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Exploring the potential of fully Biodegradable PEG-Dendrimers as Nanocarriers of Different siRNA Sequences for Dual Delivery

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Abstract

The Human genetic code encodes information for every biological process needed for life and death. Given the undeniable importance of this information, it was inevitable for investigators to turn to it in search of novel therapies; hence, arises genetic therapy. Amid the gene therapy approaches, RNA interference (RNAi) machinery is the most investigated strategy for short-term gene downregulation. Different nucleic acids (NAs) can integrate the RNAi machinery leading to gene silencing, namely, small interfering RNAs (siRNAs). Even though siRNA-based RNAi is a powerful therapeutic tool, it bears some challenges, especially when it comes to transport and delivery. Accordingly, there is a high demand for delivery vectors that can safely and efficiently deliver NAs. These NA vectors are divided into viral and non-viral vectors. While viral vectors allow high transfection efficiency, researchers have been concentrating their efforts on the development of non-viral vectors because they offer a more favourable safety profile.

Cationic dendrimers are up-and-coming candidates as non-viral vectors for NAs delivery as a result of their unique structural features and their ability to efficiently compact NAs into electrostatic complexes called “dendriplexes”. However, the use of the most common dendrimers as NA vectors still face some problems related to bioaccumulation and subsequent cytotoxicity, which is primarily caused by their nonbiodegradability.

Due to the unmet need for an effective, safe and biodegradable NA delivery vector for gene therapy applications, recently our nBTT group synthesized a new family of fully biodegradable poly(ethylene glycol) (PEG)-Gallic Acid Triethylene Glycol Ester (GATGE) dendrimers and explored their function as NA vectors. For that, they were functionalized with different amine moieties displaying different chemical properties: a single amine (fbAmin), a diamine (fbD) or a benzylamine (fbB). For now, fbB and fbD have been successfully explored as efficient vectors for single siRNA delivery of an anti-eGFP (enhanced Green Fluorescent Protein) siRNA, obtaining excellent transfection efficiencies in several cell lines.

However, very recently, in our group, it has been observed that using the same dendrimer as a vector, different transfection efficiencies were obtained when different siRNA sequences were carried. Taking this into account, it seems that the same dendrimer may complex and interact differently with distinct siRNA sequences. So, in the present dissertation, the new fully biodegradable dendritic vectors were explored as vectors of different siRNA sequences for dual delivery.

Hereby, the first main aim was to evaluate the possible different interactions in each case and the ability of each dendrimer, presenting different surface functional groups, as vehicles for the different siRNAs. Thus, the dendriplexes between the three developed dendrimers (fbAmin, fbD and fbB) and four different siRNA sequences (anti-PTEN (Phosphatase and Tensin Homolog Protein) siRNA, anti-eGFP siRNA, A and B) were prepared and biophysicochemically characterized. The three amine-terminated dendrimers proved to complex siRNA efficiently, with fbB and fbD exhibiting excellent binding profiles. Moreover, besides the observed differences in the performance between dendrimers with different peripheral modifications, it has been possible to observe some variations in the complexation of the same dendrimer with each of the four different siRNA sequences. All dendriplexes presented very small sizes (on the nanometre scale — <100 nm), with those fbB-based presenting exceptionally small nanosizes (on average 41.8 ± 7.6 nm) with very homogenous populations (average PDI of 0.26±0.07). The determinant factor for the dendriplexes size and polydispersity was mainly the dendrimer, namely its surface functional group. All developed dendrimers also showed to be capable of efficiently protecting the carried siRNA sequences against endonucleases degradation, with fbB and fbD ensuring an excellent level of protection at different time points.

Furthermore, and inspired by some of the recent therapeutic findings concerning the co-delivery of multiple siRNAs, the second main objective of the present work was to evaluate the biological performance of the developed dendrimers (namely, fbD and fbB) as carriers for single and dual delivery of different siRNA sequences (anti-PTEN and anti-eGFP siRNAs). Both dendrimers showed an excellent ability to associate/internalize both siRNAs and led to moderate downregulation of eGFP; however, an excellent silencing of PTEN was obtained using fbB as the carrier. Regarding the co-delivery of two siRNAs and the possibility of obtaining a synergistic effect, it was not possible to observe a clear synergistic effect for now.

The physicochemical properties of the fbAmin-, fbD- and fbB-based siRNA dendriplexes together with their biological performance, demonstrate the great potential of PEG-GATGE dendritic block copolymers to act as single and multiple siRNA vectors.

Resumo

O código genético humano contém as informações para todos os processos biológicos necessários para a vida e a morte. Inevitavelmente a investigação recorreu a esta informação em busca de novas estratégias terapêuticas. É esta a origem da terapia genética. Entre as diferentes 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. Diferentes ácidos nucleicos (ANs) conseguem integrar a maquinaria de ARNi induzindo o silenciamento de genes, nomeadamente, pequenos ARNs interferentes (siRNA, do inglês *small interfering RNA*). Embora o ARNi baseado em siRNA seja uma ferramenta terapêutica poderosa, esta apresenta vários desafios, especialmente no transporte e entrega. Consequentemente, há uma grande procura por transportadores, isto é, vetores de entrega que entregar AN de forma segura e eficaz. Estes vetores de ANs são divididos em vetores virais e não virais. Embora os vetores virais permitam elevadas eficiências de transfecção, os esforços da comunidade científica têm-se focado no desenvolvimento de vetores não virais porque estes oferecem um perfil de segurança mais favorável.

Dentro dos vetores não virais, os dendrímeros catiónicos são candidatos promissores para entrega de AN devido às suas características estruturais únicas e sua capacidade de compactar eficientemente os AN em complexos eletrostáticos denominados “dendriplexos”. No entanto, a aplicação das estruturas dendríticas mais comuns como vetores de NA, ainda enfrenta alguns desafios relacionados com a bioacumulação e a consequente citotoxicidade, causada essencialmente pela não degrabilidade destes materiais sintéticos.

Devido a esta necessidade de um vetor de entrega de NA que seja eficaz, seguro e biodegradável, o nosso grupo nBTT sintetizou recentemente uma nova família de copolímeros de bloco polietilenoglicol (PEG)- ácido trietilenoglicol gálico ester (GATGE) dendríticos totalmente biodegradáveis cuja utilização é direcionada para vetores de entrega de ANs. Para isso, foram funcionalizados com diferentes grupos amina apresentando diferentes propriedades químicas: uma única amina (fbAmin), uma diamina (fbD) ou uma benzilamina (fbB). De momento, fbD e fbB foram explorados com sucesso como vetores eficientes para a entrega de um siRNA contra a Proteína verde fluorescente melhorada (eGFP, do inglês *enhanced Green Fluorescent Protein*), obtendo excelente eficácia de transfecção em várias linhas celulares.

No entanto, muito recentemente, no nosso grupo, foi observado que usando o mesmo dendrímero como vetor, foram obtidas diferentes eficácias de transfecção quando diferentes sequências de siRNA foram entregues. Estas observações apontam para a possibilidade do mesmo dendrímero complexar e interagir de forma diferente com sequências de siRNA distintas. Assim, na presente dissertação, os novos vetores dendríticos totalmente biodegradáveis foram explorados como vetores de entrega de diferentes siRNAs para a entrega simultânea de dois siRNAs.

Deste modo, o primeiro objetivo principal foi avaliar as possíveis diferentes interações em cada caso (cada dendrímero com cada uma das sequências de siRNA) e a capacidade de cada dendrímero, com diferentes grupos funcionais de superfície, de atuar como vetor de entrega para os diferentes siRNAs. Consequentemente, os dendriplexos preparados entre os três dendrímeros desenvolvidos (fbAmin, fbD e fbB) e as quatro diferentes sequências de siRNA (siRNA contra PTEN (do inglês, *Phosphatase and Tensin Homolog Protein*), siRNA contra eGFP, sequência A e sequência B) foram caracterizados biofísicoquimicamente. Os três dendrímeros mostraram complexar com siRNA de forma eficaz, com fbB e fbD, exibindo excelentes perfis de complexação. Adicionalmente, além das diferenças observadas no desempenho entre dendrímeros com diferentes modificações periféricas, foi possível observar algumas variações na complexação do mesmo dendrímero com cada uma das quatro diferentes sequências de siRNA. Todos os dendriplexos apresentaram tamanhos muito pequenos (na escala nanométrica — <100 nm), sendo que os dendriplexos de fbB apresentaram tamanhos excecionalmente pequenos (em média 41,8 ± 7,6 nm) com populações muito homogéneas (PDI médio de 0,26 ± 0,07). O fator determinante para o tamanho e polidispersidade dos dendriplexos foi principalmente o dendrímero, nomeadamente, o seu grupo funcional de superfície. Todos os dendrímeros desenvolvidos mostraram também ser capazes de proteger com eficácia as sequências de siRNA transportadas contra a degradação por endonucleases, com fbB e fbD garantindo um excelente nível de proteção.

Adicionalmente, inspirado por algumas das recentes descobertas terapêuticas relativas à co-entrega de múltiplos siRNAs, o segundo objetivo principal do presente trabalho, foi avaliar o desempenho biológico dos dendrímeros desenvolvidos (concretamente fbD e fbB) como vetores de entrega de um e dois siRNAs para diferentes sequências de siRNA (siRNAs contra PTEN e contra eGFP). Ambos os dendrímeros mostraram uma excelente capacidade de associar/internalizar ambos os siRNAs e levaram a um silenciamento moderado de eGFP. No entanto, no caso do PTEN foi obtido um excelente silenciamento usando dendriplexos baseados em fbB como vetores de entrega. Em relação à co-entrega de dois siRNAs e à possibilidade de obtenção de um efeito sinérgico, por agora, não foi possível observar um efeito sinérgico claro.

As propriedades físico-químicas dos dendriplexos de siRNA baseados em fbAmin-, fbD- e fbB, juntamente com seu desempenho biológico, demonstram o grande potencial destes nanossistemas dendríticos para atuarem como vetores em aplicações de terapia genética, para a entrega de um siRNA ou de múltiplos siRNAs.

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Contents

List of Figures

List of Tables

List of Acronyms

AGO2	Argonaute 2
Bis-HMPA	2,2-bis(hydroxymethyl)propionic acid
CuAAC	Copper(I)-Catalysed Huisgen 1,3-Dipolar Cycloaddition
DC-Chol	3b[N-(N′,N′-dimethylaminoethane)-carbamoyl) cholesterol
DDAB	dimethyldioctadecyl-ammonium bromide
DLS	Dynamic Light Scattering
DMEM	Dulbecco’s Modified Eagle’s Medium
DMRIE	dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide
DNA	Deoxyribonucleic Acid
DOGS	dioctadecylamidoglycylspermine
DOPE	dioleoylphosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium
DOTAP	1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane
DOTMA	N-[1-(2, 3-dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride
dsRBP	dsRNA-binding protein
dsRNA	double-stranded RNA
EC	European Commission
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
GATGE	Gallic Acid Triethylene Glycol Ester
HEPES	2-[4-(2-Hydroxyethyl) Piperazin-1-yl] Ethanesulfonic Acid
miRNA	microRNA
MPS	mononuclear phagocyte system
mRNA	messenger RNA
NA	Nucleic Acid
NF	Nuclease-Free
NLCs	nanostructured lipid carriers
NPs	Nanoparticles
P/S	Penicillin-Streptomycin
PAGE	Polyacrylamide Gel Electrophoresis
PAMAM	poly(amidoamine)
PB	Phosphate-Buffer

PBS	Phosphate-Buffered Saline
PDI	Polydispersity index
pDNA	Plasmid DNA
PEG	polyethyleneglycol
PEI	Polyethyleneimine
PLGA	poly(D/L-lactic-co-glycolic acid
PLL	poly(L-lysine)
PLLA	poly(D/L-lactic acid
PMA	poly(methacrylate)
PPI	polypropylenimine
PTEN	Phosphatase and Tensin Homolog Protein
PTFE	Polytetrafluorethylene
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
RES	reticuloendothelial system
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RNase	Ribonuclease
RT	Room Temperature
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLNs	solid lipid nanoparticles
TBE	Tris/Borate/EDTA
TRBP	TAR RNA-binding protein
U2OS	Human bone osteosarcoma cell line
UV-Vis	ultraviolet-visible

Chapter 1

Introduction

1.1 Gene therapy

The Human genetic code, or the entirety of our DNA (Deoxyribonucleic Acid), can be compared to a biological book, written in genetic letters, the bases, which come together to form words, the codons, that in turn, create an enormous variety of sentences, our genes. In fact, our genome comprises thousands of genes which encode for the synthesis of proteins, colloquially named as the building blocks of the cell, on account of being responsible for every biological process needed for life and death [1], [2].

Given the undeniable importance of the information encoded in the genetic code, it was inevitable for investigators to turn to it in search of novel therapies. Hence, arises genetic therapy which proposes the rewriting the abovementioned book in order to prevent and treat several conditions. Having said that, gene therapy can be described as the delivery of therapeutic genetic material to defective cells or tissues with the purpose of treating diseases and disorders [3]. Even though the concept of gene therapy has existed for roughly 80 years, it's birth is commonly associated with the first heritable gene transfer conducted by Professor William Szybalski's in 1962, in which a genetic defect was corrected by delivering foreign DNA into mammalian cells [4].

The translation of gene therapy concepts to clinical testing started in the early 1990s, but its path was tortuous as it was characterised by a recurrent pattern of a period of great promise followed by discouraging clinical trial results. Early clinical studies were deemed to provide no clinical benefit or produce unanticipated severe adverse effects, that, in the most tragic instances led to widely publicised patient deaths. One of the most divulged cases was the death of an 18-year-old patient in 1999, as a result of an adverse reaction to an adenovirus vector, during a clinical safety trial for the treatment of a rare genetic disorder, ornithine transcarbamylase deficiency [5], [6]. This sense of danger, in conjunction, with development reaching a bottleneck stage, drove investigation back to basic research underlying gene therapy approaches, especially to the development of new vectors with the goal of improving their safety and efficiency. The knowledge gained from this step back set in motion a second round of clinical trials at the beginning of the 21st century. These clinical studies were able to provide confirmation of sustained genetic modification of target tissues and, in certain cases, proof of clinical benefit. Unfortunately, novel toxicity arose from the accomplished higher gene transfer efficiency, namely insertional genotoxicity, immune destruction of genetically modified cells and, also, immune reactions correlated to certain vectors, creating yet new challenges [7].

Even though the journey from concept to clinical application has had its highs and lows and has definitely been convoluted, at last, genetic therapies are gaining momentum. In the last decade, it was possible to observe considerable clinical progress mainly due to additional maturation of the fundamental science of gene therapy, safety modifications, and advancements in both the efficiency and delivery of gene transfer. Auspicious early successes in clinical studies accumulate and examples of regulatory approval for licensing are increasingly arising [3], [7].

As mentioned above, the progression of this field goes hand in hand with the advancements made in gene delivery technology. Undoubtedly, the optimisation of gene delivery tools plays a major role in bringing gene therapy from the bench to the bedside. Currently, the developed delivery tools can be divided into two main categories: engineered viruses and non-viral vectors. Both types of gene transfer tools have specific pros and cons. While, on one hand, viral vectors possess superior transfection efficiency and can provide long-term and stable expression, on the other hand, non-viral vectors have low immunogenicity, finer targeting capacity and ability to be manufactured at a large scale, for example [8], [9]. Later on, the present dissertation will delve further into this subject (section 1.4).

It is worth mentioning that gene therapy is now bringing cautious optimism for the creation of new treatment options for multiple fields of medicine. The progress in this field has, not only, inspired a great deal of hope for the treatment of a broad range of previously intractable genetic disorders, but also, regarding the treatment of complex acquired diseases. In fact, there are few serious disease entities for which gene therapy-based treatments are not being developed [3], [7].

Altogether, current achievements in this field are likely only a glimpse of the progress to come in the foreseeable future. Indeed, gene therapy has tremendous potential to shed light on human illnesses and make a great difference in patients' lives.

This expanding field comprises several different approaches, particularly: gene augmentation, gene editing and gene silencing. Gene augmentation is the most traditional form of gene therapy, in which a specific gene function is restored by the delivery of a functional copy of the corresponding gene with the goal of achieving long term expression at levels high enough to be therapeutic [3]. Conversely, the most novel type of gene therapy, genome editing can be defined as the modification of the genome through the insertion, deletion, or replacement of one or more segments of DNA [10]. Lastly, gene silencing, contrary to gene augmentation, aims to reduce the expression of a detrimental gene responsible for a disease or disorder development. Currently, one of the most promising technologies for gene silencing, due to its high level of specificity and versatility, is RNA interference (RNAi).

1.1.1 RNA Interference

RNAi is considered to be one of the major breakthroughs in the field of molecular medicine and, accordingly, is thought to have enormous potential for the treatment of numerous pathologic conditions [11], [12]. This natural post-transcriptional process is characterised by a double-stranded RNA (dsRNA)-induced regulatory pathway that culminates in the highly specific degradation of homologous messenger RNA (mRNA), inhibiting its translation [13], [14]. In this way, these small noncoding RNAs control the expression of genetic information which, in turn, results in the regulation of several significant mechanisms such as cell growth, cell proliferation and tissue differentiation. Indeed, this evolutionarily conserved phenomenon is responsible for controlling essential cellular mechanisms as it, not only, regulates gene expression but is also responsible for resisting the invasion of viral nucleic acids (NAs) [13], [15].

Notwithstanding the fact that the first hints about this gene regulation pathway were seen several decades ago, the crucial turning point came in 1998 with the pivotal studies by Andrew Fire and Craig Mello [16]. Their research, conducted in *Caenorhabditis elegans*, reported an unforeseen gene silencing event in which the trigger was unambiguously confirmed to be dsRNA. Unsurprisingly, the mentioned work earned them the 2006 Nobel Prize in Physiology or Medicine [17]–[19]. This milestone paper added a new dimension to our understanding of life, uncovering the new world of RNAi and heralding a revolution in RNA regulation. Shortly after, in 2001, further research verified that a similar response could also be observed in mammalian cells. This paved the way to the possibility that RNAi could provide new tools for medicine [12], [14], [20]. Thenceforth, RNAi became one of the most valuable and broadly used resources to study gene function [14]. Simultaneously, a lot of effort was put to prompt the steep development in the RNAi field in order to lead this technology from the experimental setting towards successful clinical application. Yet, unfortunately, the first wave of these endeavours did not succeed [12], [21]. In 2012, mainly due to advances in delivery systems and sequence selection methods, a resurgence in clinical trials using RNAi occurred. The continued positive outcomes obtained from these clinical studies encouraged further efforts to develop pertinent RNAi based treatments for a very wide range of conditions [21], [22]. At last, in 2018, the pioneer RNAi drug was approved by both the US Food and Drug Administration (FDA) and the European Commission (EC) and is now commercially available for the treatment of hereditary transthyretin amyloidosis under the name Onpattro [23]. Undoubtedly, this market authorisation was a major landmark event in the field of RNAi. Since then, in 2019, a second RNAi based treatment found its way into the market: Givosiran for the treatment of acute hepatic porphyria, a rare and life-threatening genetic condition [24]. Hopefully, these first approvals will pave the way for other RNAi based therapeutics.

