

MASTER THESIS

Biodegradable dendrimers as vehicles for siRNA delivery to treat peripheral nerve injury

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Abstract

Peripheral nerve injuries are tremendously impactful conditions for which new treatment options are urgently needed. Preclinical evidence suggests that targeted inhibition of the proteins PTEN and Rac1 through small interfering RNAs (siRNAs) are promising therapeutic strategies. However, the clinical application of siRNAs to extrahepatic tissues is presently limited by the lack of appropriate delivery technologies. Dendrimers have been put forward as efficient nucleic acid vectors due to their highly-branched architecture, tunable nanosize, low polydispersity, and multivalency. However, translation efforts have been hindered by toxicity resulting from the non-biodegradable character of most of the explored dendritic systems. To address this challenge, we recently proposed a novel family of fully biodegradable PEG-GATGE (PolyEthylene Glycol - Gallic Acid Triethylene Glycol Ester) dendritic block copolymers. These dendrimers functionalized with benzylamine moieties (fbB) showed great promise as siRNA carriers, forming nanoscale complexes (dendriplexes) which were efficiently internalized by cells (including neural) and mediated the silencing of a reporter protein.

The present dissertation explored the application of the fbB dendritic block copolymer as a carrier for therapeutic siRNAs targeting PTEN and Rac1 in neuronal cells. Efficient complexation of both siRNAs (75 to 89%) was achieved for two of three dendrimer/siRNA molar ratios tested (N/P 10 and N/P 20) and the resulting dendriplexes displayed promising physicochemical properties, including small sizes (44-54 nm), spherical morphologies, positive surface charges, and a good siRNA release profile. However, under the conditions tested, the formed dendriplexes failed to deliver enough siRNA molecules to elicit a detectable downregulation of their target messenger RNAs in the ND7/23 neuronal cell line.

Dendriplex exocytosis was also investigated through the use of fbB-dendriplexes carrying a fluorescently labeled siRNA mimic (siRNA_{mi}). Fluorometry measurements in the extracellular medium of dendriplex-treated cells revealed evidence of exocytosis, which requires future re-assessment. These fluorescently labeled dendriplexes were also employed to evaluate cellular internalization in dorsal root ganglion explant cultures, a model of higher biological complexity. High-throughput image analysis highlighted the remarkable ability of fbB-dendriplexes to mediate the association and uptake of siRNA_{mi}, without a significant impact on neurite outgrowth capacity (cytotoxicity).

Taken together, these findings expand our knowledge of the biological performance of fbB-based dendriplexes and motivate future efforts to improve their suitability for *in vitro* and *in vivo* applications.

Keywords: dendrimers, siRNA, peripheral nerve injury, PTEN, Rac1, nanomedicine, nanoparticles, gene therapy, exocytosis

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Resumo

As lesões de nervos periféricos são condições com um impacto tremendo que requerem urgentemente novas opções terapêuticas. Estudos pré-clínicos sugerem que a inibição das proteínas PTEN e Rac1 através de pequenos RNAs de interferência (siRNAs) é uma estratégia terapêutica promissora. Contudo, a aplicação clínica de siRNAs a tecidos extra-hepáticos é presentemente restringida pela falta de tecnologias de entrega apropriadas. Os dendrímeros têm sido propostos como vetores eficientes de entrega de ácidos nucleicos devido à sua arquitetura ramificada, nanoescala ajustável, baixa polidispersão e multivalência. No entanto, os esforços da sua translação para a clínica têm sido limitados por efeitos tóxicos resultantes do caráter não-biodegradável da maioria dos sistemas dendríticos explorados até à data. Para responder a este desafio, o nosso grupo propôs recentemente uma nova família de copolímeros em bloco denominada PEG-GATGE (PoliEtileno Glicol - Ácido Gálico Trietilen Glicol Ester), os quais são completamente biodegradáveis. Estes dendrímeros funcionalizados com benzilamina (fbB) mostraram grande potencial enquanto vetores para siRNA, formando nanocomplexos (dendriplexos) que são eficientemente internalizados por células (incluindo neuronais) e mostraram ser capazes de mediar o silenciamento de uma proteína repórter.

A presente dissertação explorou a aplicação do copolímero em bloco fbB como vetor para entrega de siRNAs terapêuticos contra PTEN e Rac1 em células neuronais. Uma complexação eficiente de ambos siRNAs (75 a 89%) foi atingida para dois dos três rácios molares dendrímero/siRNA testados (N/P 10 e N/P 20) e os dendriplexos resultantes mostraram propriedades físico-químicas promissoras, incluindo pequenas dimensões (44-54 nm), morfologias esféricas, carga positiva, e um bom perfil de libertação. Contudo, sob as condições testadas, os dendriplexos formados falharam em entregar moléculas de siRNA suficientes para permitir uma diminuição de expressão detetável dos seus RNAs mensageiros alvo na linha neuronal ND7/23.

A exocitose dos dendriplexos foi também investigada, através do uso de um siRNA mimético (siRNAmi) com marcação fluorescente. Medições de fluorometria no meio extracelular de células tratadas com dendriplexos revelaram evidência de exocitose, a qual requiere investigação adicional. Estes dendriplexos marcados fluorescentemente foram também aplicados para avaliar a internalização em explantes dos gânglios da raiz dorsal, um modelo de maior complexidade biológica, relativamente a linhas celulares imortalizadas. Análise de imagem de alto rendimento realçou a capacidade notável dos fbB-dendriplexos para mediar a associação e captação de siRNAmi pelas células, sem um impacto significativo no crescimento de neurites (citotoxicidade).

No seu conjunto, estes resultados expandem o nosso conhecimento relativo à performance biológica de dendriplexos baseados nos dendrímeros fbB e motivam esforços futuros para melhorar a sua aptidão para aplicações *in vitro* e *in vivo*.

