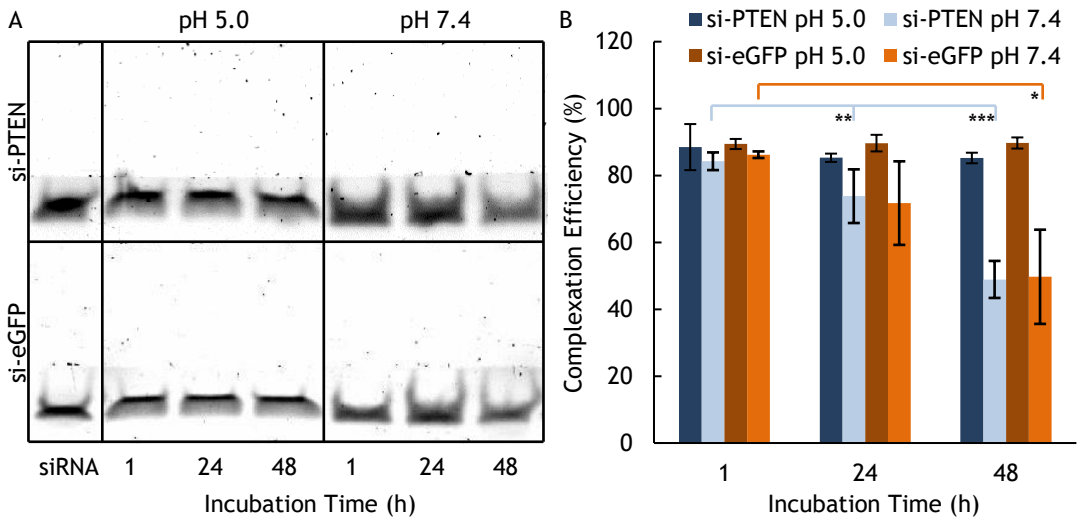

Figure 4.6 Dendriplexes degradation under endosomal pH and physiological pH. A - PAGE of both fbB/si-PTEN and fbB/si-eGFP dendriplexes at N/P 80 after incubation under acidic (pH 5.0) and physiological (pH 7.4) conditions for 1 h, 24 h and 48 h. B - SYBR Gold® exclusion assay of the same dendriplexes (n = 3 ± SD). One-way ANOVA tests were used for statistical analysis. Significant differences: * - $p<0.05$, ** - $p < 0.01$, *** - $p < 0.001$.

Overall, the results of the physicochemical characterisation of the dendriplexes demonstrated that our fbB have an excellent ability to complex siRNA, leading to well-defined dendriplexes with excellent properties for cellular uptake and siRNA delivery.

4.3. Biological Performance of Dendriplexes

The performance of the dendriplexes formed between the fbB copolymers and the siRNAs was further evaluated regarding the ability to protect the NAs from endonuclease degradation

and transfection efficiency. The transfection efficiency was evaluated in both single and dual delivery strategies.

4.3.1. Endonuclease Protection

One major barrier of NA delivery is the rapid degradation by endogenous nucleases both in the extra- and intracellular milieu. As mentioned in section 1.2.3, contrary to endogenous RNAi NAs, exogenous siRNAs are degraded within 5 min to 15 min after intravenous administration. Thus, a suitable siRNA delivery vector should ensure proper protection of the NAs from endonucleases until its biological function is complete.

Dendriplexes were prepared with the two siRNA sequences at N/P 80 and incubated with RNase I for different periods (5 min, 15 min, 30 min and 60 min). The adequate endonuclease concentration was previously determined by incubating the naked NA sequences with different concentrations of RNase I for the same periods. After incubation, the samples were subsequently analysed through PAGE shift assay (nondegraded siRNA is retained as a specific band, while small RNA fragments, resulting from siRNA degradation will migrate throughout the whole gel). The RNase I concentration used in the subsequent experiment was the minimum required to degrade the fully available siRNA within 5 min to 15 min (time in which siRNA is degraded after *in vivo* administration, as discussed in section 1.2.3). While for the sequence si-PTEN a concentration of 6 U of RNase per 0.3 µg of siRNA was enough, in the case of the sequence si-eGFP, the appropriate concentration was found to be 18 U of RNase per 0.3 µg of siRNA, as shown in Figure 4.7. These results show a higher resistance of the sequence si-eGFP to endonuclease degradation, probably due to the higher content of the more stable G-C base pairs compared with the si-PTEN sequence.