Indeed, researchers have increasingly looked towards RNAi as a novel method for the treatment of a plethora of medical conditions as this technique enables the modulation of targets, undruggable by current approaches such as antibodies, small molecular drugs or recombinant proteins [12], [25]. That is, several molecules associated with diseases, mainly proteins, are deemed as undruggable targets since they do not have an enzymatic function or their conformation is of difficult access for conventional drugs, for example [14]. RNAi circumvents these issues by acting directly on the mRNA, inhibiting its translation. Moreover, amid the continuous shortcomings of current drug technologies, RNAi represents an intriguing alternative, since it exhibits a high level of target specificity, triggers low immunogenicity and it is easy to manufacture and design (which reflects into short preclinical development times) [11], [12], [21], [25]. The latter point is of special importance to the therapeutics for infectious diseases, which usually quickly become obsolete due to mutational escape [22].

RNAi mediated gene silencing can be induced by different types of small RNA molecules, as a matter of fact, a growing number of small RNAs have been discovered and implicated in gene regulation and RNA processing since the discovery of RNAi. Some of the main examples are small interfering RNA (siRNA), microRNA (miRNA) and short hairpin RNA (shRNA). Whilst these small RNA molecules can be utilised to reach equivalent outcomes, they are intrinsically distinct, and their essential difference lies in their mechanism of action. For example, one of the main differences between siRNA and miRNA rests in the fact that while siRNA requires perfect complementarity with its target mRNA, miRNA binds to its target sequence imperfectly; thus one single miRNA may target a number of different transcripts [14], [26]. One the other hand, if one was to compare shRNA and siRNA one would find that as opposed to siRNA which can cleave its target RNA both in the cytoplasm and in the nucleus, shRNAs can only act in the nucleus, as it must interact with chromosomal DNA to work, which creates additional obstacles to its delivery [12].

Within the different techniques explored to down-regulate protein expression, siRNA is one of the most promising [27]. Not only, was siRNA the first RNAi technology to be introduced into mammalian cells with the goal of accomplishing gene silencing, the first RNAi therapy to be approved by both FDA and EC, Onpattro as stated above, was also siRNA based [23], [28]. siRNA presents numerous engaging characteristics, which herald this technology as a major player in the future of clinical therapies. First of all, siRNA is an extremely efficient and powerful tool, as it can cause efficient and sustained gene down-regulation at low concentrations; it is estimated that only a few hundred cytosolic siRNAs per cell are required [11], [26]. Additionally, if the nucleotide sequence of the target gene is known, its respective siRNA can be easily designed, which allows an unrestricted choice of targets [21], [26]. Indeed, siRNAs are now commercially available for virtually any gene for a multitude of organisms [27]. Furthermore, it has high specificity that follows from its complete complementary base pairing with its target sequence [12], [26]. Lastly, these small RNAs have a high degree of safety. While siRNA is categorised as a nucleic acid drug, it acts on the post-translational stage. Accordingly, it can modulate gene expression without wounding genomic DNA, avoiding mutation and teratogenic risks, common concerns when it comes to gene therapy [21], [26]. It is worth mentioning that siRNA's silencing effects are transient. While there are situations where a temporary down-regulation effect is desired, in cases which require a lasting effect, regular administrations are needed [27].

RNAi mediated by siRNA is a natural process that begins with the cytoplasmic identification and subsequent cleavage of endogenous long dsRNAs by an endonuclease called Dicer. (Figure 1) The resulting cleaved products, small double-stranded fragments of around 21 nucleotides generally bearing a 2-nucleotide overhang on the 3′ end of each strand, are recognised as siRNAs [26]. When formed, siRNAs are loaded by Dicer, with the aid of dsRNA-binding protein (dsRBP) such as TAR RNA-binding protein (TRBP), onto Argonaute 2 (AGO2) [15], [29]. These three proteins (Dicer, TRBP and AGO2) form a minimal RISC-loading complex which, as mentioned earlier, is in charge of creating diced dsRNA and, also, bonding it to AGO2 [15]. The referred, RNA-induced silencing complex (RISC) is a multiprotein complex which is the fundamental intermediate for the translational inhibition of target mRNA [12]. Once siRNA is presented to AGO2, it is unwound into single-stranded RNAs, and, while the guide strand (antisense) is bound to Argonaute to direct silencing, the passenger (sense) strand is cleaved and discarded [12], [15], [21], [29]. The siRNA incorporation into the RISC complex is coincident with the strand selection step and, after this step, the RISC is active and ready to catalyse the guide strand to bind with the target mRNA [12], [15]. The RISC performs cellular surveillance and whenever the Argonaute-bound guide strand pairs in a sequence-specific manner with its target mRNA sequence, it triggers site-specific mRNA cleavage, by AGO2 (the heart of the RISC complex)[15], [17], [21], [29]. The binding to the target sequence is initialised by the seed sequence, that is, the guide strand's 2–6 nucleotides [15]. As a result, the process of mRNA translation is interrupted, which culminates in reduced expression of the target gene. After the cleaved mRNA is released, the activated guide strand RNA-containing RISC binds to another mRNA and starts a new cycle of cleavage [14], [30].

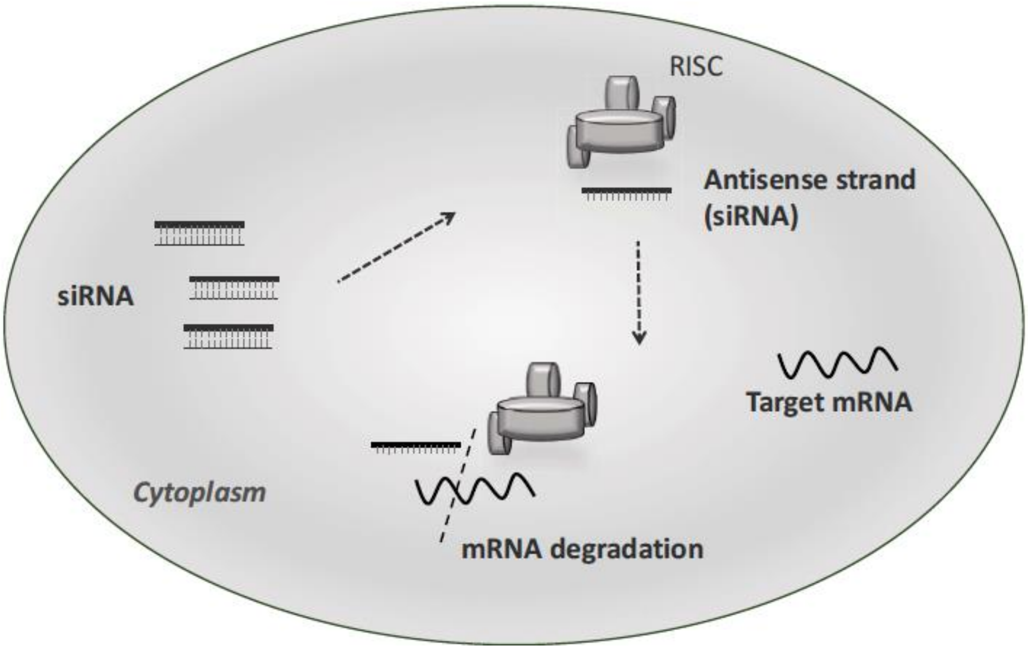


Figure 1 – Representation of the RNAi mechanism, showing the siRNA patway. From Leiro, Santos, & Pego, 2017 [27].

Therapeutically, the very same mechanism can be induced by introducing exogenous synthetic siRNAs into the cells. In this case, the siRNA will directly incorporate into RISC, where its guide-strand will bind to and cleave the complementary mRNA, accomplishing the desired specific gene silencing [11], [30]. Even though siRNAs may have differing origins, endogenous or arise from a viral infection or other exogenous sources, their pathways converge once siRNA assembles into the RISC [15].

As was mentioned above, it is possible to induce the siRNA native gene silencing pathway through the delivery of synthetic siRNA to the cytoplasm of the target cells. With this strategy, effective gene silencing with a transient effect can be induced in a simple way. This approach has numerous benefits, such as the relatively easy synthesis of small RNA molecules, the reduced probability of non-specific side effects; and, contrary to DNA-based strategies, siRNA only needs to reach the cytoplasm of the cell and is safer (it cannot integrate the genome) [31]. However, the limitations of this strategy must be kept in mind when developing siRNA-based therapies.

1.2 Main Barriers to the Therapeutic Delivery of siRNA

As mentioned in the previous section, even though siRNA-based RNAi is a powerful therapeutic tool, it bears some challenges, especially when it comes to transport and delivery. Accordingly, it is now commonly accepted that the widespread use of siRNA therapeutics will demand the use of clinically suitable, safe and effective delivery vehicles with the capability to complex and protect exposed siRNA [27].

The first point to consider when developing a delivery vector for siRNA is whether systemic or local administration is needed. This decision depends on numerous aspects: a) the accessibility of the target site, b) the least invasive technique that is still effective in delivering therapeutic amounts of the chosen agent, c) the volumes and frequency of administration, d) possible side effects, e) stability of the carrier after delivery, f) clearance through the body of the vector and g) toxicity of the delivery vehicle [27]. The benefits of local administration are, first, that it is only required a simple formulation, which results in an easier production and administration, and secondly, lower doses are needed. Notwithstanding, this route is restricted to sufficiently accessible tissues. Concurrently, the main benefit of systemic administration is prompt action and biodistribution, but also the broad range of application [31]. Hence, this section is going to focus on the most traditional route: systemic administration by intravenous (i.v.) injection.

The greatest challenge in the use of siRNA-based therapies by systemic administration is the complexity of siRNA delivery. These hurdles are described in the following section.

Stability. One significant pitfall of siRNA is related to its in vivo stability. These molecules have unfavourable physicochemical properties, when it comes to drug delivery, as they are negatively charged, large molecules with high molecular weight. The unmodified naked siRNA has a short half-life (usually >5 minutes but can up to 20 minutes) as endonucleases promptly degrade it [32]–[34]. These nucleases degrade NAs by breaking their phosphodiester backbone [35]. Hence, it can be challenging to deliver intact siRNA with preserved therapeutic activity through the circulation. Especially since siRNAs must not only survive in the serum but should also reach their target cells in the specific tissues for internalisation to occur and, consequently, for required RNAi therapeutic effect to occur [36].

Physiological Barriers. After siRNA therapeutics is administered and introduced into the systemic circulation, its course towards the desired site of action is hampered by several structural and anatomical defensive barriers. In the bloodstream, highly positively charged particles, lead to unspecific serum and blood protein adsorption, which results in aggregation or the formation of a protein corona and further clearance.

Additionally, the reticuloendothelial system (RES), also known as the mononuclear phagocyte system (MPS), poses a significant threat to the journey and integrity of genetic material. Usually, after systemic administration, clearance of gene material from the circulation occurs rapidly via opsonisation and phagocytosis. Initial opsonisation marks undesirable material in circulation for phagocytosis, then macrophages engulf and either destroy or remove it from circulation. This phagocytosis and destruction of foreign material are conducted by macrophages (Kupffer cells) in the MPS organs (lungs, spleen, and liver) [36]-[38]. Numerous factors, namely formulation size and surface electrostatic nature, may influence RES uptake and biodistribution. A formulation with a large size is undesirable considering that large particles are more susceptible to clearance through the kidney and RES (ideally to circumvent renal and RES clearance the therapeutic formulation should have a diameter ranging from 20 to 100 nm approximately) [35], [36], [38]. Moreover, excessive net charge also elicits phagocytosis because electrostatic forces tend to cause aggregation [38].

In the case of nonbiodegradable compounds, the focus should be on factors such as size and molecular weight. The reason for this is the fact that different MPS organs sequester materials bigger than the renal threshold (usually >200 nm in size), leading to accumulation and toxicity [37], [39]. Accordingly, some of the most relevant criteria to bear in mind when designing a NA delivery vector design for systemic administration should include molecular weight and biodegradability of the vector, and the size, surface charge, and stability and of the nanosystem.

Cellular Internalisation and Endosomal Escape. After overcoming these obstacles, the siRNA enters the (targeted) tissue, where new challenges present themselves, local barriers present in the extracellular matrix and the layer of endothelial cells. Within an organ, siRNA has to leave the intravascular space within a blood vessel to enter the interstitium. When in the tissue interstitium, siRNA should navigate through the interstitial space to reach the target cells.

The next crucial step is the uptake siRNA by the target cells. The cell membrane is a tightly controlled and regulated barrier and, since it is an anionic lipophilic bilayer, it obstructs the entry of exposed siRNA which is negatively charged and has a considerably high molecular weight (around 13 kDa) [31], [35], [37]. These unfavourable characteristics of the siRNA molecule can be masked through the use of a delivery vector. A fitting NA delivery system should be able to condense therapeutic NA into a size suitable for cellular internalisation, ideally inferior 200 nm in diameter. Furthermore, the overall surface charge of the gene delivery system ought to be only slightly cationic, in order to mask the anionic charge of the NA and therefore improving the interaction with cell membranes through electrostatic interactions, without inducing charge associated toxicity [37].

Other relevant factors for cellular internalisation include shape, hydrophobicity, and cell type. The cell type and respective cell membrane receptors are major determinants of cellular internalisation pathway, but one must not overlook the influence of the formulation size. 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 [40], [41].

Although there are many types of endocytosis, all forms of endocytosis involve siRNA encapsulation in endocytic vesicles that fuse with endosomes. After entering the cell, siRNA-based therapeutics are entrapped within the endosome, enclosed by its lipid bilayer and as such, kept outside the cytoplasm. Hence, the predicament of getting across the endosomal lipid bilayer is identical for all forms of endocytosis [42]. The endosomal compartment has a fundamental cell regulatory role; it is responsible for monitoring material entering the cell. The endosome will either recycle the internalised molecules to the cell surface or direct it to lysosomes for degradation.

Consequently, the endosome is one of the major impediments to effective gene silencing as it contributes to low transfection efficiency. In order to avoid destruction, the NA delivery system needs to promote escape endosome escape, which can be accomplished through different mechanisms, namely increasing osmotic pressure to induce endosome burst, or by causing leakage through the formation of pores in the endosomal membrane [37], [38]. Therefore, endosomal escape into the cytoplasm in a non-toxic manner is a critical obstacle to overcome.

After being released in the cytosol, the siRNA has to be freed of its delivery vector so it can access RNAi machinery and consequently trigger efficient gene silencing. A significant hurdle when developing a gene delivery system is ensuring that extra- and intracellular bioaccumulation and inefficient clearance of the vector does not occur after the therapeutic effect is achieved.

Off-Target Effect. Some studies have revealed that the specificity of RNAi can, in certain instances, be compromised. Some studies reported non-specific effects of small RNAs, and that siRNA treatment could lead to off-target gene silencing. The off-target effect refers to the inhibition of a genetic region other than the target gene, which may cause unwanted genetic alteration and cellular dysfunction [36]. The seed region of the siRNA sequence is responsible for the majority of off-target silencing. This region might bind to different mRNAs rather than the desired target mRNA, and it may also distort the gene expression due to interactions with the endogenous miRNAs [43], [44]. Additionally, off-target effects might also occur due to RISC's failure in distinguishing between the guide strand and the passenger strand of the siRNA [36].

Hence, the selection and sequencing of candidate siRNAs should be made carefully. The development of the siRNA sequences using appropriate algorithms (e.g. BLAST), some "rules" for siRNA design and the introduction of chemical modifications can reduce the mentioned off-target effect [27].

Saturation of RNAi Machinery. Another aspect to take into consideration when developing a siRNA-based therapy is the possibility of saturation of the RNAi machinery. As previously noted, externally delivered therapeutic siRNAs resemble intrinsic siRNA and rely on the endogenous machinery for inducing gene silencing. Thus, there are significant risks of saturating such pathways and hence disturbing the native biological systems [36].

Stimulation of the Immune System. Another limitation is the activation of the innate immune response. The siRNA might be discerned as a foreign particle through various defence mechanisms of the immune system. By way of illustration, siRNA can be recognised by cytoplasmic and endosomal Toll-like receptors expressed on the surface of different immunocompetent cells. Consequently, this induces the release of cytokines and interferon, which leads to inflammatory reactions [31], [36], [38].

1.3 Co-delivery of Multiple Nucleic Acids

Most examples found in today's literature regarding RNAi and siRNA-based systems report single delivery. Though it is still a fledgeling area, one can also find a growing number of examples of combined delivery of two or more siRNAs, or of siRNAs and other NAs, and even siRNAs with other drugs, all with the goal of achieving increased therapeutic effect [45], [46]. With this strategy, one can potentially inhibit multiple target genes and pathways via concurrent delivery of distinct small RNAs to achieve additive or synergistic effects. In concept, and as a rapidly growing body of evidence (Table 1) already suggests, coRNAi technology has far-reaching potential.

In conjunction with this, the co-delivery of multiple NAs of the same type with differing sequences, in the same delivery vehicle, follows the same design principles as delivery of a single sequence of that same type, which simplifies the application of this strategy. And even when delivering a combination of distinct types of NAs, as many of them are broadly similar in terms of physical and chemical characteristics, it is possible for them to be coloaded into the same delivery system, however, one cannot overlook the particular delivery hurdles for different types of NAs [46]. This technology certainly opens exciting new therapeutic possibilities, but for this approach to smoothly transition from bench to bedside one issue to take in consideration is the safety concern of this novel approach. This concern is derived from earlier reports, such as the ones mentioned in section 1.2, of adverse side effects from single delivery RNAi treatments, including "off-target" silencing, IFN (type I interferons) responses, and translational inhibition as it is possible that these risks might increase proportionally with the coRNAi approach [45].

On the subject of combining siRNA (or other NAs) treatment with other drugs, there are numerous reports that advocate for this strategy, with the great majority of them reporting a synergistic therapeutic effect [47]-[50]

However, regarding multiple NA administration, there are fewer works that describe this combinatory delivery. Having said that, one can find several reports in which multiple related genes or in the relation of interdependence are targeted resulting in interdependent synergistic therapeutic effect [47], [49]-[55].