Palavras chave: dendrímeros, siRNA, lesões de nervos periféricos, PTEN, Rac1, nanomedicina, nanopartículas, terapia génica, exocitose

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Abbreviations

2'-F	2'-Fluoro
2'-OMe	2'- <i>O</i> -methyl
Ago2	Argonaute 2
ASGPR	Asialoglycoprotein receptor
CNS	Central nervous system
Cy5	Cyanine-5
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DRG	Dorsal root ganglion
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EGTA	Triethylene glycol diamine tetraacetic acid
fbB	Fully biodegradable, third-generation PEG-GATGE dendritic block copolymer functionalized with alkynated benzylamine
FBS	Fetal bovine serum
FI	Fluorescence intensity
GalNAc	<i>N</i> -acetylgalactosamine
GATGE	Gallic acid triethylene glycol ester
GSK-3β	Glycogen synthase kinase 3 beta
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L2k	Lipofectamine [®] 2000
LNP	Lipid nanoparticle
Luc	Luciferase
MIP	Maximum intensity projection
miRNA	MicroRNA
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NF	Nuclease-free
NT	Non-treated
PAGE	Polyacrylamide gel electrophoresis
PAMAM	Poly(amido amine)
PBS	Phosphate buffered saline
PDI	Polydispersity index

ABBREVIATIONS

PDL		Poly(D-lysine)
PEG		Polyethylene glycol
PI3K		Phosphoinositide 3-kinase
PIP2		Phosphatidylinositol 4,5-biphosphate
PIP3		Phosphatidylinositol 3,4,5-triphosphate
PIPES		Piperazine- <i>N,N'</i> -bis(2-ethanesulfonic acid)
PNI		Peripheral nerve injury
PNS		Peripheral nervous system
PPI		Poly(propylene imine)
PS		Phosphorothioate
PTEN		Phosphatase and tensin homolog deleted on chromosome 10
Rac1		Ras-related C3 botulinum toxin substrate 1
Rho GTPases		Ras homolog family of guanosine triphosphate hydrolases
RISC		RNA-induced silencing complex
RLC		RISC-loading complex
RNAi		RNA interference
RT		Room temperature
RT-qPCR		Reverse-transcription quantitative polymerase chain reaction
SD		Standard deviation
SDS		Sodium dodecyl sulfate
shRNA		Short hairpin RNA
siGFP		Anti-eGFP siRNA
siPTEN		Anti-PTEN siRNA
siRac1		Anti-Rac1 siRNA
siRNA		Small interfering RNA
siRNA_{mi}		siRNA mimic
TBE		Tris-borate-EDTA
TEM		Transmission electron microscopy
ZP		Zeta potential

Chapter 1

Introduction

1.1 Peripheral nerve injuries

This section starts with a brief presentation of the anatomy of the peripheral nervous system (PNS) (subsection 1.1.1). This is followed by an overview of peripheral nerve injuries, which includes causes and socioeconomic impact (subsection 1.1.2), pathophysiology and clinical management (subsection 1.1.3), and novel treatment approaches (subsection 1.1.4). Finally, two relevant therapeutic targets in context of PNI are presented: PTEN (subsection 1.1.5) and Rac1 (subsection 1.1.6).

1.1.1 Peripheral nervous system anatomy

The PNS encompasses all nervous tissue that lies outside the brain and the spinal cord, which collectively form the central nervous system (CNS) [1] (Figure 1.1a). The cell bodies of PNS neurons form clusters called ganglia, whereas their axons are arranged in cable-like units called nerves, which connect ganglia to sensory receptors, muscles, and glands [1]. Within each peripheral nerve, axons (also named fibers) are organized in multiple longitudinal bundles called ‘fascicles’ and are covered by three layers of connective tissue, which provide mechanical support and irrigation (Figure 1.1b): the epineurium (the outermost layer) surrounds the whole nerve, the perineurium coats each fascicle, and the endoneurium covers each fiber [2, 3]. Other cellular components of the nerve are Schwann cells, which provide the myelin sheath for myelinated axons [3].

Regarding their anatomical location, peripheral nerves are divided into cranial nerves (12 pairs) and spinal nerves (31 pairs) [2] (Figure 1.1a). The former emerge from the brain and innervate anatomic structures of the head and neck. The latter arise from several rootlets along the spinal cord, which combine to form two main roots: the ventral root and the dorsal root [2] (Figure 1.1b). The ventral root contains the axons of motor neurons, whose cell bodies are located in the anterior horn of the spinal cord [2]. On the other hand, the dorsal root contains sensory neurons, whose cell bodies are located in the dorsal root ganglion (DRG) and whose axons split into two branches: one that extends peripherally through the spinal nerve and another that projects into the posterior

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horn of the spinal cord [2]. Each spinal nerve is formed by the combination of nerve fibers from a dorsal and a ventral root and, as such, carries both sensory and motor information [2].

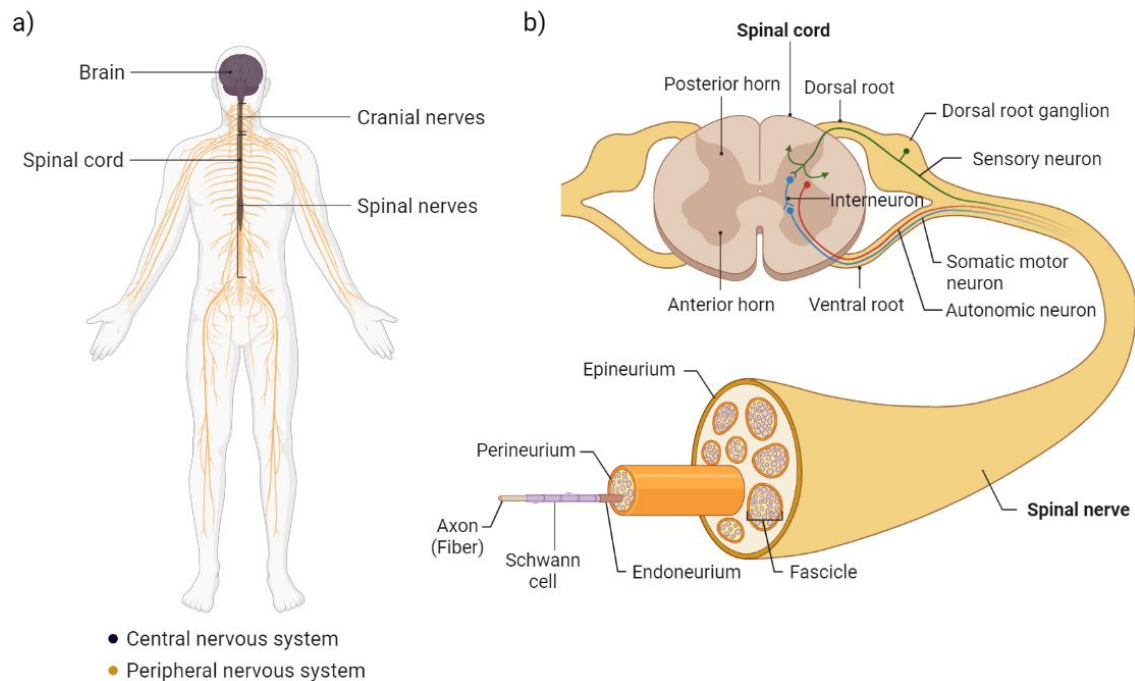


Figure 1.1 | Anatomy of the peripheral nervous system. a) The major components of the nervous system. **b)** Cross section of the spinal cord, showing the location of the dorsal root ganglion, the sensory and motor neurons, and the general nerve structure. Created with BioRender.com.