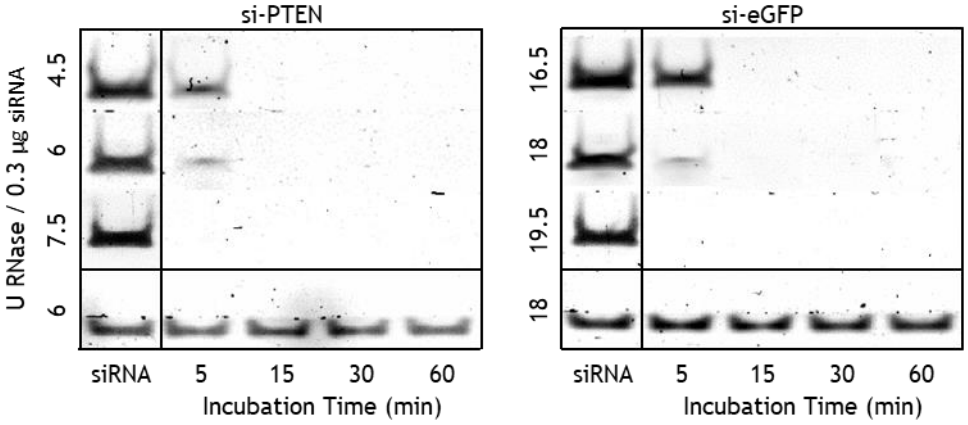

Figure 4.7 Endonuclease protection assay. The concentration of siRNA required to degrade the siRNA was determined by incubating only the siRNA in RNase I solution (3 first rows). The last row shows the ability of the fbB to protect the siRNA from RNase I degradation.

Previous studies suggested that the PEG chains of PEGylated copolymers interfere with the formation of compact condensates, leading to increased dendriplexes size and less efficient endonuclease protection compared to non-PEGylated particles [177]. However, our results regarding size and endonuclease suggest well-compacted structures, capable of efficiently protect NAs from degradation for, at least, 60 min.

4.3.2. Transfection Efficiency

The obtention of proper silencing efficiency values by the delivery of a single siRNA to the target cells is challenging. Recently some authors reported a synergistic effect upon co-delivery of multiple NAs comparing with single NA delivery (section 1.3). In the present dissertation, we proposed a therapeutic approach based on the simultaneous delivery of multiple siRNAs (si-PTEN and si-eGPF) aiming at the downregulation of different target mRNAs, using dendrimers as vectors for the first time, to the best of our knowledge. The present study aims to evaluate the synergistic effect of the co-delivery of two siRNAs to the target cells.

To reach the proposed objective, two different approaches were tested, either (i) preparing both dendriplexes independently and delivering a 1:1 mixture of both dendriplexes (Mix of Ddp), or (ii) mixing both siRNAs (1:1) previously to dendriplexes preparation (Mix of siRNA). It should be noticed that in the first strategy, each dendriplex is supposed to contain a single type of siRNA, while in the second approach, each dendriplex is expected to contain proportions of both siRNA sequences.

The ability of the fbB siRNA dendriplexes to promote an efficient transfection and to mediate a proper gene silencing was evaluated by incubating U2OS/eGFP-Luc cells in the presence of the dendriplexes, prepared at N/P ratios ranging from 5 to 80, for 24 h. Previous cell metabolic studies in our group demonstrated that fbB copolymer is non-cytotoxic at concentrations up to 2 $g \cdot L^{-1}$ in the same cells [193, 194]. In the present studies, the maximum fbB concentration in the medium was 0.52 $g \cdot L^{-1}$, far bellow cytotoxic values.