One field in which an increasing number of reports aim to achieve enhanced therapeutic effects through combinatorial delivery of NAs is the field of cancer therapy. Given that cancer is a very complex disease, its treatment with a single therapeutic agent is often inadequate for successful therapy. This stems from the fact that cancer is a very heterogeneous disease, not only between different patients but even within a single patient, which often results in a drug-resistant cell population. By delivering in combination multiple NAs, to specifically target multiple different cellular pathways at once, one can aim to circumvent the (multi)drug resistance mechanisms in heterogeneous cancer cell populations [46]. Indeed, there are several examples of strategies based on the co-delivery of NAs that are being explored for the treatment of multidrug resistant cancer [47], [50], [55]. Given a known mechanism of drug resistance by cancer cells, one can use RNAi to knock down a gene that promotes resistance and simultaneously deliver a chemotherapeutic agent. Three studies presented here have tested this strategy and obtained positive results that suggest that the co-delivery of two NAs resulted in an enhanced therapeutic effect. Firstly, Saad et al. used a cationic liposomal formulation to codeliver siRNAs against MRP1, an efflux pump that removes drugs from cells, and BCL2, which confers resistance by preventing apoptosis, along with the common anticancer agent doxorubicin [47]. Their findings suggest that the concurrent cell-death induction by doxorubicin in association with the suppression of cellular resistance by MRP1 and BCL2 siRNAs resulted in the induction of apoptosis to a level that could not be achieved by each component when applied separately [47]. Secondly, Yin et al. used bioreducible poly(β-amino esters) to codeliver shRNAs encoding siRNA against survivin and MDR-1, both with great significance for tumour cell survival and drug resistance [55]. Their in vitro studies demonstrated that the transfection of drug-resistant human breast cancer cells with either of these shRNAs increased their sensitivity to doxorubicin treatment. Additionally, in a mouse model, the combination of both shRNAs with doxorubicin was synergistic and resulted in slower tumour growth than any of the three components individually [55]. Lastly, Risnayanti et al. used poly(D/L-lactic-co-glycolic acid) (PLGA) nanoparticles to codeliver siRNAs against MDR1 and BCL2 to multidrug resistant ovarian cancer cells [50]. Their results showed that not only both genes were successfully suppressed, but also that this dual RNAi suppressor system was more efficient to overcome the drug resistance than sole RNAi suppressor system, leading to superior tumour responsiveness to two chemotherapy drugs: paclitaxel and cisplatin [50]. Briefly, all these three examples reported efficient simultaneous suppression of both target genes associated with drug resistance, which led to increased tumour responsiveness to the tested drugs.

Another authors that showed promising results regarding the benefits of small RNA combination therapy for the treatment of lung cancer was Jacks and colleagues [49]. This group delivered both miR-34a, a p53-regulated tumour suppressor miRNA, and siRNA targeting Kras in lipid-polymer hybrid nanoparticles and observed that the combination of miR-34a and siRNA targeting Kras resulted in superior antitumor response over those observed with either small RNA alone. The combination of both decreased the number of cancer cells in vitro and significantly slowed tumour growth in vivo. On top of that, this group also determined that nanoparticle-mediated small RNA delivery plus conventional, cisplatin-based chemotherapy prolonged survival in their chosen model compared with chemotherapy alone [49].

Another area in which this dual delivery strategy is being explored is the treatment of viral infections. One of the major hurdles when developing antiviral strategies is the development of resistance during treatment. This is especially true for RNAi based therapeutics due to the exquisite sequence specificity of the targeting mechanism. Thus, strategies are being developed that aim to minimise viral resistance by combining RNAi effectors with each other to raise the genetic barrier [45], [52]. Some reports already supported this hypothesis, namely one study which investigated the efficacy of combination siRNA therapy and its ability to delay or prevent viral escape against feline coronavirus [52]. Its results showed that antiviral resistance rapidly emerged with single (resistant population could be identified following a single cell passage) or dual (resistant population could be identified following two cell passages) combination siRNA treatment. However, the combination therapy with three siRNA significantly delayed the emergence of resistance as it prevented viral escape over the course of five passages [52]. Another study that successfully showed the advantages of combinatory delivery as an antiviral strategy was published in 2018 and investigated the potential of host-directed combinatorial RNAi to tackle diverse strains of influenza A virus in human respiratory epithelial cells [51]. Herein, viral inhibition was evaluated using five combinations of four siRNAs, targeting different parts of the viral replication pathway. While siRNA mediated inhibition of a single gene repressed the replication of many of the tested strains of influenza, there were numerous strains that were not inhibited, and inhibition of diverse strains was particularly weak in the absence of combinatorial RNAi. The presented results revealed that combinatorial RNAi overall performed better than single gene targets both in terms of strength and breadth of influenza inhibition [51].

Even though the majority of co-delivery examples found in the literature report efficient silencing of multiple genes, or at least comparable to that of single delivery approaches, there are also combinatory approaches that have led to antagonistic effects [56]-[58]. One interesting example explored different triple combinations of siRNAs to develop an antifibrotic treatment targeting three critical scarring genes. The study culminated in contradictory results: while one of the triple combination of siRNAs proved to be more effective showing significant gene downregulation (>80%) and reduced off-target effects, relatively to single and dual siRNA delivery; the other triple combination proved to be ineffective, even though each of the included siRNAs was potent against its intended target mRNA [56]. Some studies propose that the co-delivery antagonistic effects might be caused by the oversaturation of the endogenous RNAi machinery through the competition for critical RNAi components, such as the RISC complex [45], [56], [59].

Interestingly, some groups have observed an enhanced silencing due to a synergistic effect upon administration of multiple NAs [60]–[62]. By way of illustration, Banala et al. developed a dual siRNA-mediated system for the treatment of intervertebral disc degeneration, with the goal of achieving down-regulation of the expression of Caspase 3 and ADAMTS5 genes [61]. A synergistic effect was observed in dual siRNA delivery when compared with single siRNA delivery of both genes, which led to a reduction of the intervertebral disc degeneration – the dual siRNA delivery resulted in 2-fold less expression for Caspase 3 and 1.5-fold less expression of ADAMTS5 [61]. Moreover, some studies 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 [62]. For example, Ball et al. developed a co-formulation of siRNA and mRNA in a single lipidoid nanoparticle and found that the co-formulation of siRNA and mRNA in the same delivery system enhanced the efficacy of both drugs substantially both in vitro and in vivo. In fact, the dual delivery system with siRNA and mRNA resulted in greater gene silencing than the single delivery system only with siRNA [62].

Despite the synergistic effect of combined approaches, some gene silencing applications do not require the downregulation of multiple genes. In the above-mentioned study, Ball et al. proposed the substitution of the "helper" NA by a mimicking negatively charged polymer [62]. They found that co-formulation with the mimicking negatively charged polymer provided the same degree of potency enhancement as helper mRNA (3-fold reduction in the required amount of siRNA) [62]. In this manner, the application of single targeting systems could also benefit from the synergistic effect of the mimicking polymer.

Altogether, this is still an emergent area, but the preliminary results suggest that it deserves further investigation.

Table 1 - Co-delivery of multiple therapeutic NAs. si- stands for a siRNA, sh- stands for a shRNA m- stands for an mRNA and anti-miR stands for an anti-miRNA.
ADAMTS5 - A Disintegrin And Metalloproteinase With Thrombospondin Motifs 5, ARCN1 - Coatomer protein complex, ATP6AP1 - Proton-transporting V-type ATPase, BCL2 - B-Cell Lymphoma 2 Apoptosis Regulator, COPA - Coatomer protein complex, subunit alpha, CTGF - Connective Tissue Growth Factor, DHDEAC - N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium chloride, DOPE – dioleoylphosphatidylethanolamine, DOTAP - 1-oleoyl-2-[6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino]hexanoyl]-3-trimethylammonium propane, DSPE- 1,2-Distearoylphosphatidylethanolamine, E1A - Adenovirus Early Region 1A, EGFR - epidermal growth factor receptor, EGFR - epidermal growth factor receptor, FIPV - Feline infectious peritonitis virus, FVII – Factor VII, IVa2 – Adenovirus Packaging Protein 1, Kras - Kirsten rat sarcoma viral oncogene homolog, L2 - Leader2, Luc – Luciferase,M1 - Membrane1, MAPK - mitogen-activated protein kinases, MDR1 - Multi-Drug Resistance Protein 1, N1/2 - Nucleocapsid1/ Nucleocapsid2, NUP98 - Nuclear pore complex protein, nuclear import and export, NXF1 - nuclear RNA export factor 1, p53 – Tumour protein 53, p53-regulated miRNA miR-34a, PEG – polyethyleneglycol, PLGA - Poly(Lactic-co-Glycolic Acid), Pol – DNA Polymerase, RPS14 - ribosomal protein S14, initiation of translation, SOD1 -superoxide dismutase 1, TGFß1 – Trans-forming Growth Factor ß 1, TGFßR2 - Transforming Growth Factor ß Receptor 2, TNBC – Triple-Negative Breast Cancer, TNF – Tumour Necrosis Factor, TRAIL - tumor necrosis factor-related apoptosis-inducing ligand, VEGF - vascular endothelial growth factor (R1 - receptor 2 R2 – receptor 2)

Nucleic Acid	Vector	Condition	Outcome	Ref.
siCOPA, siATP6AP1, siNXF1, siARCN1, siPGD, siRPS14, siNUP98	Lipofectamine RNAiMAX	Influenza A virus infections	Combinatorial RNAi performed better than single gene targets in terms of both strength and breadth of influenza inhibition.	[51]
si-FIPV L2, M1, N1 and N2	Lipofectamine 2000	Feline coronavirus	Antiviral resistance rapidly emerged with single or dual combination siRNA treatment (1 passage for single delivery and 2 for dual delivery), while with combination therapy with 3 siRNA the emergence of resistance was delayed (prevented viral escape for five passages).	[52]
siKras, miR-34a	Lipid-based nanoparticle with PEI and PEG	Lung cancer	The combination of miR-34a and siRNA targeting Kras improved therapeutic responses over those observed with either small RNA alone, leading to tumour regression.	[49]
iMdr-1-shRNA, iSurvivin-shRNA	poly[bis(2-hydroxylethyl)-disulfide-diacrylate-ß-tetraethylenepentamine]	Multidrug resistance cancer	The combination of both shRNAs with doxorubicin was synergistic and resulted in slower tumour growth than any of the 3 components individually.	[55]
siMRP1, siBCL2	DOTAP based cationic liposomal formulation	Multidrug-resistant lung cancer	The concurrent cell-death induction by doxorubicin in association with the suppression of cellular resistance by MRP1 and BCL2 siRNAs led to superior induction of apoptosis when compared to each component applied separately.	[26]
siMRP1, siBCL2	PLGA nanoparticles	Ovarian cancer	Efficient simultaneous suppression of both genes, associated with drug resistance, resulting in increased tumour responsiveness to drugs.	[50]
si-E1A_4, si-IVa2_2, Pol-si2a	Lipofectamine RNAiMAX	Adenoviruses	The siRNA dual and triple mixtures did not result in increased downregulation compared to single siRNA transfections.	[57]

Nucleic Acid	Vector	Condition	Outcome	Ref.
si-TGFβ1, si-TGFβR2, si-CTGF	TransIT-TKO transfection reagent	Corneal scarring	The triple siRNA combinatory approach led to significant gene downregulation (>80%) while reducing off-target effects, relatively to single and dual siRNA delivery.	[56]
si-VEGFA, si-VEGFR1, si-endoglin	L1 peptide-based polyplexes	Cancer	The combinatorial down-regulation of si-VEGFA and si-VEGFR1 led to a synergistic anti-angiogenic effect (1.5-fold increase of anti-angiogenic properties).	[63]
si-Caspase 3, si-ADAMTS5	DHDEAC: cholesterol (1:1) liposomes	Intervertebral disc degeneration	A synergistic effect was observed in dual siRNA delivery when compared with single siRNA delivery, leading to a reduction of the intravertebral disc degeneration. The dual siRNA delivery resulted in 2-fold and 1.5-fold less expression of Caspase 3 and ADAMTS5, respectively, when compared to single delivery.	[61]
si-VEGFR2, si-EGFR	Branched PEI	Lung Cancer	Targeted knockdown by siVEGFR2 and siEGFR inhibited tumour growth effectively, and a combination of this approach with low dose chemotherapy showed a strong therapeutic effect while minimising the toxic effects.	[58]
pTRAIL, si-BCL2L12, si- SOD1	Lipid-grafted PEI	Breast Cancer	The addition of siRNA increased the pDNA transfection efficiency, but the addition of pDNA did not increase the siRNA silencing efficiency.The co-delivery of pDNA and siRNAs within the same complex displayed enhanced therapeutic effect than separate delivery.	[64]
si-p53, si-TNF	siPORT™ NeoFX™ transfection reagent	Triple-negative breast cancer	Enhanced effects observed in double gene knockdown in two TNBC cell lines (Hs578T and MDA-MB-231).	[53]
anti-miR-10b, anti-miR-21	PLGA-b-PEG polymer	Triple-negative breast cancer	Cumulative effect in TNBC tumour growth reduction in a mice xenograft model (up to 40%), even at very low doses.	[54]
si-Luc/ m-mCherryb, si-FVII/ m-Lucc	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.	[62]
si-p38α MAPK, sip65	PEG-modified liposome (DOPE, CHEM and DSPE-PEG2000 at 9.5:2:1 M ratio)	Glomerulonephritis related renal failure	The co-delivery system had better knock down efficacy for both target genes in contrast to single delivery in three types of glomerular cells (MRGEC, SV40MES13, and MPC5), even though the dose of each siRNA in the dual delivery system was halved. The co-delivery system significantly attenuated pathological related changes in model mice.	[60]

1.4 Nucleic Acid Delivery Vectors

Due to the previously mentioned therapeutic hurdles, the success of gene therapy relies significantly on the development of delivery systems that enable efficient and potent systemic delivery of NAs [63]. An ideal carrier should be biocompatibility and non-immunogenic, it should be able to compact and protect NAs, resist the premature degradation and surpass both extracellular and intracellular barriers, enabling high transfection efficacy [1], [64], [65].

Currently, the plethora of different vectors that have been already developed and optimized around the world can generally be categorized into viral and non-viral vectors [65].

1.4.1 Viral Vectors

The natural history of virus infection made viruses promising candidates to accomplish the objectives of gene therapy. Viral vectors are inspired by viruses as they are naturally able to invade host cells, deliver and exploit the cellular machinery to express its own genetic material [9], [65]. The ideal virus-based carrier would maintain all the internalization, tropism and delivery abilities of the wildtype virus while avoiding the subsequent expression of viral genes that results in replication and toxicity. This could be achieved by swapping viral components responsible for the pathogenicity, while leaving untouched all the components needed for the payload delivery, with NAs that would complement defective cellular activities [6], [9], [65].

Viral vectors are mostly known for their exceptional delivery and transfection efficiencies. However, they possess other attractive qualities: they can transduce both dividing and non-dividing cells, they can yield a long-term expression of therapeutic NAs, and do not need to be delivered in large doses [66], [67].

The number of distinct viruses that are under development as NAs vectors is growing; however, there are five main groups of clinically applicable viral vectors: retroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses. Each of these groups of vectors has a unique set of characteristics that make it suitable for some applications and unsuitable for others (see in Table 2)[6], [14].

Notwithstanding the successes of viral delivery, it is crucial to bear in mind that the use of a viral vector is associated with significant drawbacks, especially when it comes to safety. The 'Achilles heel' of viral vectors is that many of the immunological defence systems that tackle wild-type infections can be activated against these carriers. The presence of residual viral elements can potentiate an inflammatory response (especially in the case of adenoviruses), which can be highly dangerous and even fatal. Namely, a case reported in a 1999 trial, in which the inflammatory response induced by an adenovirus vector led to the unfortunate death of a patient (previously mentioned in section 1.1) [6]. Another major concern with these delivery systems is the insertional mutagenesis risk, especially in the case of lentiviruses [6], [66]. Additional obstacles of the use of viral vectors include low carrying capacity (especially in Adeno-associated virus) and expensive and challenging production in large volumes [65], [66].

While substantial progress has been made in surpassing these shortcomings, there are still fundamental obstacles to overcome, which have limited both the in vivo use of viral vector and its translation into the clinic [27]. Consequently, researchers have been compelled to search for safer alternatives.

Table 2 – Properties of the main groups of viral vectors

Vector	Packaging capacity	Gene Expression	Inflammatory Potential	Advantages	Limitations
Retrovirus	8 kb	Stable	Moderate	Persistent gene transfer in dividing cells (chromosomal integration)	Only transduces dividing cell; Insertional mutagenesis
Lentivirus	8 kb	Transient or stable	Low	Low cytotoxicity; Persistent gene transfer in most tissues (chromosomal integration)	Insertional mutagenesis
Herpes simplex virus	>30 kb	Transient	High	Large packaging capacity; Low toxicity; Long term expresssion (epissome)	Immunogenecity
Adenovirus	<7.5 kb	Transient	High	Extremely efficient transfection; Short term transient expression	High immunogenicity
Adeno-associated virus	<5 kb	Transient or stable	Low	Reduced immune response; Stable transgene expression	Small packaging size; Immune response (repeated administration)

References: [6], [14], [67]

1.4.2 Nonviral Vectors

Although initially, the development of non-viral vectors had lagged behind that of viral vectors, mainly due to their inferior transfection efficiency, currently are being increasingly trialled due to their superior safety profile [62], [64].

Among the nonviral vectors being developed, inorganic, lipid, polymer, and dendrimer based vectors (Figure 2) are the most common ones. Electrostatic interactions between negatively charged siRNA and cationic carriers are the basis, in the vast majority of cases, for the nonviral vector-mediated siRNA delivery [27], [68]. But, moreover, the positive charges of the cationic carriers also interact with the negatively charged glycoproteins and proteoglycans of the cell membrane playing a vital role in cellular uptake. These systems are usually characterised by the ratio between the positively charged amine groups of the vector (N) and the number of phosphate groups of the NA backbone (P), i.e., the N/P ratio. Despite the great number of possibilities of nonviral vectors, their transfection efficiency is still far from the obtained with their viral counterparts. Hence, researchers are working towards the design of an efficient, biocompatible, biodegradable and targeted NA delivery system with the final goal of obtaining improved transfection efficiencies [65].

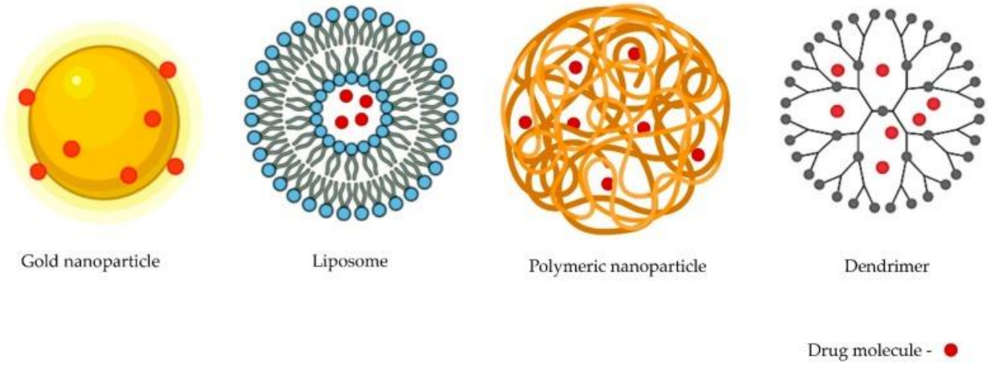


Figure 2 - Schematic representation of nanoparticles based on inorganic, lipid, polymer and dendrimer nanostructures applied as delivery vectors. From Spencer et al., 2020 [63].