1.1.2 Causes and socioeconomic impact

The PNS is particularly vulnerable to injury, which can be due to traumatic insults (e.g. vehicle collisions, lacerations, falls, fractures, etc.) or nontraumatic causes (e.g. diabetes, immune system diseases, tumors, drug toxicity, etc.) [4]. Traumatic peripheral nerve injuries, often simply referred to as peripheral nerve injuries (PNI), are particularly impactful because they commonly affect young adults and can lead to significant morbidity and lifelong reductions in quality of life [5, 6].

A recent study analyzed a cohort of over 1.2 million United States patients aged 18 to 64 who suffered extremity trauma between 2010 and 2013 [7]. It found that the overall incidence of PNI within two years after trauma was 2.3%. Moreover, extremity trauma patients diagnosed with PNI were more likely to suffer from chronic pain and to use physical therapy and occupational therapy services more than 3 months after the injury. Another study analyzed data from the US National Inpatient Sample (NIS) database from 2001 to 2013 and estimated that approximately 171 000 patients experienced upper extremity PNI during this period, corresponding to an average annual incidence of 43.8 per 1 million people [6]. The mean age of patients was 38.1 years, reflecting the disproportionate impact of this condition on the young and middle-aged adult population. Furthermore, cost analysis revealed that the average inpatient charge was \$47 004, with a mean

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increase of \$4623 (9.6%) per year. This figure rises significantly if other potential costs are considered, such as long-term treatment and rehabilitation, sick leave, and disability pensions [8].

1.1.3 Pathophysiology and clinical management

According to Sunderland's classification system (Figure 1.2), PNIs can be classified into one of five grades, based on the increasing severity of nerve damage [9–11]. Grade I, the least severe, usually corresponds to nerve compression, where the myelin sheath is damaged but axonal continuity is preserved. Grades II, III, and IV involve loss of axonal continuity and are typically associated with crush or stretch injuries. These grades are distinguished based on the continuity of the endoneurium (compromised in grades III and IV) and the perineurium (compromised in grade IV). Grade V injuries correspond to complete nerve transection, where all three layers of connective tissue (endoneurium, perineurium, and epineurium) are disrupted. More recently, Sunderland's classification was updated with a sixth-degree injury, which corresponds to mixed injuries within the same nerve [9, 10].

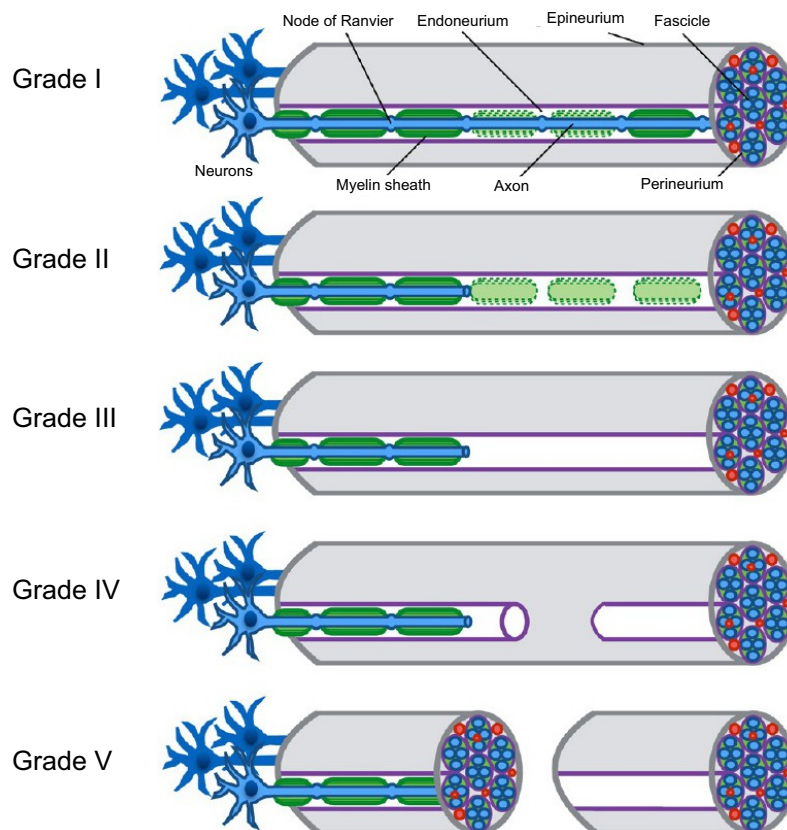


Figure 1.2 | Sunderland's classification of nerve injuries. Grade I: the myelin sheath is temporarily damaged but axonal continuity is preserved. Grade II: the axon is damaged, but the three layers of connective tissue of the nerve (epineurium, perineurium, endoneurium) are preserved. Grade III: the axon and endoneurium are damaged, but the epineurium and perineurium are intact. Grade IV: disruption of the axon, endoneurium, and perineurium, but not the epineurium. Grade V: complete nerve transection. Reprinted from Caillaud et al. (2019) [10] with permission from Wolters Kluwer Medknow Publications.

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The extent of nerve damage will largely determine the intrinsic capacity of recovery and the need for surgical intervention [9]. If axonal continuity is preserved (grade I injuries), a fast and complete recovery without surgery is expected [9, 12]. On the other hand, if there is axonal disruption, spontaneous recovery will be slow at best (grades II and III) and non-existent at worst (grades IV and V) [12]. The main reason for this is that axonal disruption will trigger the degeneration of the axon segment distal to the injury site, a process called Wallerian degeneration [10, 13]. This is typically followed by a regeneration stage, where a growth-promoting microenvironment will stimulate the regrowth of axons from the proximal injury end [10, 13]. Whether this leads to complete recovery is dependent on several factors. In certain cases, the distance between the injury site and the denervated target might require several months (or even years) of elongation, considering the typical axonal regeneration rate of 1-2 mm per day [12]. Since the microenvironment's capacity to support regeneration usually declines after 1-2 months [13], complete recovery in those cases is very unlikely. Furthermore, the state of preservation of the connective tissues of the nerve is critical to ensure effective axonal regrowth because these tissues will act as scaffolds for the regenerating axons [10]. Thus, while a grade II injury is likely to culminate in complete recovery without surgical intervention, spontaneous recovery in grade V injuries is nearly impossible [9, 10, 12].