The two genes targeted herein presented considerable differences. PTEN is a tumour-suppressor endogenous gene, and one of the most commonly mutated and downregulated genes in tumour cell lines [199]. This gene was selected because both efficient siRNAs (leading to considerable silencing in other cell lines) and primers were already developed in our group, with excellent results. The expression of this gene was evaluated by quantifying the amount of mRNA by one-step qRT-PCR. Conversely, eGFP was cloned into U2OS cells and, due to incompatibilities inherent to the method of obtention of the mRNA, its expression cannot be evaluated through the same methodology used to evaluate the expression of the previous gene. Thus, the silencing of eGFP was assessed through FACS, by measuring the median fluorescent intensity, expressed relative to the median fluorescence intensity of untreated cells.

In general, there was a moderate to high downregulation of PTEN in all conditions tested, being higher than the control transfection reagent (Lipofectamine® 2000), as depicted in Figure 4.8. Moreover, the silencing effect increased with increasing N/P ratio. However, considerable differences were observed between lower N/P ratios (5 and 10) and higher N/P ratios (20, 40 and 80). This effect was expected since, at higher N/P ratios, the increased capacity of dendriplexes to complex and protect the siRNA leads to higher amounts of siRNA being internalised and, consequently, higher transfection efficiency. The observed silencing effect demonstrates that the fbB was able to promote the siRNA internalisation allowing the siRNA to enter the RNAi machinery of the cells.

Interestingly, it can be seen that the premise posed in this study, regarding the synergistic effect of the co-delivery of multiple siRNAs was confirmed in the case of PTEN. The dual siRNA delivery strategy led to a gene silencing close to 90%. The PTEN silencing increased in both dual siRNA delivery strategies as compared with the single siRNA delivery approach, being especially notable the synergistic effect at lower N/P ratios (5 and 10). Taking into account that it has been showed that an excess of vectors favours the NA internalisation, giving higher