1.4.2.1 Inorganic Nanoparticles

Inorganic nanoparticles (NPs) are arising as NA vectors. These inert vectors are highly sought due to their reduced sizes (10 nm to 100 nm), low cytotoxicity at low concentrations, low immunogenicity, low polydispersity, high surface-to-volume ratio and proper storage stability [13], [69], [70]. Additionally, currently, it is possible to tune their porosity, shape and size precisely, and their surface can be easily modified. Thus one can tailor these carriers to achieve biocompatibility and controlled release [71].

One common example of inorganic NPs is **Gold NPs**. These NPs have emerged in the late 1990s and have been broadly investigated due to their attractive proprieties, such as their small size (as small as 2 nm) and spherical morphology. NAs are mostly loaded by gold nanoparticles through thiol linkages or electrostatic interaction with cationic gold NPs. NAs can be modified and attached to gold nanoparticle cores in a selective manner, thiolated siRNA, for example, can also be stably conjugated directly to the gold core or attached to polymer-modified gold cores [65], [72], [73]. Another type of inorganic nanocarriers that have been gaining relevance is **iron oxide NPs**. This NPs feature inherent magnetic properties coupled with tunable size and functionality. Most of the strategies for iron oxide nanoparticle-mediated delivery of NAs rely on surface-engineered cationic iron oxide NPs that can electrostatically interact with anionic NAs [73]. The magnetic properties of iron-oxide based nanocarriers have been applied for remote NA delivery through magnetofection [74], [75].

Nevertheless, inorganic carriers are prepared synthetically from inorganic materials which are hard, water-insoluble and non-biodegradable. Due to these limitations, inorganic delivery systems are less used for siRNA delivery than their organic counterparts. These carriers are often coated with several biocompatible, biodegradable, natural or synthetic polymers or lipids to increase their biocompatibility and loading capacity. Additionally, inorganic NPs are often cytotoxic at higher concentrations, and their stability under biological fluids ought to be addressed with caution since they are prone to aggregation [36], [38], [76].

1.4.2.2 Lipids

Lipids are a group of biological molecules known by their amphiphilic nature, which confers them the ability to form vesicles make lipid-based systems into appealing delivery vectors. As the major constituents of the cell membrane are lipids and phospholipids, lipidic carriers naturally interact well with the cell membrane favouring cellular uptake [12], [70]. Lipid-based delivery systems are among the most broadly utilised nonviral gene carriers [77]. Even though anionic, cationic, or neutral lipids can be employed to deliver NAs into cells, cationic lipids are preferred because of their capability to adsorb onto the anionic cellular membrane and to facilitate complex formation with the polyanionic NAs. Consequently, cationic lipids/NAs complexes (lipoplexes) allow greater transfection efficiency [70]. Lipid-based NPs are currently the leading nonviral RNAi delivery systems [12]. In fact, various classes of lipid-based NPs have been developed for RNA delivery.

Liposomes are extensively researched nanocarriers. The liposome's structure is based on a bilayer lipid membrane with an aqueous compartment inside. Contrary to other lipid-based systems, that are almost exclusively used for loading lipophilic compounds, liposomes can entrap hydrophilic and ionic molecules. As analogues of biological membranes, liposomes can enter the cells by fusing with their membranes [12], [14], [65]. Cationic lipids are more efficient than neutral liposomes because, as the lack of positive surface charges results in low transfection efficiency. N-[1-(2, 3-dioleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioctadecylamidoglycylspermine (DOGS), 3b[N-(N′,N′-dimethylaminoethane)-carbamoyl) cholesterol (DC-Chol), 2,3-dioleyloxy-N-[2-(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamin-ium (DOSPA), dimethyldioctadecyl-ammonium bromide (DDAB), dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE) and 1-oleoyl-2-[6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino]hexanoyl]-3-trimethylammonium propane (DOTAP) have been proven to be successful for RNAi in vitro delivery. The disadvantage of incorporating a positive charge is that it can cause dose-dependent cytotoxicity, inflammatory effects and unwanted interactions with negatively charged serum proteins [12], [14]. To minimize these effects, cationic lipids are normally combined with neutral lipids, such as 1,2-dioleoylphosphatidylethanolamine (DOPE) or cholesterol, while improving the complex's stability, prolongate the blood circulation time, enhance endosomal escape, and promote the release of the NAs from the vector [14], [78], [79]. Among them, Lipofectamine is a commonly used cationic liposome-based reagent. Its derivative composed of a 3:1 w/w mixture of DOSPA and DOPE [77], Lipofectamine 2000, is effective at transducing siRNA and has become a standard transfection reagent for siRNA delivery in vitro [14].

An alternative to traditional liposomes may be other lipid-based nanocarriers, such as **solid lipid NPs (SLNs)**. These NPs, with a size range of 50–1000 nm, are comprised by a solid lipid core enveloped by a layer of surfactants in aqueous dispersion, with various potential combinations of lipids and surfactants. The lipid elements of SLNs are in solid-state at both physiological and ambient temperatures. SLNs are one of the most effective vectors for hydrophilic and hydrophobic drugs owing to their inclusion of cationic lipids, that provide a positive surface potential that promotes binding to NAs. Given that, SLNs can be used as NAs delivery system. On the other hand, **nanostructured lipid carriers (NLCs)**, have a matrix composed of solid and liquid lipid blends. These lipid vectors are next-generation lipid NPs that combine the benefits of diverse nanocarriers. When compared with SLNs, NLCs have superior loading capacity and less water in the dispersion, which makes them more stable for storage [12], [65].

Notwithstanding the significant advantages of these vectors and their extensive use as NA vectors with high transfection efficiency in vitro, some issues limit their applicability to some extent. Their high cytotoxicity, limited blood circulation time, low solubility, poor stability and low encapsulation efficiency are obstacles that still need to be addressed. Due to these facts, the in vivo performance of most lipid-based delivery vectors is not satisfactory [13], [65], [66], [80].

1.4.2.3 Polymers

Polymeric systems offer an attractive platform to deliver NAs. Polymers present broad versatility regarding composition, molecular weight and side chains density (in the case of branched polymers), allowing the adjustment of the physicochemical properties of the delivery system. Due to this, polycationic polymers have arisen as one of the most auspicious candidates for developing efficient NAs delivery vectors [36], [79]. Negatively charged NAs, such as siRNAs, can form complexes with positively charged polymers through electrostatic interactions, forming structures called “polyplexes”. 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 [12], [78], [81]. The preparation of polyplexes is simple, and the nanosized polyplexes can facilitate cellular uptake through endocytosis. Moreover, polymers that exhibit high proton buffering capacity can augment endosomal escape, and therefore help to evade NAs degradation [66]. One of the first polymers used in genetic therapy was poly(L-lysine) (PLL) [82]. From then on, a significant number of other polymers have been used to engineer gene delivery systems, including chitosan, poly(ethylene imine) (PEI), poly(methacrylate) (PMA), poly(D/L-lactic acid) (PLLA) and PLGA, among others [78]–[80]. Polymers can be divided into two types based on their sources: natural and synthetic.

Regarding natural polymers, **chitosan** has probably been the most widely investigated. Chitosan is a natural, biocompatible, and biodegradable polysaccharide with low immunogenic properties. It has been applied in diversified fields, particularly in pharmaceutics [12], [14], [65]. The positively charged chitosan has been employed to bind and form polyplexes with siRNA and has been applied in both in vitro and in vivo systemic delivery of this NA [14], [36]. Nonetheless, naked chitosan is incompatible with biologic fluids, which results in degradation and reduced efficiency [12]. Regarding synthetic polymers, **PEI** is the most extensively investigated for NA delivery [31], [66]. PEI has dense cationic charge, which promotes encapsulation of siRNA. Furthermore, PEI has an extensive pH buffering capacity, which allows it to work as a proton sponge to induce the release of polyplexes from the endosome to the cytoplasm. Notwithstanding, some caution surrounds PEI use in vivo, as due to their high charge density, cationic PEI is often associated with high toxicity [14], [66].

All in all, it is crucial to bear in mind that cationic polymers have significant charge-related toxicity, which results in low transfection efficiency (particularly in vivo) and polymer polydispersity. Nevertheless, because of the overabundance of synthetic chemical possibilities when creating a polymer-based NA vector, it is possible to mask their surface charge with different ligands and moieties (as poly(ethylene glycol (PEG)) [65], [70], [80], [81].

1.4.2.4 Dendrimers

More recently, dendrimers have emerged as a promising class of nonviral carriers due to their unique structural properties: a globular, highly branched, controlled and nanosized structure, very low polydispersity, and various controllable multifunctionalization possibilities on account of an extraordinary number of terminal groups [79], [83]. The first dendritic structures were synthesized in 1978 and arose as a novel class of highly branched polymers labelled as “cascade molecules” [68], [84]. Throughout the subsequent decade, Tomalia’s group further developed the level of complexity of these branched molecules, originating larger dendritic structures that were later renamed as “dendrimers” [68], [85]. The term dendrimer stems from the Greek dendron (“tree” or “branch”) and meros (“part”) and refers to the distinctive design of their branched building blocks [86]. Dendrimers are comprised of three main components (Figure 3):

i) a central core with two or more reactive groups,
ii) interior layers – generations – of branched repeating units or monomers,
iii) terminal functional groups on the outer surface.

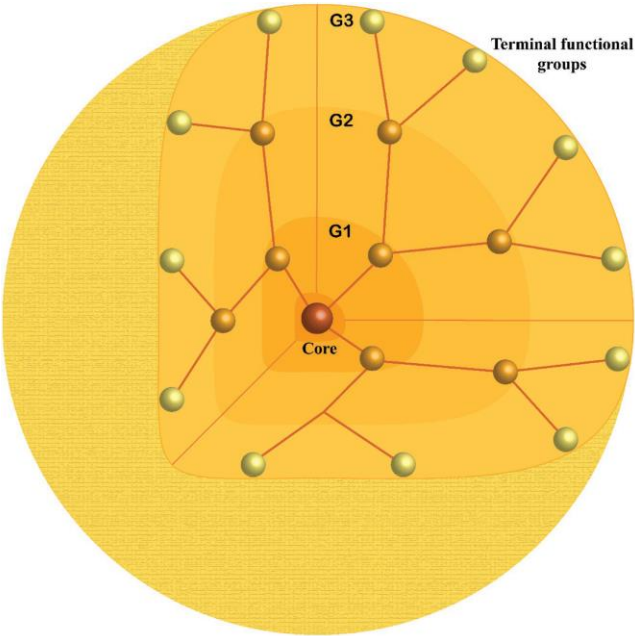


Figure 3 - Schematic representation of a dendrimer structure with three generations (G). From Leiro et al. 2018 [86].

Dendrimers are labelled per their number of generations (G), that is, the number of layers between each cascade point (subdivision point of each branch). With the addition of generations of a dendrimer, its number of terminal functional groups increases exponentially, its diameter tends to increase linearly, and its shape becomes more globular [87], [88]. Moreover, the abundant terminal groups of these promising carriers can be functionalized in a specific and controlled way with a great variety of bioactive ligands emulating the multivalency existing in various biological systems. This multivalency is the greater virtue of dendrimers [68], [86].

1.4.2.4.1 Dendrimers' synthesis

The synthesis of dendrimers is achieved through an iterative synthetic methodology that comprises a series of repetitive growth and activation steps. There are two traditional methods to synthesize dendrimers: divergent and convergent.

The first method described is known as the **divergent method**. It encompasses the growth of the dendrimers, layer by layer, from a multifunctional core site towards the periphery. The core molecule reacts with monomeric molecules (repeating units) containing one reactive and at least two inactive branched sites, generating the first-generation dendrimer (G1). After, the surface of this G1 is activated for reaction with more repeating units. This process can be repeated numerous times until the dendrimer of the desired generation and size is obtained. Some limitations of this synthetic method include the fact that it is susceptible to defective monomeric molecule assembly as a result of the high number of reactions performed concurrently, and consequently, purification is usually required after each step [27], [86].

In the convergent method, synthesis is initiated on the perfect branched dendrons (dendrimer wedges), which are connected to a multifunctional core after activation/deprotection of their focal point. As only a limited number of groups are active per reaction, the probability of structural flaws of this approach is lower than in the divergent methodology. Nevertheless, a significant disadvantage of this method is its low ability to produce dendrimers of higher generations as a result of the steric hindrance between increased dendrons in the last step [27], [86].

In this regard, it is worth mentioning that novel synthetic strategies, which allow higher reaction efficiency and step number reduction, have emerged to overcome the shortcomings and the tedious purification intensive iterative processes of the classical strategies. Some examples of these user-friendly methods include “Lego” and “click” chemistry [89], [90].

1.4.2.4.2 Dendrimers as Small Interfering RNA Delivery Vectors

Dendrimers have superior efficiency with limited cell toxicity when compared to linear polymers; accordingly, they are promising synthetic macromolecular carriers for NA delivery [91]. Similarly to other cationic NAs delivery vectors, the primary interaction between cationic dendrimers and siRNA is electrostatic. Nevertheless, some studies also pointed to the non-negligible contribution of hydrophobic interactions and hydrogen bonds [91], [92]. Through these interactions, the cationic dendrimers are able to complex and protect NA in compact structures, named “dendriplexes” (dendrimer-NA complexes) [27]. These nanostructures can shield NAs from non-specific interactions and degradation [93], [94].

Of the wide range of dendrimers that have been synthesized to date, as a result of their commercial availability, the poly (amido amide) (PAMAM), poly(propylene imine) (PPI), and PLL cationic classes have been mainly used for delivery of siRNAs. PAMAM was the first class of dendrimers synthesized and have been used in a wide range of applications, due to their commercial availability [98]. Both PAMAM and PPI dendrimers have been broadly used in drug and NA delivery applications because of their water solubility, non-immunogenicity, and relatively proper biocompatibility [99]-[102]. PAMAM dendrimers are the base of some commercially available reagents for in vitro NA transfection, namely PolyFect® and SuperFect®, that have revealed to mediate efficient siRNA delivery [27]. PLL dendrimers are also widely used for drug and gene delivery. They are built from the naturally occurring monomer L-lysine and hence, present good biocompatibility, and water solubility. Despite this, PLL dendrimers capability to complex NAs is relatively weak, and thus its transfection efficiency is reduced due to this together with an insufficient endosomal escape [103], [104]. In principle, one important advantage of PLL systems would be their biodegradation under the action of proteases and, consequently, low cytotoxicity. Nevertheless, the ability of proteases to degrade PLL dendrimers (branched structures) in vivo has not yet been clarified [98].

From the studies presenting dendrimers for siRNA delivery, one can conclude that the dendriplex stability and NA delivery profile usually improve with the dendritic generation. Nonetheless, it has also been observed that higher generations do not always result in higher transfection efficiencies. This may be due to the fact that dendrimers of lower generation may interact with NAs more efficiently because of their more flexible structure. Thus, as high generation dendrimers are more rigid but have more charges and, lower generations might show higher flexibility, but lack of necessary positive charges, a balance between generation and flexibility should be sought when designing optimal dendrimers for siRNA delivery [27], [91].

Even though strong complexation between the dendrimers and NAs is desired, one should keep in mind that an efficient gene delivery carrier should be able to release the siRNA once in the cytoplasm of the target cells so it can execute its function. Hence, to design an optimal delivery system, one should aim for a balance between efficient complexation and NA release.

Overall, the mechanisms involved in the binding between cationic dendritic vectors with siRNA during complexation process is not as straightforward as previously thought. As already mentioned, many distinct interactions (electrostatic, hydrophobic and hydrogen bonds) are involved, and the different properties of the dendrimer (generation, flexibility, charge, etc.) play major roles complexation process. Interestingly, it has even been hypothesised that the same dendrimer interacts differently with different types of NAs (siRNA and DNA, for example) [95]-[98]. To design highly efficient dendritic NA carriers, it is necessary to have a detailed understanding of the interactions between the vector and its cargo [91]. Hence it is interesting to study the dendrimer

– siRNA interactions and explore whether and how the interactions might change the delivery process.

1.4.2.4.3 PEGylation and Biodegradable Dendrimers: Overcoming Dendrimers' Toxicity

Several dendrimers have already been used in in vitro trials as potential future delivery vectors for nanomedicine. However, problems related to cytotoxicity have constrained their use of dendrimers in the in vivo biological system. Nonspecific dendrimer toxicity is associated with its internal chemical composition, size, generation, and concentration, and mainly influenced by the nature of the surface terminal groups. The high number of positive charges belonging to their groups makes cationic dendrimers more cytotoxic than the anionic ones. The cationic systems interact strongly with the negatively charged cell membranes, which might destabilize its integrity followed by leakage of intracellular components that lead to cell death and toxicity. Consequently, higher concentrations and generations of unmodified cationic dendrimers present an inherently greater cationic surface charge and, accordingly, a marked cytotoxic and hemolytic effect. However, there is still some disparity in the literature concerning the influence of the generation of a dendrimer on its toxicity profile [86], [93].

Due to these toxicity issues related to cationic dendrimers, much effort has been put into improving their cytotoxicity profile. Several studies have already shown less cytotoxicity due to modifications on this cationic surface, such as acetylation [99] or the conjugation of PEG chains that partially shield the positive charges of the dendritic structure surface. Indeed, it has already been possible to decrease the cytotoxicity of cationic dendrimers for NA delivery, such as PAMAM dendrimers for example, while maintaining efficient NA delivery through the modification with PEG [100]–[102].

PEG has been the most commonly used polymer in many biomedical applications. It is often employed in the development of delivery nanocarriers as it can be easily modified at its terminal hydroxyl group with different reactive groups, enabling crosslinking and conjugation chemistries. Due to the hydrophilic nature of PEG, PEG-dendritic block copolymers based on hydrophobic dendrons lead to amphiphilic macromolecules very suitable to act as vectors for different bioapplications. Hence, PEGylation enhances the solubility and biocompatibility of the copolymers and sterically stabilises the complexes, due to sequestration and masking of positive charges. Furthermore, due to the shielding effect, the interaction of the complexes with the plasma components is suppressed, which results in increased circulation times [103]. Nevertheless, in some instances, excessive PEGylation can lead to larger dendriplexes with higher polydispersity and lower NA protection [104], [105]. Additionally, steric hindrance due to the presence of PEG chains may hamper the interaction of dendriplexes with cell membranes, which can result in inefficient internalization and/or endosomal escape. Hence, a fundamental problem associated with the nanoparticle PEGylation arises. The solution to this issue comes from finding a balance between an appropriate biocompatibility and efficient transfection. Also, a hydrolyzable linkage between the linear polymer and the focal point of the dendron is a promising strategy, proposed in the nBTT group [106], [107], to overcome low endosomal escape. Because, while the system can still benefit from the PEG chain, under the acidic conditions of the endosome, the disruption of the PEG-dendritic block leads to charges unmasking and promotes the endosomal escape.