To enhance the recovery potential after PNI, several surgical approaches are available. These aim to aid axonal regrowth across the injury site by reestablishing continuity between the connective tissues of the nerve [12]. For small nerve gaps (< 1 cm), the most common intervention is direct nerve repair, which involves suturing the proximal and distal nerve stumps [3, 12]. For larger nerve gaps, where direct tensionless repair is not possible, the gold standard intervention is autologous nerve grafting [3, 9, 12]. This involves using a 'less important' functioning nerve to bridge the gap between the damaged nerve ends. However, this has some drawbacks, such as donor site morbidity and the potential for scarring due to size and fascicle mismatch [3, 9]. If no adequate nerve autografts are available, nerve allografts from cadaveric donors can be used instead [3, 9]. In this case, no healthy donor nerve is sacrificed but long-term immunosuppression is required to avoid rejection [9]. Another alternative to autologous nerve grafting is nerve guide conduits, which are commercially available hollow tubes made from natural (collagen) or synthetic biomaterials (poly lactic-co-glycolic acid, polycaprolactone) [9, 14]. The problem with current nerve guide conduits is that they act merely as physical scaffolds and do not provide Schwann cells or neurotrophic factors needed for effective nerve regeneration [9, 14]. So far, their application is mostly restricted to small sensory nerve repair [9].

The decision of whether to operate is not straightforward, especially in cases when the full extent of nerve damage is unknown [9]. On the one hand, relying solely on spontaneous regeneration may culminate in incomplete recovery and permanent loss of nerve function. On the other, unnecessary surgery can lead to increased pain and prolonged recovery times. Furthermore, surgery outcomes are often unpredictable, as they are influenced by a myriad of factors such as the age of the patient, the location and type of injury, the timing of surgery, etc. [9, 11]. In many cases, functional recovery after PNI is unsatisfactory [3, 11]. Thus, there is a clear clinical need for new therapeutic

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approaches that can be used alone or in combination with surgical intervention to improve functional outcomes after PNI [11].

1.1.4 Novel treatment approaches

There are several non-surgical approaches under development for the treatment of PNI, including pharmacological treatments, stem cell-based therapies, and gene therapies.

In regards to pharmacological agents, several have been proposed to improve nerve regeneration, including neurotrophic factors (e.g. NGF, BDNF), steroid hormones, erythropoietin, among others [3, 11, 14]. The application of neurotrophic factors is particularly attractive due to their endogenous roles in promoting axonal regrowth after PNI [3]. However, clinical administration of neurotrophic factors (as well as other pharmacological agents) is complex due to their multiple biological actions, short half-lives, and the need to ensure spatially and temporally controlled delivery for optimal therapeutic action [14–16].

Stem cell-based therapies have also been shown to promote recovery after PNI through cell-to-cell contact, differentiation into Schwann-like cells, and release of signaling cues that promote axonal regeneration [3]. However, these therapies are still in an early stage of development. Clinical translation is currently limited by a lack of robust cell expansion and differentiation protocols, high production costs, and safety concerns [14].

Lastly, gene therapies are also promising in the context of PNI [15–17]. Here, the pharmacological agent is a nucleic acid (DNA or RNA) that modulates the expression of a target gene. These therapies are highly versatile and often have a longer-lasting effect than conventional drug approaches [16]. DNA or messenger RNA (mRNA) can be delivered to increase the production of proteins that support axonal regeneration, such as neurotrophic factors or transcription factors that drive the expression of regeneration-associated genes [15, 17]. Conversely, nucleic acids such as microRNAs (miRNAs) or small interfering RNAs (siRNAs) can be used to downregulate genes that inhibit regenerative processes [18, 19]. Either way, the goal is to support axon regeneration until complete reinnervation is achieved.

The unraveling of the pathophysiological mechanisms underlying PNI constantly identifies new signaling pathways and molecular targets with key roles in this condition. The modulation of these players (either through conventional drugs or gene therapy) can constitute innovative treatment routes for PNI. In the following sections, two targets whose modulation has proven particularly promising are presented: PTEN and Rac1.

1.1.5 Phosphatase and tensin homolog deleted on chromosome 10

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was first identified in 1997 as a tumor suppressor gene mutated in multiple human cancers [20] and was later shown to play a pivotal role in neuronal growth and regeneration.

Early *in vitro* studies revealed that PTEN overexpression in hippocampal neuron cultures hampers axon formation [21, 22], whereas PTEN inhibition induces axon elongation [23]. The

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first *in vivo* study to explore PTEN deletion in the context of neuron regeneration was reported in 2008 by Park et al. [24]. PTEN knockout following optic nerve crush injury resulted in improved survival and robust axon elongation in adult CNS neurons (retinal ganglion cells), a result that was corroborated by later studies [25, 26]. Additionally, deletion or siRNA-mediated inhibition of PTEN was also associated with improved regeneration and functional recoveries in several spinal cord injury models [27–29].

Christie et al. [18] were the first to investigate the role of PTEN in the context of PNI. Using cultures of adult sensory neurons from rats previously subjected to nerve injury, they found that there is a post-injury downregulation of PTEN mRNA in neuronal cell bodies, but the active PTEN protein remains present and is detrimental to regeneration. Pharmacological or siRNA-mediated inhibition of PTEN accelerated neurite outgrowth, both in cultured neurons and in an animal model of sciatic nerve transection injury. In line with these results, a miRNA expression profiling study found that miR-222, a miRNA that targets PTEN, is upregulated in DRGs following sciatic nerve injury [19]. Overexpression of miR-222 increased neurite elongation, and a similar effect was observed using siRNA-mediated PTEN knockdown. Chen et al. [30] also reported the endogenous inhibition of PTEN expression during remyelination after PNI. *In vivo* pharmacological inhibition of PTEN following sciatic nerve crush injury had a positive effect on several measures of functional recovery, including nerve fiber myelination, nerve conduction velocity, and compound muscle action potential amplitude. Finally, PTEN knockdown also enhanced functional recovery in injured neurons in diabetic mice [31], as well as motor neuron survival and axonal growth in a mouse model of spinal muscular atrophy [32].

The main reason why PTEN acts as a roadblock for axon regeneration is its negative regulation of the PI3K/Akt signaling pathway, which is schematically represented in Figure 1.3. This pathway is initiated by growth factor-mediated activation of tyrosine receptor kinases, which, in turn, activate the phosphoinositide 3-kinase (PI3K) [33]. Activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP3), whose accumulation leads to membrane recruitment and phosphorylation of Akt [33]. Activated Akt phosphorylates other downstream targets, including the glycogen synthase kinase 3 beta (GSK-3 β) and the mammalian target of rapamycin (mTOR) [23, 33]. Phosphorylation inactivates GSK-3 β , which favors axon elongation and growth cone remodeling by modulation of cytoskeleton dynamics [22]. Conversely, phosphorylation activates mTOR, resulting in the induction of protein synthesis [24].