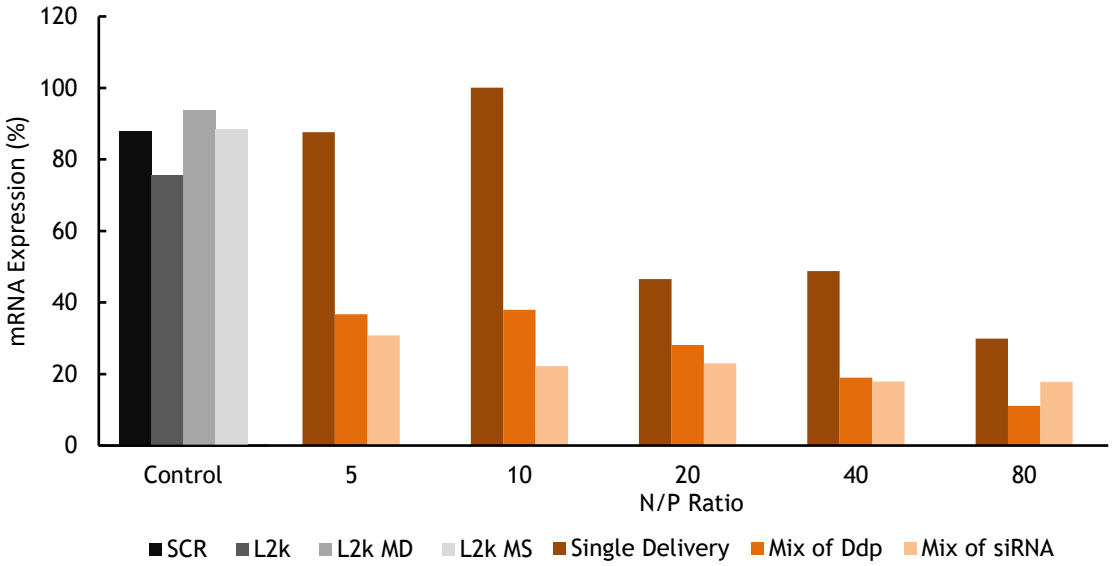

Figure 4.8 PTEN silencing. The silencing efficiency of PTEN in both single and dual siRNA delivery mediated by fbB was assessed through qRT-PCR. The silencing effect was determined based on the expression of the PTEN mRNA relative to non-treated cells (n = 1). SCR – fbB/Scramble dendriplexes (N/P 80), L2k – si-PTEN transfected with Lipofectamine® 2000, L2k MD – transfection of a 1:1 mixture of L2k/si-PTEN and L2k/si-eGFP; L2k MS – transfection of a si-PTEN/si-eGFP 1:1 mixture with Lipofectamine® 2000.

transfection efficiencies, one could conclude that this effect could be due to the 2-fold amount of fbB copolymer deliver at the same N/P ratio [182]. However, the silencing effect reached in dual delivery strategies at N/P 5 was higher than that of the single delivery strategy at N/P 40. The same happened at the corresponding N/P 10 and N/P 80. Thus, similar or higher silencing was observed when 4 times less of fbB copolymer was used in both dual delivery strategies, suggesting the synergistic effect of the "helper" siRNA.

Globally, the silencing effect spectrum of the eGFP gene was considerably different from that of the PTEN gene, as one can see in Figure 4.9. The negative control, consisting of the transfection with a Scramble siRNA, and the positive controls of the single and dual delivery strategies, mediated by Lipofectamine® 2000, resulted in a silencing ranging between 34% and 42%. However, the single siRNA delivery mediated by the commercial transfection agent resulted in the highest gene downregulation observed (62%).

Furthermore, neglectable silencing was observed in cells transfected with the fbB copolymers at low N/P ratios (5 and 10). At higher N/P ratios (20, 40 and 80), however, the reduction in fluorescent intensity was moderate, reaching 45% at N/P 80 in the single delivery strategy. In cells transfected with the single siRNA delivery system, the reduction of the expression of eGFP increased in a N/P ratio-dependent manner. Again, this effect can be explained by the increased dendriplexes stability with increasing N/P ratios, resulting in increased siRNA protection and higher amounts of siRNA delivered to cells.

Conversely to the observations of the silencing of PTEN, the dual siRNA delivery strategies did not result in reduced fluorescent intensity in the case of eGFP, comparing to the single delivery strategy. In fact, in both dual siRNA delivery strategies, the fluorescent intensity consistently ranged between 25% and 30% between N/P ratios 10 and 80 (except in one of the cases at N/P 10). Moreover, the dual delivery formulations from the siRNA mixture resulted in slightly inferior silencing, discording with the results observed with PTEN. These results can be explained by a mechanism in which both siRNAs compete to enter the RNAi machinery, specifically

for binding to the RISC, as theorised by Koller *et al.* [200]. Nevertheless, the absence of a synergistic effect in dual siRNA delivery has also been reported by other research groups [60].

Overall, fbB provided an outstanding capacity to protect NAs from endonuclease degradation and promoted efficient silencing in both single and dual siRNA delivery approaches. Although the synergistic effect was only observed in one siRNA sequence, the obtained results constitute promising preliminary data for further studies. In fact, the present study was the first approach using the GATGE dendritic family in multiple siRNA delivery.