Another crucial characteristic responsible for dendrimers toxicity is their non-biodegradable nature. Most of the currently used dendrimers for biomedical applications are very stable under physiological, and this can lead to cytotoxicity or complications induced by the accumulation of non-degradable synthetic materials in the organism. Additionally, the non-biodegradability of the carrier can hinder the intracellular release of the siRNA, and as a result, lead to low transfection efficiency [27], [108]. Accordingly, the ideal delivery vector ought to be biodegradable.

Various factors affect the degradation rate of dendrimers, namely [68]:

i. Type of cleavable chemical bond;

ii. Location of the cleavable linkage (structures presenting internal hydrolyzable linkages have faster degradation rates).

iii. Hydrophobicity of the repeating units; (hydrophilic ones result in faster degradation rates of compounds);

iv. Size (larger dendrimers, presenting increased packaging, have slower degradation rates).

Taking these parameters into account, one can modulate the features of biodegradable dendritic structures to achieve the desired degradation rate. Regarding i), the functionalities more susceptible to hydrolytic cleavage are based on anhydrides, esters and phosphoesters [68].

Currently, the majority of biodegradable dendrimers reported so far are based on ester bonds. This is due to their biocompatibility and good compromise between biodegradability trait and relative ease of synthetic manipulation [109], [110]. Even so, the preparation of ester-based vectors is challenging due to undesired or premature degradation observed during synthesis, purification, and subsequent functionalization and application [111], [112]. Accordingly, there are still few works of dendritic structures containing esters and reported for specific functions in biomedicine. In the area of NA delivery, these are restricted to very few examples with amine-terminated bis-HMPA [2,2-bis(hydroxymethyl)propanoic acid] dendrons for the encapsulation of DNA, described mainly by Smith and Coworkers [113]–[116].

Chapter 2

Scope, Aim and Objectives

Due to the unmet need for an effective, safe and biodegradable NA delivery vector for gene therapy applications, recently our nBTT group synthesised and explored the biological function as NA vectors, of two new families of biocompatible and biodegradable PEG-Gallic Acid Triethylene Glycol Ester (GATGE) dendritic block copolymers/PEG-GATGE dendrimers: one hybrid and one fully biodegradable. These new dendritic copolymers/dendrimers present azides as terminal groups enabling the straightforward functionalisation with different ligands, via Copper(I)-catalysed Huisgen 1,3-dipolar cycloaddition (CuAAC) - "click" chemistry.

In the present dissertation, the new fully biodegradable (fb) PEG-GATGE dendrimers of generation 3 (G3) were explored as vectors of different siRNA sequences. These dendrimers present 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 [106]. The fully degradable nature of these dendritic vectors was expected to favour the siRNA release from the dendriplexes, rendering higher transfection efficiencies.

To employ the developed PEG-GATGE as siRNA vectors, they were functionalised with amine groups, which will be protonated under physiological conditions allowing the complexation of the NA. Considering the utmost importance of knowing the interactions between the vector and its cargo, to design highly efficient dendritic NA carriers, fb PEG-GATGE were functionalised with different amine moieties exhibiting different chemical properties: a single amine, a diamine [106]–[108] or a benzylamine [107], [117], [118] (Figure 4). The functionalisation with a single amine provides the basic positive charge can ensure the complexation of the siRNA. The functionalisation with diamine is intended to increase the positive multivalency of dendritic systems, hence boosting the strength of the electrostatic interactions with the NAs, without increasing the dendron generation. The decoration with benzylamine is intended to strengthen the hydrophobicity of the system since hydrophobic interactions contribute to the stability of dendriplexes (as mentioned in section 1.4.2.4.2).

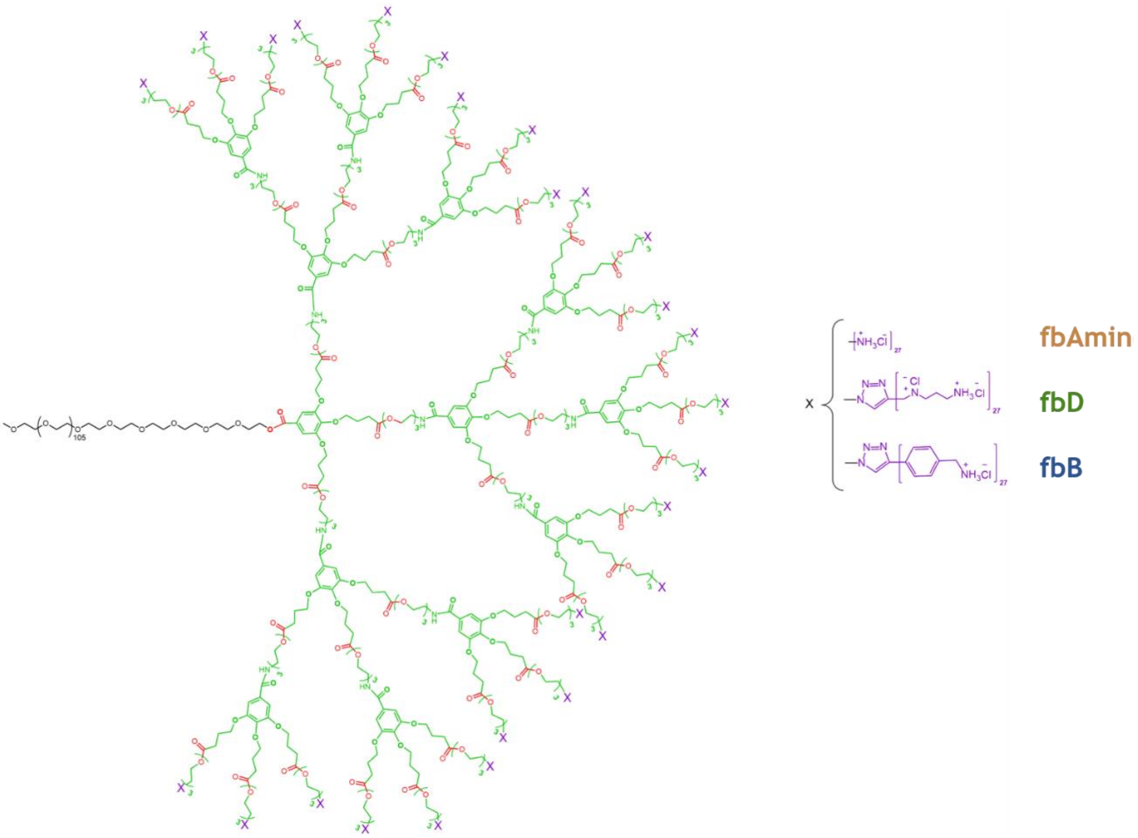


Figure 4 - Amine, Diamine and Benzylamine terminated fb G3 PEG-GATGE dendritic block copolymers (fbAmin, fbD and fbB).

For now, the new fb G3 PEG-GATGE, benzylamine- (fbB) and diamine-terminated (fbD) have been successfully explored as vectors for single siRNA delivery of an anti-eGFP (enhanced Green Fluorescent Protein) siRNA, obtaining excellent transfection efficiencies in several cell lines.

However, very recently, in our group, it has been observed that using the same dendrimer (namely, fbB) as a vector, different transfection efficiencies were obtained when different siRNA sequences were carried/delivered. Taking this into account, it seems that the same dendrimer may complex and interact in a different way (weaker/stronger interactions; more or less stable dendriplexes) with different siRNA sequences. Hence, in the present dissertation, the dendriplexes between the three developed dendrimers (fbAmin, fbD and fbB) and four different siRNA sequences (anti-PTEN (Phosphatase and Tensin Homolog Protein) siRNA, anti-eGFP siRNA and sequences A and B) were prepared and biophysicochemical characterised. In this way, the first main aim of this work was to evaluate the possible different interactions in each case and the ability of each dendrimer, presenting different surface functional groups, as a vehicle for the different siRNAs (Figure 5A).

Moreover, and inspired by the recent findings concerning the co-delivery of multiple siRNAs (section 1.3), the second main objective of the present work (Figure 5B) was to evaluate the biological performance of the developed dendrimers (namely, fbD and fbB) as carriers for single and dual delivery of different siRNA sequences (si-eGFP and si-PTEN).

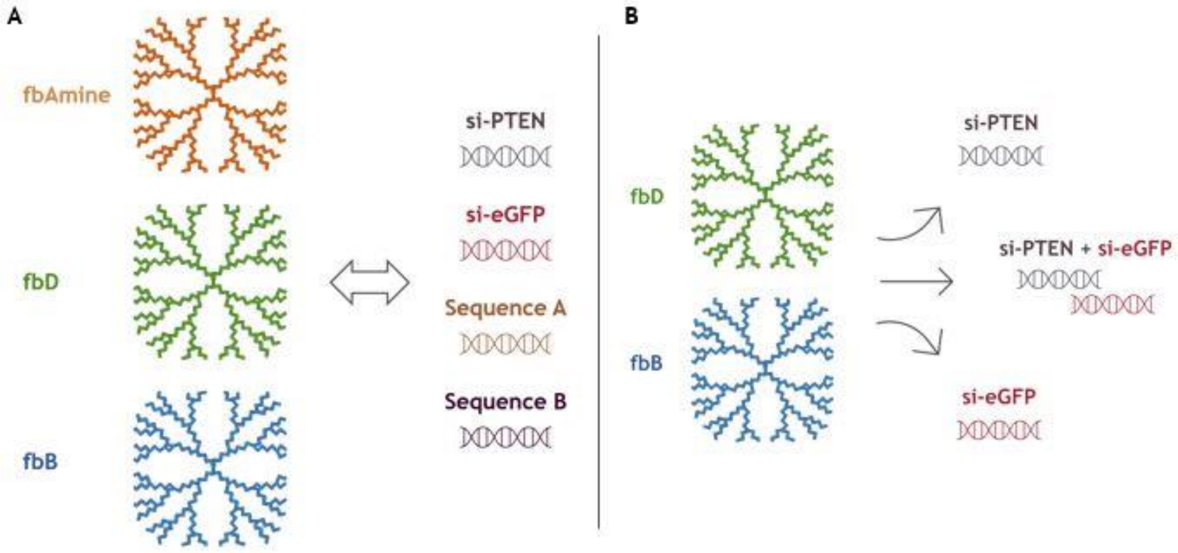


Figure 5 - Schematic representation of the main aims of the present dissertation. A) Evaluation of how different PEG-dendrimers, developed in our lab (fbB, fbD and fbAmin), complex/interact with distinct siRNA sequences. The selected sequences include: si-PTEN □ targeting the mRNA coding the Phosphatase and Tensin Homolog Protein (PTEN); si-eGFP□ targeting the mRNA coding the enhanced Green Fluorescent Protein (eGFP); sequence A and sequence B. **B)** Evaluate the biological performance of the developed dendrimers (namely, fbD and fbB) as carriers for single and dual delivery of different siRNA sequences (si-eGFP and si-PTEN).

Chapter 3

Materials and Methods

The research work developed during the present dissertation accounts the physicochemical characterisation of dendriplexes between dendritic block co-polymers with three different surface modifications with four 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 central material of the present dissertation is the fully biodegradable dendritic block co-polymers: fbAmin, fbD and fbB. The dendritic copolymers consist of a PEG chain connected at the focal point of a GATGE repeating unit dendron, with -amine, -diamine and benzylamine-functionalized terminal groups. The biodegradable dendritic copolymers were synthesised by our group (nanoBiomaterials for Targeted Therapies – nBTT, INEB/i3S).

The efficiency of the developed dendrimers as NA vectors was tested using four siRNA sequences, si-PTEN, si-eGFP and sequences A and B, respectively, all with 21 bp. The sequences si-PTEN and si-eGFP were supplied by Integrated DNA Technologies and are present in Table 3. The nucleotide content of the siRNA sequences A and B is unknown, as they are confidential, however, it is known that they are very similar.

The transfection efficiency of the dendriplexes 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 - siRNA sequences

siRNA	Sequence 5' to 3'	Modifications
si-PTEN	Sense: CGA CUU AGA CUU GAC CUA UAU Antisense: AUA GGU CAA GUC UAA GUC GAA	2' O-Methyl RNA bases in the AntiSense strand (10 out of 21 bp)
si-eGFP	Sense: GGC UAC GUC CAG GAG CGC ACC Antisense: UGC GCU CCU GGA CGU AGC CUU	-

The remaining reagents and materials used throughout the experimental procedures and the respective suppliers are presented in Table 4.

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

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®
10x 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®
6x NZYDNA loading dye (MB13101)	NZYTech
96-well Polystyrene Black Flat Bottom Plates (655090)	Greiner Bio-One International
D-(+)-Glucose (G7528)	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (DMEM) GlutaMAX™ (10569010)	Gibco™
Fetal Bovine Serum (12657011)	Gibco™
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
Potassium phosphate dibasic (P8281)	Merck
Potassium phosphate monobasic (P5379)	Merck
RNase I (AM2294)	Ambion™
Sodium dodecyl sulphate (817034)	Merck
SYBR® Gold Nucleic Acid Stain (10000x in DMSO, S11494)	Molecular Probes, Invitrogen™
Trypsin (T-0646)	Sigma-Aldrich

3.2 Dendriplexes Preparation

Dendriplexes between the fully biodegradable dendritic block co-polymers and siRNA were prepared at different N/P ratio. Firstly, the dendritic copolymer (fbAmin or fbD or fbB) was dissolved in Nuclease-Free (NF) water at a concentration of 6 g·L-1 using a vortex for 30 s followed by thermomixer for 3 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) to the corresponding volume of dendrimer solution (N/P ratios = 5, 10, 20, 40, and 80), in 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) + 5% Glucose for benzylamine-terminated and in Phosphate Buffer (PB) 10 mM for diamine-terminated and amine-terminated dendrimers. Afterwards, the samples were vortexed for 10 s and incubated for 30 min at RT before subsequent experiments to allow complex stabilisation.

The required volume of the dendritic copolymer, Vfb, for a given N/P ratio and final dendriplex solution volume, Vtotal, is given by Equation 1,

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

Equation 1

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

where Nfb is the number of primary amines per dendritic copolymer molecule, cfb is the concentration of filtered dendrimer solution, Mfb is the molecular weight of the dendrimer (16451.25 g·mol-1 for fbAmin, 21180.14 g·mol-1 for fbD and 20814.5 g·mol-1 for fbB), bpsiRNA is the average number of bp of the siRNA sequence, and csiRNA is the molar concentration of the siRNA solution (usually 20 µM).

3.3 *Physicochemical Characterization*

3.3.1 Spectrophotometry

The mass loss upon filtration of the three dendrimers (fbAmin, fbD and fbB) was quantified through absorbance reading (λ = 250 nm and 210nm), in a spectrophotometer (LAMBDA 35 UV-Vis Spectrophotometer, PerkinElmer, USA) using quartz cells. This was possible due to the intrinsic ultraviolet-visible (UV-Vis) absorption of PEG-GATGE. To obtain the calibration curve, the absorbance of different concentration of unfiltered dendrimer solution (0.0025 to 0.05 g·L-1) was read at the abovementioned wavelengths. The filtered solution was diluted 200× prior to analysis. The presented data were obtained as a mean of the concentration obtained for each wavelength and are expressed as a mean ± standard deviation (SD) of two independent sample measurements.

3.3.2 SYBR Gold® intercalation assay

An SYBR Gold® intercalation assay was performed in order to access the dendrimers' (fbAmin, fbD and fbB) binding ability to the studied siRNAs, si-PTEN, si-eGFP, and sequences A and B. The complexation efficiency was studied by quantifying the uncomplexed siRNA. Dendriplexes were prepared as previously described and then incubated in 96-well black plates in TBE buffer with 1:100 SYBR Gold® 10000x and a final volume of 200 µL, for 10 min to allow the intercalation of the free siRNA. After incubation, the fluorescence was measured (λexc = 485 nm, λem = 540 nm) using a microplate reader (SynergyMx, BioTek Instruments, Inc., USA). The results correspond to the percentage of complexed siRNA of duplicates, where 100% represents the complete siRNA complexation. The presented data are expressed as a mean ± SD of one independent sample measurement for fbAmin si-PTEN, Sequence A and B dendriplexes and two independent sample measurements for the remaining.

3.3.3 Size measurements

Size and polydispersity index (PDI) of the dendriplexes (formed between fbAmin, fbD and fbB and the four studied siRNAs, si-PTEN, si-eGFP and sequences A and B) were measured at 633 nm by a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., UK), following the manufacturer specifications. Size and PDI were determined at RT (25ºC) with a detection angle of 173º using ZEN0040 cells in the automatic mode, using solutions with a final volume of 80 µL and N/P ratios of 5, 10, 20, 40 and 80 without dilution. The mean hydrodynamic diameters were determined by cumulative analysis (Z-average mean). The data were analysed using the Zetasizer Software, version 7.13, supplied by the manufacturer (Malvern Instruments, UK). The presented data correspond to the mean ± SD of one independent sample measurement for fbB and fbD si-PTEN dendriplexes and fbAmin si-eGFP dendriplexes and two independent sample measurements for the remaining.

3.4 Biological Performance

3.4.1 Endonuclease Protection Assay

Endonuclease protection assay was conducted to evaluate the ability of fbAmin, fbD and fbB to protect the siRNA (si-PTEN and si-eGFP) from enzymatic degradation. The adequate RNase I concentration used in this experiment was the minimum required to degrade the naked siRNA within 5 to 15 min, and was previously determined in the nBTT Group by incubating the naked NA sequences with different concentrations of RNase I at different time periods (5 to 60 min). For the si-PTEN sequence a concentration of 6 U of RNase per 0.3 µg of siRNA was used, while for the si-eGFP sequence, a concentration was 18 U of RNase per 0.3 µg of siRNA was used.

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 0.3 µM). The solutions were incubated with 0.1% SDS for 30 min to inactivate the RNase and dissociate the complexes. The siRNA degradation was evaluated through PAGE shift assay. Polyacrylamide gels were prepared in Tris/Borate/Ethylenediaminetetraacetic 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). Afterwards, loading dye (1x) 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® 10000x NA stain, diluted in TBE buffer (1:10000), for 10 min, visualised using GelDoc™

XR+ Imager (Bio-Rad Laboratories, Inc., USA) and analysed using Image Lab 6.0.1 (Bio-Rad Laboratories, Inc., USA).

3.4.2 Cells

3.4.2.1 Cell Culture

U2OS/eGFP-Luc was cultured in (Dulbecco's Modified Eagle's Medium) DMEM medium supplemented with 10% V/V heat-inactivated FBS (56 °C for 30 min) and 1% V/V Penicillin-Streptomycin (P/S) and incubated (CCL-170A-8-SS, Esco) at 37 °C, 5% CO2.