Through its lipid phosphatase activity, PTEN counteracts PI3K signaling, converting PIP3 into PIP2 [33, 35]. This prevents downstream activation of Akt and mTOR, thereby inhibiting axon growth. Releasing this “PTEN-induced brake” is emerging as a promising strategy to promote regeneration following PNI [33, 36]. Nevertheless, due to the tumor suppressor role of PTEN, a valid clinical concern is that systemic PTEN knockdown may promote tumorigenesis [35]. Furthermore, prolonged PTEN inhibition during the regenerative process might lead to inappropriate regenerative responses, including axon hypermyelination [36, 37]. Thus, PTEN inhibition must be spatially and temporally controlled.

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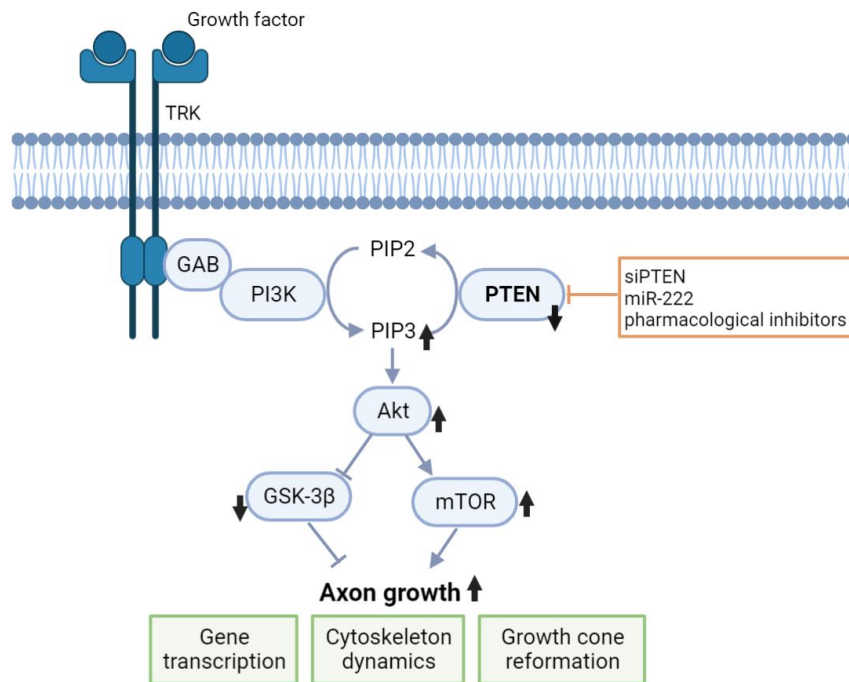


Figure 1.3 | Role of PTEN in the PI3K/Akt signaling pathway, which regulates axon growth. The pathway is induced after activation of a tyrosine receptor kinase (TRK) by a growth factor (e.g. nerve growth factor), which activates the adaptor protein GRB2-associated binder (GAB) [33, 34]. GAB activates the phosphoinositide 3-kinase (PI3K), which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3), whose accumulation leads to membrane recruitment and phosphorylation of Akt. Activated Akt inactivates the glycogen synthase kinase 3 beta (GSK-3 β) and activates the mammalian target of rapamycin (mTOR), collectively promoting axon growth. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) reverts the phosphorylation of PIP2 to PIP3, thereby counteracting PIP3K signaling. PTEN inhibition through the use of anti-PTEN siRNA (siPTEN), miRNA-222 (miR-222), or pharmacological inhibitors increases PI3K/Akt signaling, thereby amplifying the axon growth response (schematically represented by the black arrows). Created with BioRender.com.

1.1.6 Ras-related C3 botulinum toxin substrate 1

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small intracellular protein (~21 kDa) that belongs to the Ras homolog family of guanosine triphosphate hydrolases (Rho GTPases) [38]. These proteins function as ‘molecular switches’, cycling between an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state [39]. In the active conformation, Rho GTPases interact with their numerous downstream effectors, resulting in the propagation of specific intracellular signaling pathways [39, 40].

Rho GTPases play a major role in controlling actin and microtubule dynamics, which explains their importance in numerous neuronal processes [39]. Rac1, in particular, is critical for neuronal development, survival, outgrowth, migration, and plasticity [39, 40]. Unsurprisingly, Rac1 signaling has also been implicated in the response to several nervous system injuries, including PNI [38]. Lorenzetto et al. [41] reported that intravitreal injections of constitutively active Rac1 mutants in a mouse model of optic nerve crush injury improved neuronal survival and axon regeneration, whereas inhibition of Rac1 signaling by injection of a dominant negative form of this protein

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impaired neuronal survival. Cheung et al. [42] reported that overexpression of FE65 (an adaptor protein) and ARF6 (a Ras superfamily GTPase) stimulated neurite outgrowth in rat cortical neurons. This effect was linked to the activation of Rac1: overexpression of Rac1 further enhanced neurite outgrowth in neurons cotransfected with FE65 and ARF6, whereas Rac1 knockdown blocked the outgrowth-inducing effect of the FE65+ARF6 cotransfection. Moreover, in agreement with the results by Lorenzetto et al., expression of a constitutively active Rac1 was sufficient to stimulate neurite outgrowth. More recently, Wang et al. [43] also reported a regeneration-promoting role of Rac1 in the context of PNI. They found that overexpression of the protein Vav1 in rats subjected to sciatic nerve crush injury enhanced axon outgrowth and functional recovery, an effect that was linked to a downstream increase in Rac1 activity. Pharmacological inhibition of Rac1 in adult DRG neurons using the small molecule NSC23766 reduced the total neurite length and longest axon length, both as an isolated treatment and after Vav1 overexpression.

Despite the aforementioned, there is also an increasingly large body of literature suggesting the benefits of inhibiting Rac1 signaling in the context of nervous system injuries [44–48]. This is related to the link between Rac1 and neuropathic pain, as explained below.

Neuropathic pain is defined as pain arising directly from a lesion of the somatosensory system, and it is highly prevalent among PNI patients [5, 49]. Emerging evidence suggests that PNI can induce aberrant structural changes in excitatory synapses in the spinal cord, which lead to the hyperexcitability of nociceptive neurons and the subsequent development of neuropathic pain [50]. A key hallmark of this synaptic plasticity is the remodeling of dendritic spines, which are small, actin-rich protrusions that arise from neurons' dendrites and are involved in excitatory synaptic signaling [51]. In both spinal cord injury [44, 46–48] and PNI models [45], a similar pattern of dendritic spine changes was observed, which includes: (i) increased density of dendritic spines, especially of mature morphology (mushroom-shaped spines), and (ii) redistribution of spines toward the dendritic branches closer to the cell body [44–48] (Figure 1.4). Consistent with the role of Rac1 as a master regulator of dendritic spines [51, 52], inhibition of Rac1 was able to revert injury-induced dendritic spine dysgenesis, which resulted in improved behavior measures of neuropathic pain [44, 45].