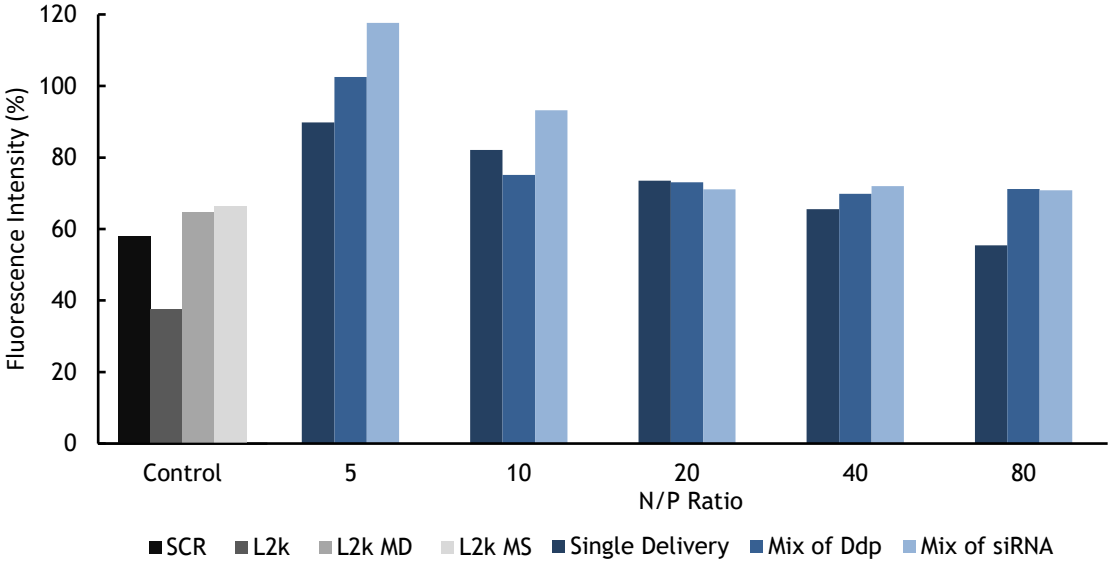

Figure 4.9 eGFP silencing. The silencing efficiency of eGFP in both single and dual siRNA delivery mediated by fbB was assessed through FACS. The silencing effect was determined based on the fluorescence intensity of treated cells relative to non-treated cells (n = 1). SCR – fbB/Scramble dendriplexes (N/P 80), L2k – si-eGFP transfected with Lipofectamine® 2000, L2k MD – transfection of a 1:1 mixture of L2k/si-PTEN and L2k/si-eGFP; L2k MS – transfection of a si-PTEN/si-eGFP 1:1 mixture with Lipofectamine® 2000.

4.4. Future Perspectives

The silencing effect results obtained in the present studies regarding the eGFP silencing are lower than those reported by our group using the same cells, siRNAs and vectors (60% of silencing at all N/P ratios studied, unpublished data). Thus, the next step in this project will be to conduct more experiments in order to increase the robustness of the result herein reported. Moreover, additional transfection replicates will be carried out in order to confirm the silencing results obtained in the case of PTEN.

Furthermore, although the internalisation of fbB-based siRNA dendriplexes has been consistently confirmed in our group for single siRNA delivery, the internalisation mediated by the fbB copolymers in these dual delivery strategies has not been assessed yet. Thus, the internalisation of both siRNAs will be evaluated by flow cytometry and imaging flow cytometry.

The biological *in vitro* characterisation of these (dual) siRNA delivery systems will also be performed in other cell lines, such as a neuronal cell line and primary cortical neurons. Moreover, in the future, *in vivo* evaluation, using an appropriate animal model according to the scope of our group (nervous system neuroprotection and neurodegeneration) will be carried out. This gene therapy strategy directed to the nervous system will require the functionalization of our fbB dendriplexes with a neuronal-specific targeting moiety.

Chapter 5

Concluding Remarks

Gene therapy has emerged as a new therapeutic field that carries the promise to bring hope to the treatment and prevention of several hard to cure diseases, such as cancer, cystic fibrosis or hereditary disorders. Among the gene therapy approaches, gene silencing taking advantage of the naturally occurring RNAi machinery is the most studied approach for short-term gene downregulation, so far. This strategy consists of the delivery of small RNAs, usually a miRNA or a siRNA, to hamper the expression of an upregulated gene. Moreover, in recent years, there has been evidence of the synergistic effect of multiple therapeutic NAs delivery. Despite the potential of this approach, these small therapeutic RNAs present reduced stability in the bloodstream and are promptly recognised by the MPS, or degraded by endonucleases present in extra- and intracellular milieu. Moreover, due to the size and negative charge of miRNAs and siRNAs, they lack the ability to cross the cell membrane and enter the cell.