3.4.2.2 Cellular Uptake

U2OS/eGFP-Luc cells were seeded in 24-well plates at a density of 2.5 x 10^4 viable cells per cm^2 and incubated for 24 h in supplemented DMEM medium at 37 ºC, 5% CO2, and grown to reach 70-80% confluence prior transfection. The medium was substituted by non-supplemented DMEM 1h before transfection. Three different sets of the two dendriplexes (fbD and fbB) were prepared at N/P ratios of 5, 20 and 80: with Cy5-labeled siRNA, with Cy3-labeled siRNA, and with a 1:1 mixture of siRNAs (Dual delivery - Mix of siRNA). Dendrimer solutions with the amount of fbD and fbB corresponding to the N/P ratios in both mixes were also prepared in PB 10mM and HEPES 20 mM Glu 5%, respectively. Positive controls consisted of Lipofectamine® 2000 lipoplexes prepared according to manufacturer instructions in Opti-MEM® (final concentration 0.15% V/V in Cy5-labeled siRNA or Cy3-labeled siRNA only transfected cells and 0.30% V/V in mixture of siRNAs).

Cells were transfected using 50 µL in a final volume of 300 µL in the case of single siRNA dendriplexes and 100 µL in the case of mix of siRNAs (each siRNA at 0.1 pmol·µL-1). Cells were then incubated at 37°C, 5% CO2 for 24 h.

After 24 h incubation, cells were rinsed twice with 1x Phosphate Buffer Saline (PBS), trypsinized, centrifuged, resuspended in 1x PBS containing 2% FBS and analysed by flow cytometry (BD Accuri™ C6 Plus Cytometer). Untreated cells and cells transfected with Lipofectamine® 2000 (Invitrogen) were used as negative and positive controls, respectively. Lipofectamine® 2000 was used according to the manufacturer instructions. The resulting data were analysed using FlowJo software (FlowJo X 10.0.7r2, FlowJo, LLC). The present data correspond to the mean of two technical replicates.

3.4.2.3 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 medium and incubated at 37°C, 5% CO2 for 24 h to reach 70% to 90% confluence (prior to transfection). At transfection time, the medium was replaced with non-supplemented DMEM medium (rinsed twice with 1x PBS). Three different sets of the two dendriplexes (fbD and fbB) were prepared at N/P ratios of 5, 10, 20, 40 and 80: with si-PTEN, with si-eGFP, and with a 1:1 mixture of siRNAs (Dual delivery - Mix of siRNA). Dendrimer solutions with the amount of fbD and fbB corresponding to the N/P ratios in both mixes were also prepared in PB 10mM and HEPES 20 mM Glu 5%, respectively.

Positive controls consisted of Lipofectamine® 2000 lipoplexes prepared according to manufacturer instructions in Opti-MEM® (final concentration 0.15% V/V in si-PTEN or si-eGFP only transfected cells and 0.30% V/V in mixture of siRNAs).

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 siRNAs (each siRNA at 0.1 pmol·µL-1). Cells were then incubated at 37°C, 5% CO2 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 fluorescence-activated cell sorting (FACS)/flow cytometry (24-well plate, for eGFP quantification) or quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (96-well plate, for PTEN mRNA quantification), as described in sections 3.4.2.4 and 3.4.2.5. Non-treated cells were used as the negative control, and cells whose transfection was mediated by Lipofectamine® 2000 was used as the positive control.

3.4.2.4 Flow Cytometry

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

3.4.2.5 Quantitative Reverse Transcriptase Polymerase Chain Reaction

96 h post-transfection, the medium was discarded, and cells were rinsed twice with 1x PBS (100 µL). Afterwards, 30 µL of Cell-Lysis-Buffer (10 mM Tris-HCl, pH7.4, 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™ Prime-Script™ 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 1x 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 5. 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, and mRNA expression levels were computed by the relative quantification method based on the exponential transformation of ΔCt values ($2^{-\Delta\Delta Ct}$). PTEN mRNA expression was normalised against the endogenous control HPRT1 and calibrated to untransfected cells. Cells which transfection was mediated by Lipofectamine® 2000 was used as the positive control. The presented data are expressed as the mean of the three technical replicates.

Table 5 - PCR primers for PTEN and HPRT1 mRNA.

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

Chapter 4

Results and Discussion

4.1 Dendriplexes Preparation

Dendriplexes between fully biodegradable amine-terminated dendrimers (fbAmin, fbD and fbB) and four different siRNA sequences, si-PTEN, si-eGFP, sequence A and sequence B, were prepared at different N/P ratios ranging from 5 to 80.

While fbD and fbAmin were prepared in Phosphate Buffer (10 mM, pH 7.4), fbB was prepared in HEPES-Glucose buffer (20 mM + 5% w/w, pH 7.4). These buffers were chosen for each of the available dendrimers after an exhaustive physicochemical evaluation of the physicochemical performance of the said dendrimers in a variety of universally applied buffers. The phosphate buffer is used ubiquitously in biological experiments, as it can be adapted to a variety of pH levels, is highly water-soluble and has a high buffering capacity. The buffer is one of the most popular currently used and is commonly employed in molecular and cell biology, chemistry, and material science, among many others. 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 bondages. 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 dendrimers were solubilised in NF-water at 6 g·L-1 by vortexing for 30 s and agitating at 1000 rpm. Afterwards, the copolymer solution was filtered in a 0.45 µm PTFE filter. The dendrimers concentration after filtration was determined by spectroscopy, taking advantage of the intrinsic UV-Vis absorption of the copolymer. The average concentration of the dendrimers was the following: 5.0 g·L-1 ± 0.37 g·L-1 for fbAmin, 5.8 g·L-1 ± 0.05 g·L-1 for fbD and 5.5 g·L-1 ± 0.34 g·L-1 for fbB. This slight mass loss, corresponding to 17%, 3% and 9% of the total mass of fbAmin, fbD and fbB respectively, 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. In our group, it has been observed that for the same dendrimer different transfection efficiencies were obtained for different siRNA sequences. Taking this into consideration, it was proposed that the same dendrimer may complex and interact differently (more or less stably) with different siRNA sequences. Hence, the interactions between the different available dendrimers and the four studied siRNA sequences were evaluated.

In order to evaluate the ability of our fully biodegradable amine-terminated dendrimers to complex, protect and act as vectors of different sequences of siRNA (si-PTEN, si-eGFP, sequence A and sequence B), the corresponding dendriplexes were assessed and compared regarding their physicochemical properties.

4.2.1 Small Interfering RNA Complexation Efficiency

A suitable NA vector must be able to bind, compact and protect NA to allow its efficient delivery. The formation of dendriplexes between cationic dendritic molecules and NAs is similar to other cationic molecules, namely polymers, and is mostly driven by electrostatic interactions, as previously stated. Nevertheless, as mentioned before, other types of interaction, such as hydrophobic interactions and hydrogen bonds, play essential roles in the complexation process (Section 1.4.2.4.2).

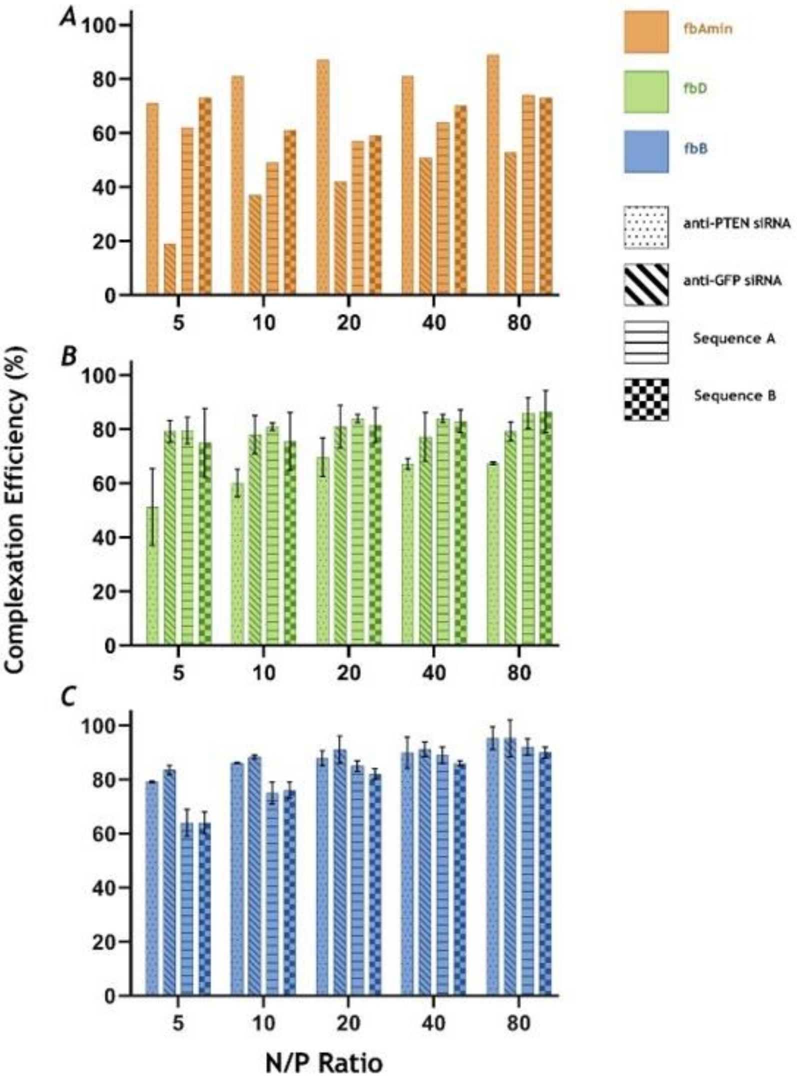
The complexation efficiency of the dendrimers as a function of both N/P ratio, and siRNA sequences, was studied by a nucleic acid dye (SYBR Gold®) accessibility assay. SYBR Gold® is a highly sensitive fluorescence dye that presents a substantial fluorescence enhancement (approximately 1000-fold) when bounding to NAs; thus, it can be used to distinguish between free and complexed (inaccessible) NAs.

All dendrimers were able to bind to the siRNA molecules efficiently. Nevertheless, it is possible to observe a clear variation in the performance of the three amine-terminated dendrimers. As can be observed in Figure 6, benzylamine-terminated dendrimer (fbB) displayed higher complexation efficiency (ranging between 64% and 100 % with an average of 85±8%), followed right after by diamine-terminated dendrimer (fbD) (ranging between 41% and 96 % with an average of 76±10% complexation efficiency). The amine-terminated dendrimer (fbAmin) came in third place (ranging between 19% and 89 % with an average of 63±17% complexation efficiency). It was expected that fbB and fbD would provide better complexation efficiencies when compared with fbAmine, as fbD possess a divalent character which doubles the points of connection for siRNA and fbB has an extra hydrophobic group that may positively contribute to this interaction strength [90], [91].

In general, as seen in Figure 6, there seems to be an increase in complexation efficiency with the increase of the N/P ratio, for all cases: the three copolymers with the four siRNA sequences. This is an expected result since increasing the amount of dendrimer per siRNA molecule promotes the higher complexation efficiency.

Interestingly, it was possible to notice differences in how the same dendritic vector complexes and interacts with the distinct siRNA sequences. These differences are especially clear in the case of fbAmin, followed by fbD. In the case of fbB, while it is possible to note some differences, they are not as evident. Regarding fbAmin (Figure 6A), as mentioned above, while it shows good complexation efficiencies, its results fall short of those of fbB and fbD, probably due to its lower hydrophobicity and positive charge density, compared with fbB and fbD, respectively. This leads to lower overall interactions between the dendrimer and siRNA. Surprisingly, this dendrimer showed a higher si-PTEN affinity than fbD (> 80% complexed siRNA for N/Ps > 10 VS <70% for all N/Ps). fbD showed excellent binding ability (Figure 6B), yet, this dendrimer appears to have lower affinity for anti-PTEN siRNA when compared to the other three sequences (ranging from 41% to 75 % for si-PTEN and for the other three sequences it ranges from 66% to 87%, as seen in Figure 6D). The fact that si-PTEN is modified with 2' O-methyl RNA bases in the antisense strand (10 out of 21 bp) might contribute to the lower affinity of fbD, as CH_3 hydrophobic character might not interact as favourably with the divalent character of this dendrimer when compared with fbAmin which has a lower surface positive charge. Both for fbAmin and fbD sequences A and B presented comparable complexation profiles, probably due to the high similarity between them (Figure 6A and 6B). Regarding fbB, it exhibited excellent complexation efficiency, for all four siRNA sequences (Figure 6C), displaying a superior complexation efficiency with anti-PTEN siRNA and anti-GFP siRNA (>85% for N/P ≥ 10 and even percentages over 90% for N/P ≥ 40). Even though fbB showed lower bonding ability with the sequences A and B, it still exhibited great binding (> 80% for N/Ps > 10) with very similar results for these A and B sequences.

All in all, regarding the dendrimer surface functionalities, the three studied amine-terminated dendrimers proved to complex siRNA efficiently, with fbD and fbB exhibiting excellent binding profiles. Interestingly, besides the differences in performance between dendrimers with different peripheral modifications, it has been possible to observe variations in the interaction of the same dendrimer with each of the four different siRNA sequences. While fbAmine shows a clear preference for anti-PTEN siRNA, fbD appears to bind more efficiently to anti-GFP siRNA and sequences A and B and, lastly, although it displays an overall excellent complexation with all sequences, fbB seems to interact more efficiently with anti-PTEN and anti-GFP siRNA.



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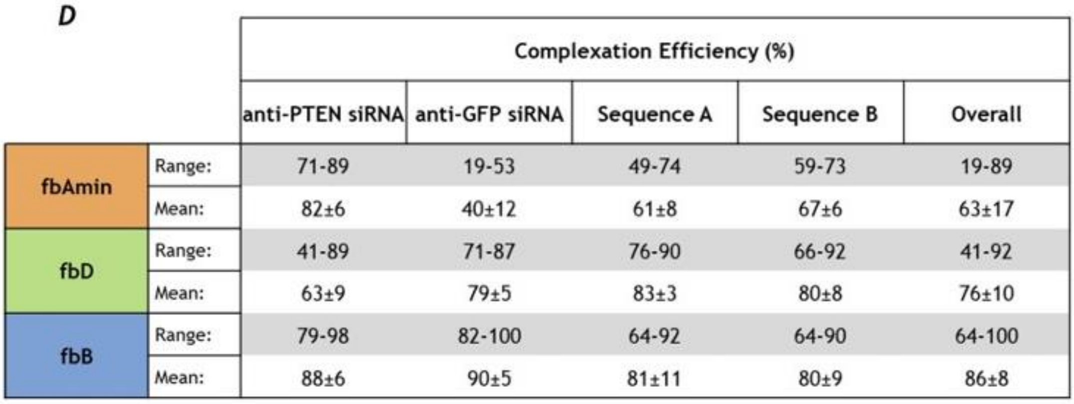


		Complexation Efficiency (%)				
		anti-PTEN siRNA	anti-GFP siRNA	Sequence A	Sequence B	Overall
fbAmin	Range:	71-89	19-53	49-74	59-73	19-89
	Mean:	82±6	40±12	61±8	67±6	63±17
fbD	Range:	41-89	71-87	76-90	66-92	41-92
	Mean:	63±9	79±5	83±3	80±8	76±10
fbB	Range:	79-98	82-100	64-92	64-90	64-100
	Mean:	88±6	90±5	81±11	80±9	86±8

Figure 6 - SYBR Gold® exclusion assay of fbAmin (6A), fbD (6B) and fbB (6C) for all four studied siRNA sequences (si-eGFP, si-PTEN, sequence A and sequence B). Overview of the complexation efficiency obtained with fbAmin, fbD and fbB based dendriplexes (6D). Data represent the percentage of inaccessible siRNA (n = 1 for fbAmin si-PTEN, Sequence A and B dendriplexes, n = 2 for the remaining, mean ± SD).

4.2.2 Size

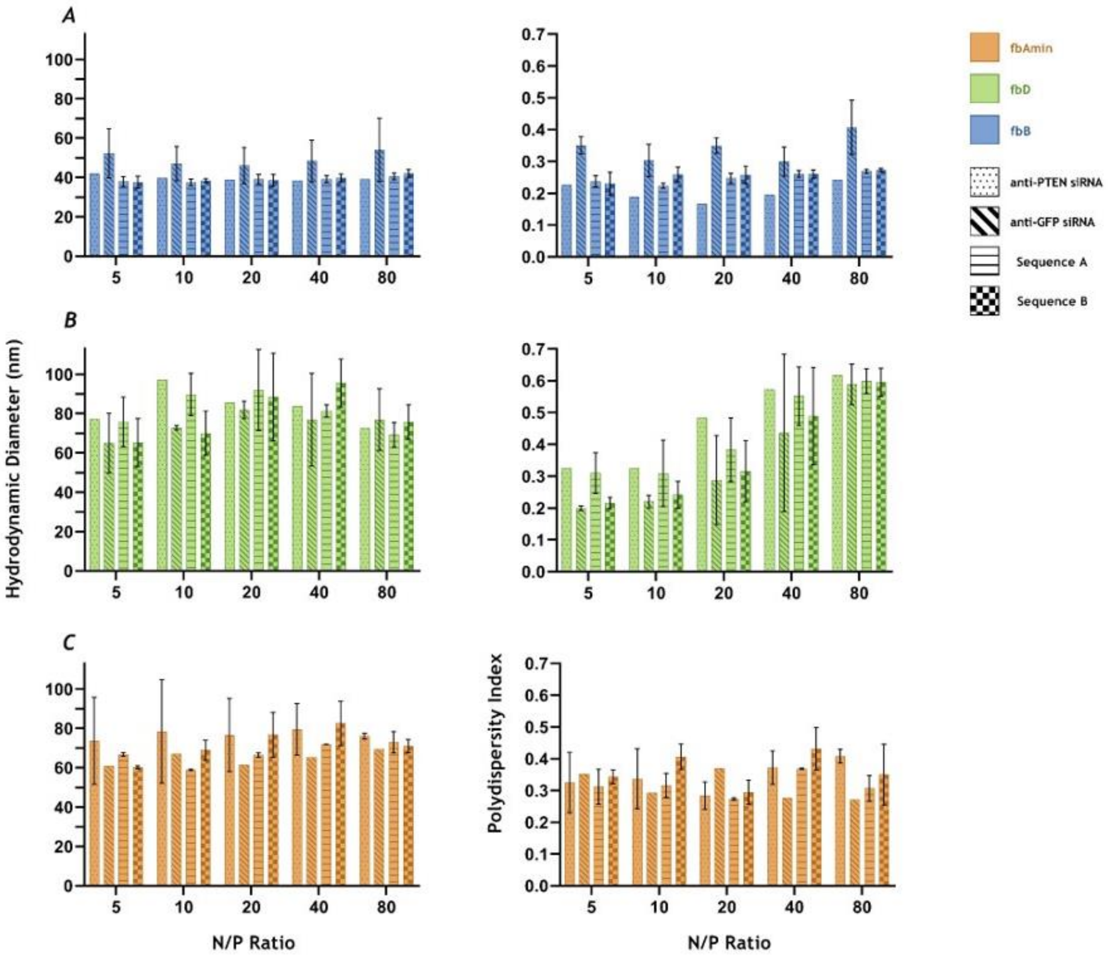
As addressed in section 1.2, the size plays an essential role in cellular internalisation, as well as in escaping renal and RES clearance. Therefore, dendriplexes size was studied through DLS. As dendriplexes will be administered in solution in the corresponding in vivo and clinical application, hydrodynamic diameter obtained through DLS is an accurate representation of the size of the dendritic nanoparticles. DLS measurements consider non-covalent interactions between the solvent and the particles and include any molecule adsorbed or attached to the particle surface.