In short, Rac1 signaling seems to have a dual role in the pathophysiology of PNI: on the one hand, it is involved in signaling pathways that promote axon outgrowth; on the other hand, it mediates maladaptive changes in dendritic spines that can contribute to neuropathic pain. Even though most studies seem to consider only one of these two conflicting roles, *in vivo* regeneration studies tend to manipulate Rac1 signaling at an early stage post-injury [41, 43], whereas pain studies usually inhibit Rac1 one or several weeks after [44–46, 48]. Recent work by Zhao et al. suggests that early Rac1 inhibition is not effective in reducing neuropathic pain, presumably because dendritic spine dysgenesis is not an immediate response to injury [47].

Summary

PNIs are traumatic lesions to the PNS that carry tremendous individual and societal costs. Especially in cases of severe nerve damage, the intrinsic regenerative capacity of the PNS is often

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insufficient to guarantee adequate functional recoveries. Current clinical management is centered around surgical interventions, which aid the regenerative process by reestablishing continuity between the connective tissues of the nerve. However, since surgical outcomes are frequently unsatisfactory, new therapeutic options for PNI are long awaited. In this regard, PTEN and Rac1 are two targets whose inhibition has the potential to constitute a valuable therapeutic approach to enhance regeneration and reduce neuropathic pain after PNI.

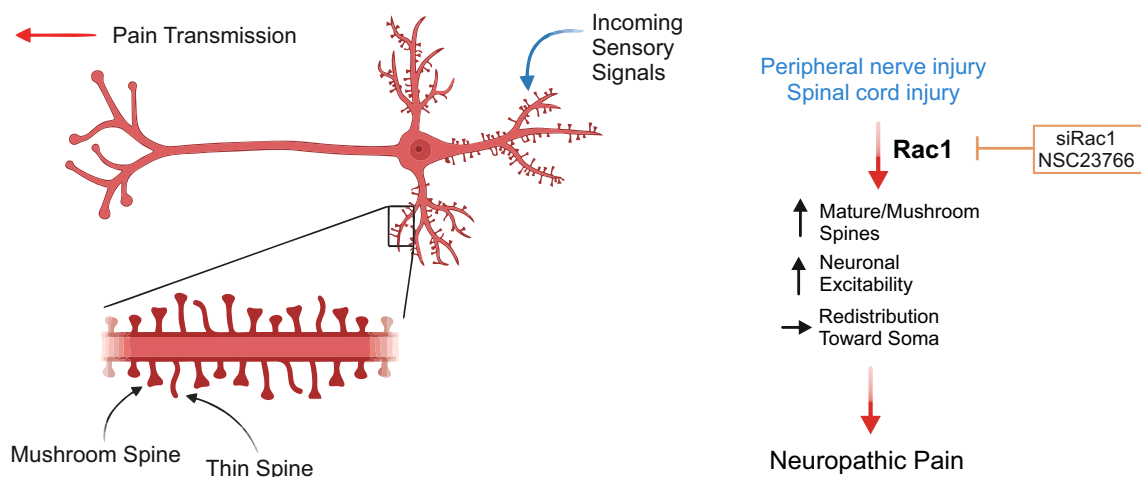


Figure 1.4 | Schematic representation of the link between Rac1 and neuropathic pain arising from peripheral nerve injury or spinal cord injury. Spinal dorsal horn neurons receive nociceptive input at excitatory dendritic spines located at the distal dendritic branches. In the aftermath of a nerve injury, structural changes in these dendritic spines are induced, namely: increased density of mature/mushroom spines, increased neuronal excitability, and redistribution of spines toward the soma. These changes, which are mediated by the Ras-related C3 botulinum toxin substrate 1 (Rac1), ultimately contribute to the development and maintenance of neuropathic pain. Rac1 inhibition through specific siRNA (siRac1) or by administration of a pharmacological inhibitor (NSC23766) can revert these changes and, consequently, ameliorate neuropathic pain. Adapted from Stratton and Khanna (2020) [51] (Creative Commons License CC BY-NC-SA 3.0).

1.2 siRNA therapeutics

One emerging strategy to inhibit therapeutic targets is through the use of siRNA drugs. In this section, this class of therapeutics is discussed in detail, starting with a historical perspective of siRNAs (subsection 1.2.1), followed by a description of their mechanism of action (subsection 1.2.2), their advantages over other drug classes (subsection 1.2.3), and the challenges inherent to their clinical development (subsection 1.2.4). Then, the two main approaches used to solve these challenges are discussed: chemical modifications (subsection 1.2.5) and the use of delivery systems (subsection 1.2.6).

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1.2.1 The history of RNA interference: from biological mechanism to new drug class

In 1998, Andrew Fire, Craig Mello, and coworkers [53] reported that the injection of double-stranded RNA (dsRNA) molecules into the nematode *Caenorhabditis elegans* led to the silencing of the corresponding endogenous gene, producing visible phenotype changes in the worms. Such genetic interference was not observed upon injection of either individual RNA strand. Moreover, the injection of dsRNA was accompanied by a reduction in the level of the homologous mRNA. This phenomenon – coined RNA interference (RNAi) – was not only sequence-specific but also potent, as only a tiny amount of injected dsRNA was required to produce an effect. These findings were a major breakthrough that would later be recognized with the 2006 Nobel Prize in Physiology and Medicine [54].

In the years that followed Fire and Mello's discovery, several studies elucidated the mechanisms underlying the RNAi phenomenon. In 2000, Hammond et al. [55] reported that extracts prepared from dsRNA-transfected *Drosophila melanogaster* S2 cells were able to specifically degrade synthetic mRNA homologous to the transfected dsRNA. This indicated that RNAi acts at the post-transcriptional level and involves the generation of sequence-specific nuclease activity. After purification, this sequence-specific nuclease co-fractionated with small RNAs (~25 nucleotides) homologous to the transfected dsRNA, which were essential for nuclease activity. Taken together, their results suggested that RNAi was mediated by a ribonucleoprotein, which was termed RNA-induced silencing complex (RISC). Follow-up work by the same laboratory identified a separate enzyme, Dicer, which processes the dsRNA into small guide RNA sequences that are then incorporated into the RISC [56].

Up to this point, reports of RNAi had been mostly restricted to plants and invertebrates. The introduction of dsRNA into mammalian cells led to nonspecific responses, including the induction of interferon synthesis [57, 58]. In 2001, two landmark papers showed that the introduction of small dsRNAs (21-25 nucleotides) into mammalian cell lines was able to produce potent and specific RNAi whilst avoiding the activation of nonspecific pathways observed when longer dsRNAs were used [57, 58]. Such discovery offered the possibility of using these small RNAs – termed siRNAs – as a therapeutic tool. Shortly after, in 2003, a pioneer *in vivo* study demonstrated that the intravenous injection of siRNA targeting the gene *Fas* could inhibit Fas expression on mouse hepatocytes and protect the liver from fulminant hepatitis and fibrosis [59]. In 2006, the first report on the systemic efficacy of siRNA in non-human primates was made public [60]. A single intravenous injection of siRNA targeting the *APOB* mRNA resulted in dose-dependent silencing in the liver of cynomolgus monkeys, accompanied by a long-lasting reduction in the levels of apolipoprotein B, serum cholesterol, and low-density lipoprotein.