Therefore, researchers have been joining efforts towards the development of an appropriate vector to efficiently protect and deliver the small therapeutic RNAs to the target cells. NA vectors are divided into viral and non-viral vectors. The former class usually leads to high transfection efficiency but lacks safety validation. Thus, researchers have been focusing on the development of non-viral vectors that present a better safety profile but currently lack efficient transfection. Among them, cationic dendritic structures arouse enormous interest due to their unique structural characteristics together with their ability to efficiently complex and protect NAs in compact and tuneable dendriplexes. Moreover, the presence of multiple functional groups allows further functionalization with different ligands. However, the use of these positively charged molecules as NA vectors still faces several difficulties regarding cytotoxicity. The positive surface charge density required to complex the NAs can lead to cell membrane destabilisation and cell death. Also, the lack of degradation of the vast majority of the structures reported so far can lead to bioaccumulation of these synthetic materials. Hence, it urges the development of dendritic structures that degrade into small fragments under physiological conditions, which can be easily excreted from the organism. However, the desired biodegradability makes these structures susceptible to undesirable degradation during their development or even to a premature degradation during their application. This additional challenge reflects on the limited number of studies reporting biodegradable dendrimers for specific applications in nanomedicine, especially for gene therapy applications.

Due to the gap regarding the description of biodegradable dendritic structures, our group proposed a new family of fully biodegradable (fb) copolymers, based on the repeating unit GATGE, to serve as vectors in nanomedicine. This dissertation explored the new fbB as vectors of different siRNAs (si-PTEN and si-eGFP). The corresponding fbB/siRNA dendriplexes were studied at different N/P ratios (ranging between 5 and 80), not only in the context of single siRNA delivery but also dual siRNA delivery. Furthermore, two different dual siRNA delivery approaches were tested, either (i) using a mixture of two types of dendriplexes previously and independently formed with the different siRNAs, or (ii) using dendriplexes formed from a mixture of both siRNAs.

The fbB copolymer showed an excellent ability to bind and complex siRNA. The increase in the N/P ratio resulted in increased siRNA binding strength and increased siRNA complexation efficiency (up to 93% of complexed siRNA). Moreover, the size, surface charge and morphology of the resulting dendritic nanoparticles were exceptionally ideal for cellular uptake. The fbB copolymers successfully condensed the siRNAs forming globular dendriplexes, presenting a narrow hydrodynamic diameter distribution with a mean of 39 nm. The nanosystems complexing both siRNA presented similar sizes and complexation efficiencies. The surface charge density of all developed dendriplexes increased with increasing N/P ratio reaching a plateau of +7.4 mV ± 1.2 mV at N/P ratios above 20. This plateau was attributed to charge masking by the presence of the PEG chain.

Furthermore, dendriplexes demonstrated a suitable compromise between the stability in the presence of serum (after 4 h of incubation) and sustained siRNA release in the presence of an anionic polymeric competitor (after 2 h of incubation). Also, these dendritic nanoparticles revealed suitable stability under endosomal pH during the first hours (up to 48 h), and led to continuous siRNA release under physiological pH due to biodegradable character of the fbB copolymer (after 48 h of incubation). The dendriplexes also showed an excellent siRNA protection against endonucleases degradation and led to excellent silencing of PTEN and moderate downregulation of eGFP. Interestingly, the premise of this work, *i.e.*, the possibility to observe a synergistic effect on the co-delivery of two siRNA, was only observed in the case of PTEN. Conversely, the synergistic effect was not observed in the case of eGFP silencing mediated by dual siRNA delivery.

Concluding, both the physicochemical properties of the fbB-based siRNA dendriplexes as well as their biological assessment demonstrated the high potential of PEG-GATGE dendritic block copolymers as both single and multiple siRNA delivery vectors. Therefore, the new family of fb PEG-dendritic block copolymers developed by our group is of great interest not only in the engineering of NA delivery systems but also in the development of drug delivery systems in general.

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