As can be seen in Figure 7, all of the developed dendriplexes present very suitable nanosize for cellular internalisation (< 100 nm), [37], [38]. Nevertheless, one can observe some differences in the sizes of the dendriplexes depending on the dendrimer surface functional groups, particularly different, is the case of fbB-based dendriplexes (Figure 7A). These dendriplexes display exceptional small sizes (with an average of 41.8 ± 7.6 nm) and homogenous populations (PDI with an average value of 0.26±0.07) when compared with fbD and fbAmin. These excellent results can probably be attributed to the extra hydrophobic contribution provided by the peripheral aromatic benzylamines in the fbB dendrimer, which lead to a higher siRNA compaction in homogeneous populations of dendriplexes. fbD and fbAmin based dendriplexes present very similar size and PDI values, the hydrophobicity of these dendrimers might explain this similarity in structure (Figure 7B and 7C). These dendriplexes present the larger hydrodynamic diameters (with an average of 79.6±12.9 nm and 71.3±10.2 nm, for fbD and fbAmin, respectively) and slightly less homogenous populations (PDI with average values of 0.40±0.16 and 0.35±0.06) when compared with fbB.

For the three types of dendriplexes, the size profile seems to be independent of the N/P ratio, and also the PDI values, except for the fbD dendrimer that shows larger PDI values at higher N/Ps (N/P 40 and 80), as seen in Figure 7B. These results might be explained by the excess of this divalent dendrimer at these higher N/P ratios, which increases in the positive surface charges. This might lead to higher electrostatic repulsions, resulting in less homogenous populations.

Different from what was observed in the siRNA complexation experiments, in this case, the size and PDI of the studied dendriplexes seems to be independent of the complexed siRNA sequences. That is, although small variations can be observed for all siRNA sequences there does appear to be a clear pattern. Nevertheless, in the case of fbB si-eGFP dendriplexes consistently exhibit slightly superior PDI values when compared to fbB based dendriplexes formed with the other sequences (Figure 7A).

Overall, it can be concluded that the dendriplexes size and PDI were mainly determined by the dendrimer, namely, its surface functional group.



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		anti-PTEN siRNA		anti-GFP siRNA		Sequence A		Sequence B		Overall	
		SIZE (nm)	PDI	SIZE (nm)	PDI	SIZE (nm)	PDI	SIZE (nm)	PDI	SIZE (nm)	PDI
fbB	**Range:**	38.4 - 41.9	0.17 - 0.24	39.6 - 65.4	0.27 - 0.47	37.5 - 40.6	0.22 - 0.27	37.5 - 42.0	0.23 - 0.27	37.5 - 65.4	0.17 - 0.47
	Mean:	39.6±1.2	0.20±0.03	49.5±8.9	0.34±0.05	38.8±1.1	0.25±0.02	39.2±1.6	0.26±0.01	41.8 ± 7.6	0.26±0.07
fbD	**Range:**	72.33 - 97.18	0.33 - 0.62	54.2 - 93.5	0.19 - 0.64	64.7 - 106.5	0.24 - 0.63	56.6 - 104.2	0.20 - 0.63	54.2 - 104.1	0.19 - 0.63
	Mean:	83.2±8.4	0.46±0.12	74.6±11.7	0.35±0.17	81.58±12.11	0.43±0.14	79.0±15.1	0.37±0.16	79.6±12.9	0.40±0.16
fbAmin	**Range:**	58.1 - 96.9	0.25 - 0.42	60.9 - 69.5	0.27 - 0.37	59.1 - 84.1	0.27 - 0.42	59.6 - 90.5	0.32 - 0.49	58.1 - 96.9	0.27 - 0.49
	Mean:	76.8±13.2	0.35±0.06	64.8±3.3	0.31±0.04	71.3±6.6	0.34±0.04	72.2±9.6	0.40±0.05	71.3±10.2	0.35±0.06

Figure 7 - Size distribution and Polydispersity index (PDI) of the three siRNA dendriplexes, fbAmin (7A), fbD (7B) and fbB (7C) with all four studied siRNA sequences (si-eGFP, si-PTEN and sequences A and B) measured by DLS at different N/P ratios. Overview of the sizes and PDI obtained with fbAmin, fbD and fbB based dendriplexes (7D). (n = 1 for fbB and fbD si-PTEN dendriplexes and fbAmin si-eGFP dendriplexes, n = 2 for the remaining, mean ± SD).

4.3 Biological Performance of Dendriplexes

The biological performance of the dendriplexes was evaluated regarding the ability to protect the NAs from endonuclease degradation, ability to mediate cellular uptake of siRNA and transfection efficiency. For this end, the present work concentrated on the anti-PTEN and anti-GFP siRNA sequences.

Both cellular uptake and transfection efficiency were assessed in both single and dual delivery strategies. For these studies, we focused on fbB and fbD due to their superior performance both in terms of complexation efficiency (section 4.2.1), the formation of compact and homogenous dendriplexes populations (section 4.2.2) and endonuclease protection (as described in the following section - section 4.3.1).

4.3.1 Endonuclease Protection

One major barrier of siRNA delivery is the rapid degradation by endogenous nucleases (5 min to 15 min); thus, the siRNA protection from endonucleases is a critical parameter in the development of new NA vectors (section 1.2). To study this aspect, three types of dendriplexes were prepared between the three developed dendrimers and two siRNA sequences (si-PTEN and si-eGFP) at N/P 80 and incubated with RNase I for different periods (5 min, 15 min, 30 min and 60 min). Then, the enzyme was deactivated, siRNA was displaced from the dendriplexes with sodium dodecyl sulfate, and samples were analysed by Polyacrylamide Gel Electrophoresis (PAGE). In this assay, nondegraded siRNA is retained as a specific band (Figure 8).

The adequate RNase I concentration used in this experiment was the minimum required to degrade the naked siRNA within 5 min and was previously determined in the nBTT Group by incubating the naked NA sequences with different concentrations of RNase I at different time periods (5 to 60 min), as can be seen in Figure 8A and 8B. While for the sequence si-PTEN a concentration of 6 U of RNase per 0.3 µg of siRNA was enough to observe complete degradation; in the case of the sequence si-eGFP, the appropriate concentration was 18 U of RNase per 0.3 µg of siRNA.

As can be seen in Figure 8, while naked siRNA is completely degraded within 5 min (for these RNase I concentrations), various degrees of siRNA protection were observed for the different dendriplexes. Comparing to fbD and fbB, fbAmin showed the lowest siRNA protection against endonucleases. It was able to efficiently protect the siRNA molecules especially until 15 min, however, after that, only a small amount of siRNA remained protected from RNase I. This result was expected taking into account the fbAmin chemical surface: when compared to fbB, it does not provide the extra hydrophobic interactions; when compared with fbD, it provides half of the surface positive charges. These results go hand in hand with those obtained in the siRNA complexation efficiency assay, in which fbAmin provided a good ability for complexing the different studied siRNA sequences, but the lowest complexation efficiency performance of the three studied dendrimers (section 4.2.1).

Regarding fbD, although it did not protect siRNA in the same excellent degree as fbB, it still ensured a very good level of siRNA protection, even after 60 min of incubation. In this case, the higher surface positive charge density of fbD allows, not only great complexation efficiency (section 4.2.1) but also provides very good protection against endonucleases. It should be noted that fbD seems to provide superior protection to si-eGFP. This could be expected since the complexation efficiency and, therefore, the affinity of fbD for si-eGFP is superior to that for si-PTEN (section 4.2.1).

fbB showed the highest protection capacity with an exceptional level of unaffected siRNA (for both si-PTEN and si-eGFP, no apparent discrepancies can be observed) even after 60 min of incubation (Figure 8C and 8D). This points again to the relevance of the extra hydrophobic contribution provided by the peripheral aromatic benzylamines in this dendrimer, which together with the hydrophobic spacers in GATGE provides excellent protection of siRNA from degradation. Once again, these results are consistent with the findings for both the excellent siRNA complexation efficiency and the small dendriplexes with very homogeneous populations obtained with fbB (section 4.2.1 and 4.2.2).

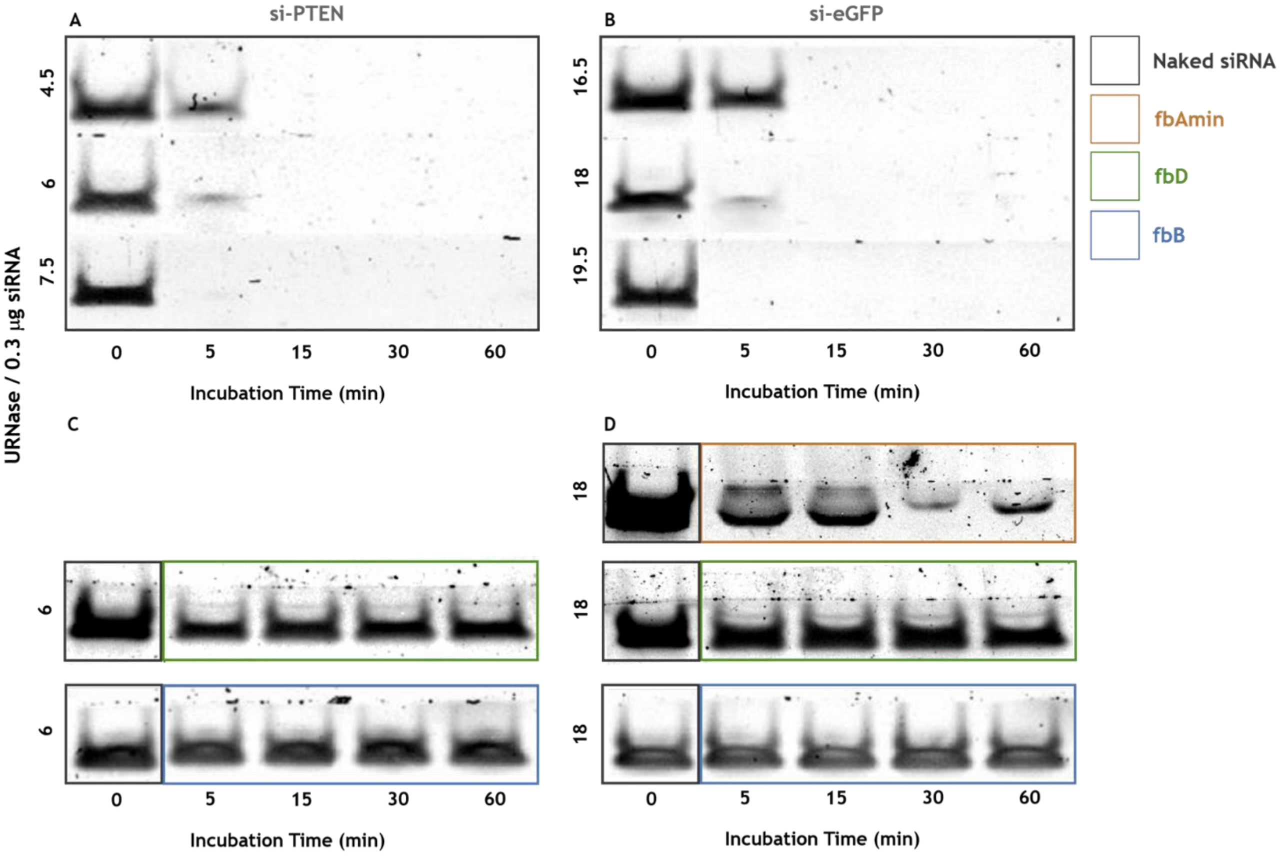

Figure 8 - Endonuclease protection assay. The concentration of siRNA required to degrade the siRNA was determined by incubating only the siRNA in RNase I solution (si-PTEN and si-eGFP, 8A and 8B respectively). Evaluation of the ability of the three dendrimers at N/P 80 (fbAmin, fbD and fbB) to protect siRNA, si-eGFP (8C) and si-PTEN (8D), from degradation determined by PAGE after 0, 5, 15, 30 and 60 min of incubation with RNase I (n=1).

Previous studies suggested that the PEG chains of PEGylated copolymers interfere with the formation of compact condensates, leading to less efficient endonuclease protection compared to non-PEGylated particles [103]. Nevertheless, in this study, our results suggest that the three dendrimers are capable of efficiently protecting the assessed siRNA sequences from degradation, with fbB and fbD ensuring an excellent level of protection even after 60 min.

4.3.2 Cellular association and uptake

Previously, in our group, the cellular association/internalisation of both fbD-based and fbB-based siRNA dendriplexes was consistently confirmed for single siRNA delivery. These studies were performed in U2OS with fbB and fbD dendriplexes carrying Cy5-labeled siRNA, and their ability to associate/internalise the membrane was evaluated by flow cytometry. These studies showed that both dendriplexes exhibited a very good ability to associate/internalise siRNA (especially fbB), with an increase of the association/cellular uptake with the increase of the N/P ratio [108].

In the context of the present dissertation, the cellular association/internalisation mediated by the fbD and fbB dendritic copolymers in dual delivery strategies has been assessed for the first time. This ability was also investigated by flow cytometry. U2OS eGFP/Luc cells were incubated at 37 ºC for 24 hours with dendriplexes carrying 1:1 mixture of Cy5-labeled siRNA and Cy3-labeled siRNA (Mix siRNA). Cy5 and Cy3 are cyanine fluorescence markers that yield bright and stable fluorescence which are commonly combined for two colour detection. Then, after the 24-hour incubation, the cells were analysed through flow cytometry.

As seen in Figure 9A and 9C, regarding Cy5 fluorescence intensity (FL), cells treated with dendriplexes showed a great shift to higher FL compared with untreated cells (NT). This result indicates the dendriplex cell association/internalisation and, therefore, the Cy5-siRNA association/internalisation. Both dendrimers showed to have an excellent ability to associate/internalise siRNA, as they present FL values very similar to those of Lipofectamine® 2000 (L2K, which is a standard agent for in vitro transfection), this is especially true at N/Ps ≥ 20. For all dendriplexes tested, fluorescence was detected in 100% or near 100% of the cells (Table S 1). While fbD and fbB have very similar performances, fbD showed to have a slightly higher FL swift, when compared with fbB, at lower N/Ps (5 and 20). This result could be explained by a higher cell membrane association/interaction of fbD dendriplexes, probably due to their higher positive surface charge which allows a more favourable interaction with the negatively charged cell membrane (in the near future the net surface charge of these delivery systems will be investigated with Laser Doppler Electrophoresis to confirm this premise). It is worth mentioning that no differences between single and dual delivery at N/P 80 were observed.

Regarding Cy3 FL, as can be observed in Figure 9B and 9D, it was not possible to observe a shift to higher FL of the cells treated with either the dendriplexes or L2K when compared with untreated cells. However, untreated cell presented a high FL value; accordingly, one can conclude that there might have been some interferences of the cells GFP fluorescence in the Cy3 detector. Nevertheless, the cells treated with either fbD or fbB based dendriplexes presented a slightly superior percentage of cells with Cy3 fluorescence when compared with untreated cells (table S1). In order to confirm the association/internalisation of both NAs, additional experiments should be done using U2OS cells without the expression of the eGFP-Luciferase fusion protein.

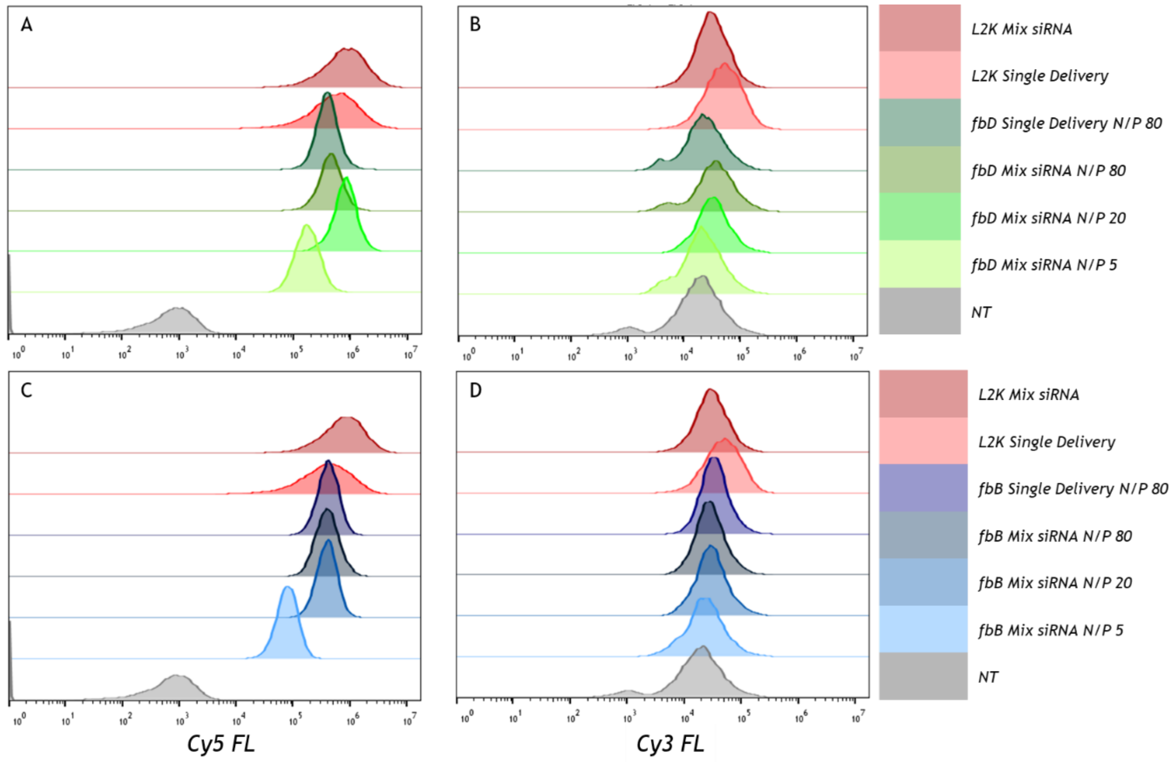

Figure 9 - Flow cytometry characterization of the cellular association of dendriplexes. Dendriplexes carrying a 1:1 mixture of Cy5-labeled siRNA and Cy3-labeled siRNA (Mix siRNA) were incubated for 24 hours with U2OS eGFP/Luc cells at a final NA concentration of 0.1 µM. The highlighted area corresponds to populations of cells with high relative FL Lipofectamines 2000 (L2k) was used as a control according to the manufacturer's instructions. (n=1) Mix siRNA - 1:1 mixture of Cy5- and Cy3-labeled siRNA, L2k Mix siRNA – 1:1 mixture of Cy5- and Cy3-labeled siRNA transfected with Lipofectamine® 2000, L2k Single Delivery – transfection of either Cy5- and Cy3-labeled siRNA with Lipofectamine® 2000, NT – untreated cells

4.3.3 Transfection Efficiency

As discussed earlier, to obtain proper transfection efficiencies values by delivery of a single siRNA to the target cells is challenging due to the previous describes barriers and obstacles (section 1.2). Most examples found in today's literature regarding siRNA-based systems report single delivery. However, even though it is still a fledgeling area, one can also find a growing number of examples of combined delivery of two or more siRNAs or of siRNAs and other NAs. Recently, some authors reported a synergistic effect upon co-delivery of multiple NAs compared with single NA delivery (section 1.3). Thus, in the present dissertation, we propose the study the simultaneous delivery of two siRNAs (si-PTEN and si-eGFP) aiming at downregulating different target mRNAs, using dendrimers as delivery vectors for the first time. We aim to evaluate the impact of this dual siRNA delivery on gene silencing efficiency when compared with the single delivery.