The initial excitement around RNAi in the early 2000s led to massive investments in the field but also created unrealistic expectations about a technology still in its youth [61, 62]. By 2009, eight clinical trials using siRNAs were ongoing [63]. However, the absence of mature delivery platforms and the emergence of off-target and immunostimulatory effects caused the initial optimism to

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fade and led large pharmaceutical companies to reduce their RNAi portfolio in the early 2010s [62–64]. Nonetheless, the evolution of RNAi therapeutics proceeded, with innovations in chemical modification and delivery technologies, combined with more rational approaches for therapeutic target selection [65]. In 2018, exactly 20 years after Fire and Mello’s discovery, the US Food and Drug Administration and the European Medicines Agency gave regulatory approval to patisiran, a siRNA drug developed by Alnylam Pharmaceuticals for the treatment of the polyneuropathy of hereditary transthyretin-mediated amyloidosis [66]. Since then, three other siRNA drugs have reached the clinics: givosiran (2019), for the treatment of acute hepatic porphyria; lumasiran (2020), for primary hyperoxaluria type 1; and inclisiran (2021), for hypercholesterolemia [67]. Additionally, several candidates are currently undergoing phase III clinical trials [67]. However, for reasons that will later become clear, most siRNA drug development has focused on hepatic gene targets, particularly those involved in rare conditions [67]. Thus, most of the therapeutic potential of siRNA remains untapped.

1.2.2 Mechanism of action of siRNAs

The mechanism underlying the biological action of siRNAs is the RNAi pathway. In RNAi, small RNA guides interact with specialized effector proteins to induce sequence-specific post-transcriptional gene silencing [68]. This is a naturally occurring cellular process, with important roles in the modulation of gene expression and the defense against viral infection and transposons [69]. Several small RNAs can direct RNAi, including siRNAs, miRNAs, and Piwi-interacting RNAs [68, 70, 71]. These RNA classes differ in their structure, biogenesis, and function, resulting in a high diversity of RNAi pathways [68, 70].

In terms of biogenesis, siRNAs usually derive from longer dsRNA precursors, which are recognized and cleaved by the RNase III enzyme Dicer [56, 71] (Figure 1.5a-1). These precursors can be exogenously provided (like in the early RNAi experiments) but can also be produced endogenously (e.g. from the bidirectional transcription of specific loci) [68, 72]. In any case, Dicer processing typically originates siRNAs consisting of two ~21 nucleotide strands, paired in a way that leaves two-nucleotide overhangs at the 3’ ends [73, 74] (Figure 1.5b). The Dicer-siRNA complex then associates with the TAR RNA-binding protein (TRBP) and the Argonaute 2 protein (Ago2) to form the RISC-loading complex (RLC) [75, 76] (Figure 1.5a-2). The RLC ensures the efficient transfer of the siRNA from Dicer to Ago2, which is necessary for the assembly of the RISC, the effector complex of RNAi [75–77]. Once bound to the siRNA duplex, Ago2 cleaves one of the strands (termed the ‘passenger strand’) and the remaining strand (the ‘guide strand’) is incorporated into the active RISC [78] (Figure 1.5a-3).

Once assembled, the RISC can recognize its target mRNA within the cell based on complementary binding between the guide strand and the substrate mRNA [79] (Figure 1.5a-4). A stable association between the two induces cleavage of the target mRNA by Ago2 [77, 79]. The cleavage occurs precisely between nucleotides 10 and 11 of the target mRNA, counting from the nucleotide paired to the 5’-end of the RNA guide strand and moving upstream (3’ to 5’ in the mRNA) [73,

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80] (Figure 1.5b). The resulting fragments are then degraded by the general cellular mRNA degradation machinery [81] (Figure 1.5a-5). Since target cleavage only occurs in the case of a high degree of complementarity between the guide strand and the mRNA, siRNA induces specific gene silencing [79].