To carry out the study, fbD and fbB were used as carriers, and their performances were compared. The dendriplexes were prepared, at N/P ratios ranging from 5 to 80, with either one of the two siRNAs (si-PTEN and si-eGFP) — Single Delivery —, or with a 1:1 mixture of both siRNAs (Mix of siRNA) — Dual Delivery. The ability of the developed dendriplexes to transfect and mediate gene silencing was evaluated by incubating U2OS/eGFP-Luc cells in the presence of the dendriplexes for 24 h.

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 [119]. 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. Hence, the silencing of eGFP was assessed through FACS, by measuring the median fluorescence intensity, expressed relative to the median fluorescence intensity of untreated cells.

Regarding the silencing effect on the eGFP gene, one can observe that for both dendriplexes, there was a decrease in fluorescence intensity compared to untreated cells (Figure 10). In both cases, the percentage of GFP silencing seems to be relatively constant for all N/P ratios, with fbD dendriplexes reaching a maximum in silencing of 26%, whereas fbB dendriplexes had a slightly superior silencing effect (reaching its highest at 29 % of GFP silencing). Nevertheless, the GFP silencing profiles of the two dendriplexes were very comparable, as expected, these findings are in accordance with those obtained in the cellular association/internalisation studies, in which one can observe that the fbD and fbB based dendriplexes accomplished very similar results related to siRNA association/internalization (section 4.3.2).

Altogether, it is worth mentioning that the FL percentage values of both dendrimers still fall far from the results obtained using the in vitro standard control, L2k, as vector (Figure 10). And also, far from those gene silencing values previously obtained by our group in the single delivery of the same siRNA to the same cells (U2OS/eGFP-Luc) using the same vectors (fbD and fbB). In these studies, a si-eGFP 60% and 50% of silencing were obtained using fbB and fbD, respectively, at all N/P ratios. One possible reason for this difference in the eGFP silencing values obtained in the present work is the fact the used cells presented high passage number, which might change gene expression profile, diminishing the percentage of expression (especially in cloned genes such as GFP). Additionally, excessive passaging is likely to negatively affect transfection efficiency.

Regarding the impact of a dual siRNA delivery system on gene silencing efficiency when compared with single delivery, the performance of both fbD and fbB based dual delivery dendriplexes was very comparable to their respective single delivery performance (except in the case of fbD dendriplexes, at N/P 5 and N/P 20, in which the dual delivery strategy showed a slightly inferior silencing effect). Overall, it was not possible to observe a clear synergistic effect for now.

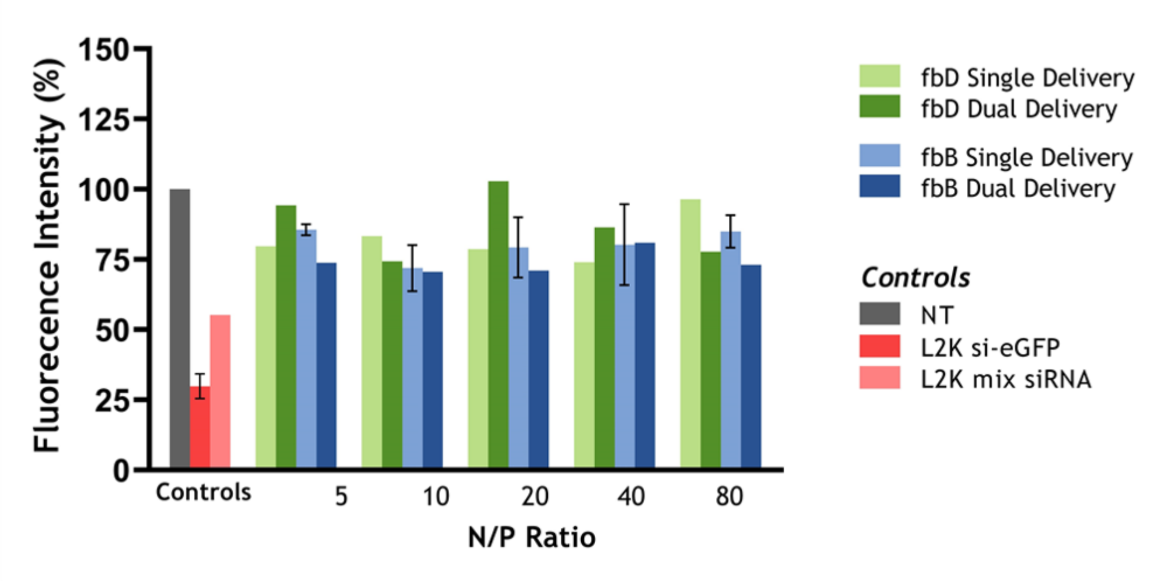

Figure 10 - eGFP silencing. The silencing efficiency of eGFP in both single and dual siRNA delivery mediated by fbD and fbB was assessed through FACS. The silencing effect was determined based on the fluorescence intensity of treated cells relative to non-treated cells (n = 3 for fbB Single Delivery and n=1 for the remaining). NT - non-treated cells, L2k si-eGFP – si-eGFP transfected with Lipofectamine® 2000, L2k mix siRNA –transfection of a si-PTEN/si-eGFP 1:1 mixture with Lipofectamine® 2000.

Regarding the downregulation of PTEN, it was only possible to study the fbB based single delivery system (Figure 11) due to a time limitation. Overall, the obtained PTEN silencing was superior to that of si-eGFP, reaching a maximum silencing of 71%, at N/P 80. The percentage of PTEN silencing remains relatively constant for all N/P ratios. Remarkably, fbB allowed a higher downregulation of PTEN than the control transfection reagent, Lipofectamine® 2000 (71% vs 50%). This seems to indicate that fbB is an excellent delivery vector for anti-PTEN siRNA.

All in all, both fbD and fbB showed to be able to protect NAs from endonuclease degradation efficiently, promoted efficient cellular uptake and induced silencing in both single and dual siRNA delivery approaches to some extent. Although, for now, it was not possible to observe a clear synergistic effect for now in the case of e-GFP, only one independent experiment was conducted in the case of si-eGFP silencing studies with dual delivery systems, thus more replicates/studies have to be done in order to confirm the lack of synergistic effect. Additionally, considering the excellent si-PTEN downregulation results obtained with fbB based single delivery, si-PTEN silencing efficiency experiments for dual delivery will carry out to determine whether or not synergistic effect can be observed with these dual siRNA delivery systems.

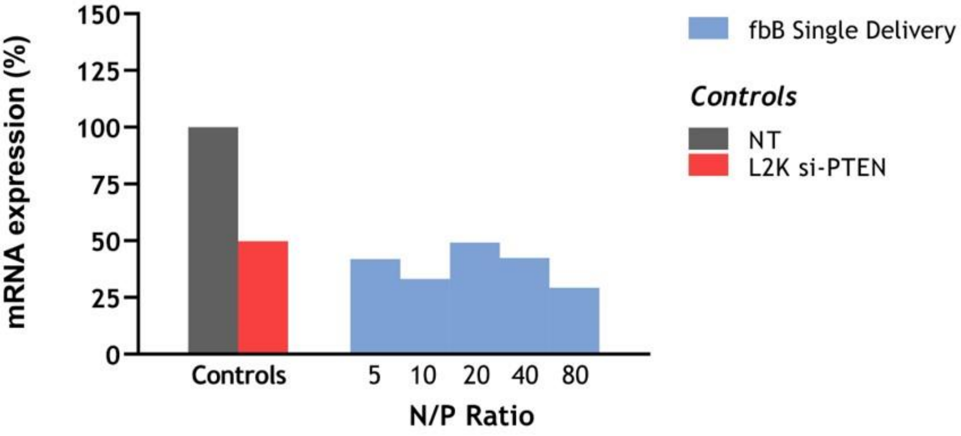

Figure 11 - PTEN silencing. The silencing efficiency of PTEN in single siRNA delivery mediated by fbB was assessed through qRT-PCR. The silencing effect was determined based on the expression of the PTEN mRNA relative to non-treated cells (n = 1). NT - non-treated cells; L2k si-PTEN – si-PTEN transfected with Lipofectamine® 2000.

4.4 Future Perspectives

The physicochemical properties of the fbAmin-, fbD- and fbB-based siRNA dendriplexes together with their biological performance, demonstrated the potential of these dendriplexes to act as single and multiple siRNA vectors. Nevertheless, there are still some studies to be done regarding the physicochemical characterisation dendriplexes and a lot to investigate regarding the biological performance of the dendriplexes for dual siRNA delivery.

Since the net surface charge of a delivery system is a significant factor determining cellular interaction and uptake efficiency (section 1.2), the zeta potential of the fbD- and fbB-based dendriplexes complexing a si-eGFP mimic was previously evaluated by laser Doppler electrophoresis in our group, obtaining a net positive charge in general [108]. However, it will be interesting to evaluate the surface charge for all developed dendriplexes (between the proposed dendrimers and all the studied siRNA sequences), to understand if the complexation between the same dendritic vector with the distinct siRNA sequences affects the zeta potential of the delivery system. Thus, in the near future, the zeta potential of all developed dendritic delivery systems with all the studied siRNA sequences will be determined.

Thinking of intravenous administration, another interesting propriety to be assessed is the stability of the dendriplexes in blood circulation and ability to penetrate the ECM to reach the target cells as the vectors can be destabilised leading to premature NA release. Additionally, after reaching the cytoplasm of target cells, the carrier has to be able to release siRNA, so this can execute its function. Accordingly, a balance between efficient complexation and NA release is necessary. To evaluate these mentioned abilities, the stability of dendriplexes in the presence of heparin, a polyanion commonly used to study the destabilisation and release of NAs from dendriplexes, will be assessed.

Additionally, as we study the dual delivery with a si-PTEN/si-eGFP 1:1 mixture, it might be interesting to investigate the complexation efficiency of the dendriplexes prepared with a mixture of the studied siRNAs. Since we have shown that the same dendrimer may interact differently with different siRNA sequences, it is very likely that it might also be the case for mixtures of these sequences.

Regarding the biological performance, although the internalisation of both fbB-based and fbD-based siRNA dendriplexes has been consistently confirmed in our group for single siRNA delivery, the dual siRNA internalisation mediated by the fbB and fbD copolymers has been assessed for the first time in the present work with a pilot study. Thus, additional internalisation experiments will be performed with previously mentioned alterations to the protocol (using U2OS cells without the expression of the eGFP/Luciferase fusion protein).

As mentioned before, the silencing results obtained in the present studies regarding the single delivery of anti-eGFP siRNA delivery, are lower than those reported by our group using the same cells, siRNAs and vectors (si-eGFP silencing of 50 % and 60% of at all N/P ratios studied using fbB and fbD, respectively). Thus, the next step in this project will be to perform additional si-eGFP silencing efficiency experiments, for both single and dual delivery, using cells with a lower passage number (due to the reasons mentioned in Section 4.3.3) in order 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 si-PTEN single delivery, and si-PTEN silencing efficiency experiments following a dual delivery strategy will be carried out.

A long-term, and when reached the above-mentioned main objectives of the present work, the biological in vitro characterisation of these (dual) siRNA delivery systems will also be performed in other cells, such as a neuronal cell line and, later, in primary cortical neurons. Moreover, in the future, the 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 functionalisation of our dendriplexes with a neuronal-specific targeting moiety.

Chapter 5

Concluding Remarks

The Human genetic code encodes information for every biological process needed for life and death. Given the undeniable importance of this information, it was inevitable for investigators to turn to it in search of novel therapies; hence, arises genetic therapy. Amid the gene therapy approaches, currently, gene silencing, taking advantage of the naturally occurring RNAi machinery, is the most investigated strategy for short-term gene downregulation. Within the different RNAi techniques, siRNA is one of the most promising. Notwithstanding the potential of this approach, these small therapeutic RNAs exhibit reduced stability in the bloodstream and are promptly recognised by the RES or MPS, or degraded by endonucleases. Furthermore, due to the size and negative charge of siRNAs, they are unable to cross the cell membrane and enter the cell.

Gene therapy demands a detailed design of delivery systems that can safely and efficiently deliver NAs to cells. These NA vectors are divided into viral and non-viral vectors. While viral vectors allow high transfection efficiency, researchers have been concentrating their efforts on the development of non-viral vectors because they offer a more favourable safety profile. Dendrimers are up-and-coming candidates as non-viral vectors for NAs delivery due to their unique features as globular, well-defined, and very branched structure, tuneable nanosizes, low polydispersity and the presence of several functional groups that enable the surface multifunctionalization according to the desired properties. Most dendrimers used as NAs vectors are cationic dendrimers due to their ability to efficiently compact NAs into electrostatic complexes called “dendriplexes”. However, the use of the most common dendrimers as NA vectors still face some problems related to bioaccumulation and subsequent cytotoxicity, which is primarily caused by their nonbiodegradability. Consequently, the focus has been turned to engineering biodegradable dendritic structures that degrade into small fragments under physiological conditions and, thus, can be readily excreted from the organism. Nevertheless, to develop biodegradable dendrimers is a very challenging task, since one can have undesirable and/or premature degradation during synthesis, purification, functionalisation or even during subsequent application steps. Accordingly, a reduced amount of biomedical studies has been performed using biodegradable dendrimers.

Due to the unmet need for an effective, safe and biodegradable NA delivery vector for gene therapy applications, our nBTT group proposed two new families of fully biodegradable PEG-dendrimers, based on the repeating unit GATGE, to serve as vectors in nanomedicine. They were functionalized at their surface with three different amine groups: a single amine, a diamine and a benzylamine. In this dissertation, we have started to explore the potential of these fully biodegradable PEG-GATGE dendrimers as nanocarriers of different siRNA sequences for dual delivery.

The ability of the three different proposed dendrimers (fbAmin, fbD and fbB) to complex four distinct siRNA sequences (si-PTEN, si-eGFP, Sequence A and Sequence B) was investigated. The three amine-terminated dendrimers proved to complex siRNA efficiently, with fbB and fbD exhibiting excellent binding profiles. Moreover, besides the differences in performance between dendrimers with different peripheral modifications, it has been possible to observe some variations in the complexation of the same dendrimer with each of the four different siRNA sequences. While fbAmin shows a clear preference for anti-PTEN siRNA, fbD appears to complex more efficiently to anti-GFP siRNA and sequences A and B. Although it displays an overall excellent complexation with all sequences and at all N/P ratios, fbB seems to interact more efficiently with anti-PTEN and anti-GFP siRNA, especially at low N/Ps. Regarding the sizes of the resulting dendriplexes, it could be inferred that all of them present very suitable nanosize (< 100 nm) for cellular internalisation and, therefore, have a great interest for many biomedical applications. Especially, fbB-based dendriplexes showed exceptionally small sizes and homogenous populations, probably due to the extra hydrophobic contribution provided by the peripheral aromatic benzylamines in the fbB dendrimer. In this case, the determinant factor for dendriplexes size and PDI was mainly the dendrimer, namely its surface functional group.

All developed dendriplexes also showed to be capable of protecting the carried siRNA sequences against endonucleases degradation, with fbD and fbB ensuring an excellent level of protection even after 60 min.

Related to fbD and fbB biological performance as vectors for single and dual siRNA delivery (si-PTEN and si-eGFP), both dendrimers showed to have an excellent ability to associate/internalise both siRNA (no differences between single and dual delivery were observed) and led to moderate downregulation of eGFP, while an excellent silencing of PTEN was obtained using fbB as carrier. Regarding the former (eGFP silencing), the transfection efficiency performance of both dendrimers (fbD and fbB) were very similar (which is in accordance with the cellular uptake findings), but fbB showed a slightly superior gene silencing. Concerning the co-delivery of two siRNAs and the possibility of obtaining a synergistic effect, it was not possible to observe a clear synergistic effect for now.

Concluding, both the physicochemical properties of the fbAmin-, fbD- and fbB-based siRNA dendriplexes together with their biological performance, demonstrate the potential of PEG-GATGE dendritic block copolymers to act as single and multiple siRNA 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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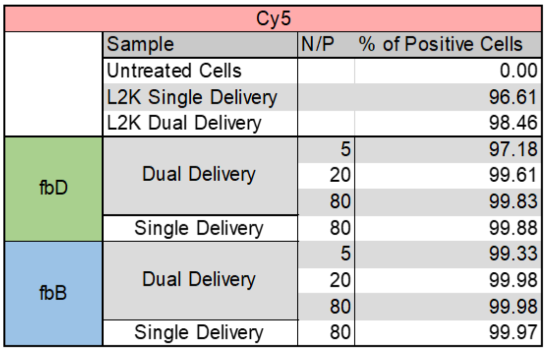
Supplementary Information

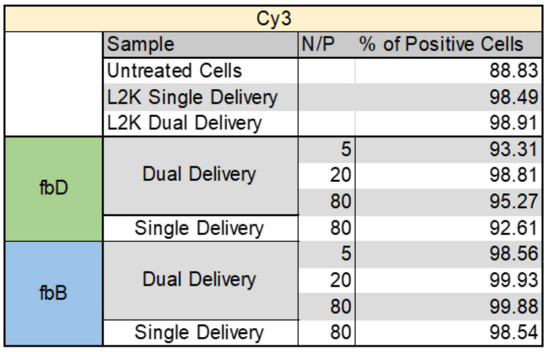
Table S 1 - Percentage of cells with Cy5 (S1A) and Cy3 (S1B) fluorescence, 24 hours after incubation with dendriplexes or Lipofectamine complexes.

A



Cy5			
	Sample	N/P	% of Positive Cells
	Untreated Cells		0.00
	L2K Single Delivery		96.61
	L2K Dual Delivery		98.46
fbD	Dual Delivery	5	97.18
		20	99.61
		80	99.83
	Single Delivery	80	99.88
fbB	Dual Delivery	5	99.33
		20	99.98
		80	99.98
	Single Delivery	80	99.97

B



Cy3			
	Sample	N/P	% of Positive Cells
	Untreated Cells		88.83
	L2K Single Delivery		98.49
	L2K Dual Delivery		98.91
fbD	Dual Delivery	5	93.31
		20	98.81
		80	95.27
	Single Delivery	80	92.61
fbB	Dual Delivery	5	98.56
		20	99.93
		80	99.88
	Single Delivery	80	98.54