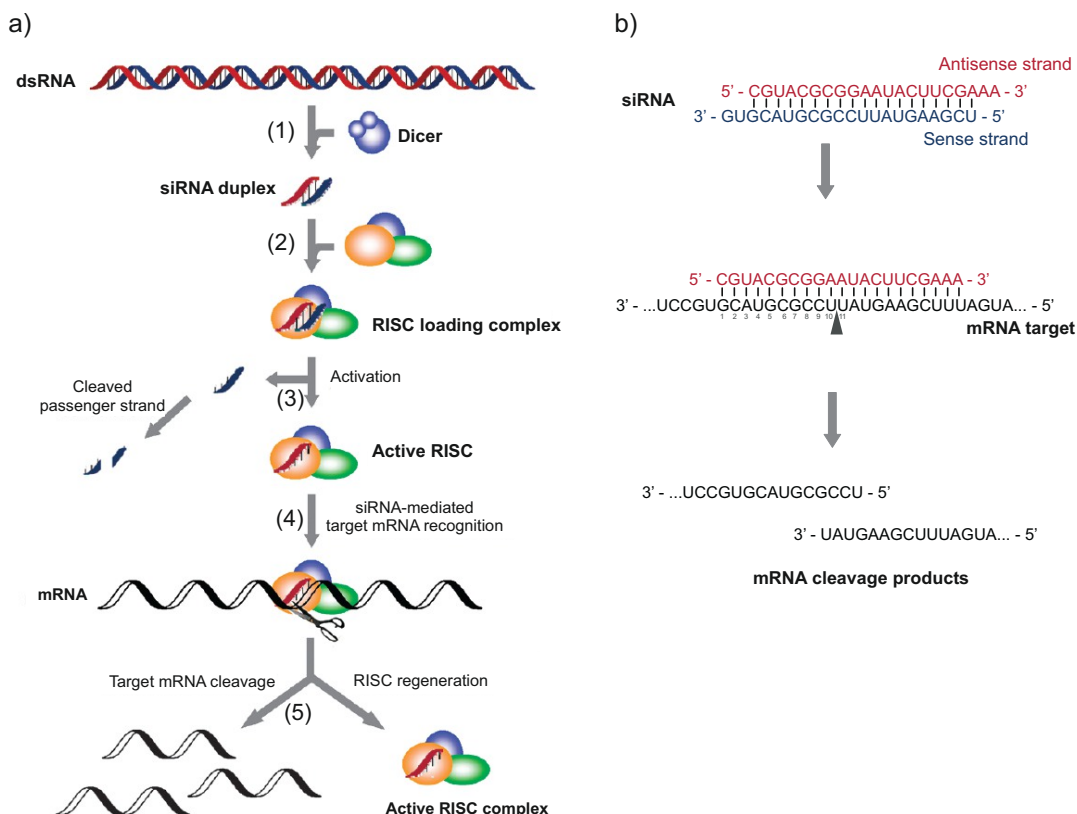


Figure 1.5 | The mechanism of RNA interference. **a)** A long double-stranded RNA (dsRNA) precursor is cleaved by Dicer to originate a small interfering RNA (siRNA) (1), which then associates with other proteins to form the RISC loading complex (RLC) (2). The passenger strand of the siRNA duplex is cleaved and dissociates from the complex, yielding the active RNA-induced silencing complex (RISC) (3). The RISC can then recognize and induce the cleavage of its target messenger RNA (mRNA) (4-5). **b)** Detail from (a) showing the canonical 21-mer siRNA structure and indicating the cleavage site within the target mRNA. Adapted from Liu et al. (2012) [82] with permission from the Royal Society of Chemistry.

1.2.3 Promise of siRNA therapies

Having a thorough knowledge of how siRNAs mediate post-transcriptional gene silencing is critical for the rational design of siRNA drug candidates with potent and specific activity. Obviously, synthetic siRNAs must be able to engage efficiently with the RNAi machinery. A straightforward way to achieve this is by using the canonical 21-mer structure (i.e. 21-bp RNA duplex with two-base 3'-overhangs). These siRNAs can bypass Dicer processing and directly associate with the RISC [83]. Nevertheless, non-canonical siRNAs with different structural motifs and/or chemical modifications have been developed to overcome pharmacokinetic and pharmacodynamic-related challenges posed by conventional siRNAs [65]. If carefully designed, these non-canonical

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siRNAs can retain a high level of compatibility with the RNAi machinery and even improve RNAi activity and guide strand selection [65]. For example, Dicer substrate siRNAs are longer RNA duplexes (25-29 bp) that are cleaved by Dicer before being incorporated into the RISC [84, 85]. There is evidence that some of these siRNAs induce more potent gene silencing than their sequence-matched 21-mers, whilst remaining under the dsRNA length threshold that would elicit an interferon response [84, 85].

Conventional small molecule drugs and monoclonal antibodies typically engage directly with protein targets. Because proteins can have a myriad of structural features and physicochemical properties, these drugs must have a high level of structural precision, resulting in long and sometimes unfruitful development processes [67, 86]. By contrast, designing siRNA molecules that can efficiently induce target mRNA degradation is much simpler, allowing for faster research and development [67]. This is primarily because mRNA-siRNA binding depends mainly on Watson-Crick base pairing, which is easily predictable [86]. Provided that the mRNA sequence is known, virtually any gene can be silenced using an appropriate siRNA, including genes encoding for proteins previously deemed ‘undruggable’ [86, 87]. This offers numerous novel therapeutic possibilities for many diseases.

Furthermore, while small molecule drugs designed for different target proteins can differ significantly in their pharmacokinetic properties, different siRNA sequences are expected to have similar pharmacokinetics [65]. Consequently, once effective delivery of siRNA to a specific cell type or tissue has been achieved, the same delivery platform can be repurposed to carry other siRNAs targeting different disease-promoting genes within the same cell type or tissue. Additionally, it is possible to co-deliver multiple siRNAs to achieve simultaneous knockdown of more than one therapeutic target; this is particularly important in diseases where drug resistance is a problem (e.g. cancer) [87]. Lastly, carefully designed siRNAs can provide a potent, specific, and long-lasting effect. In fact, some siRNAs are capable of more than 95% gene silencing [88]. Once inside target cells, siRNAs are very stable, leading to durable gene knockdown [89]. As a result, monthly, quarterly, or even bi-annual administrations are enough to achieve a therapeutic effect [67]. This is a considerable advantage over monoclonal antibodies, which generally require more frequent administrations [90].

1.2.4 Challenges in the development of siRNA therapies

Sequence selection

Ensuring optimal silencing of the target gene requires careful selection of the siRNA sequence. The most basic premise of sequence selection is that one of the strands of the siRNA duplex must be complementary to a region of the target mRNA [91]. This strand is termed the antisense strand and is the main determinant of the potency and specificity of gene silencing [73]. Given that most mRNA sequences are readily available, this requirement is very easy to fulfill. Nevertheless, there are some caveats.

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The first caveat relates to guide strand selection. In theory, both antisense and sense strands can be incorporated into an active RISC as the guide strand (see subsection 1.2.2). However, optimal on-target silencing requires the antisense strand to be exclusively selected as the guide strand, while the sense strand should constitute the passenger strand (i.e. it should be cleaved during RISC assembly) [92]. This is a critical consideration during siRNA sequence design. The prevailing rule is that the siRNA should have a higher AU content at the 5' end of the antisense strand than at the 5' end of the sense strand [65]. This is based on the theory that the strand with the thermodynamically less stable 5' end is preferentially selected as the guide strand [93, 94]. Nevertheless, more recent evidence has put this general rule into question, as other factors also appear to influence guide strand selection [95, 96].

Secondly, even though efficient RISC-mediated cleavage of target mRNAs requires perfect base-pairing to the siRNA guide strand, other non-target mRNAs can still be silenced to some degree [97, 98]. This off-target silencing typically affects transcripts with 3' untranslated regions complementary to the siRNA seed region (bases 2-8 from the 5' end of the guide strand [65, 97, 98]. This is usually referred to as miRNA-like off-target effect, as it resembles miRNA-mediated silencing [98]. In order to guarantee maximal siRNA specificity, it is important to screen candidate sequences for partial matches [65].

Finally, a further complication in siRNA design is that siRNAs complementary to different regions of the same target mRNA can exhibit a wide range of knockdown efficiencies [88]. Evidence suggests that numerous factors determine the potency of siRNAs, including nucleotide frequencies, thermodynamic stability, accessibility of the mRNA target site, among others [79, 88, 99, 100]. Using experimental data, several tools have been developed to predict siRNA knockdown efficacy [99, 100]. Nevertheless, since no algorithm offers perfect performance prediction, experimental validation is still essential to identify the most potent siRNA drug candidates.

Delivery and uptake

Irrespective of the intrinsic potency of the siRNA sequence, therapeutic efficacy will only be achieved if enough siRNA molecules are delivered to the cytosol of target cells, where the RNAi machinery is present. Historically, ensuring effective delivery and uptake of siRNAs has been the biggest obstacle preventing the widespread translation of therapeutic siRNAs into the clinic [62, 63, 83, 89, 91, 101–103].

The first delivery challenge is getting siRNAs from the administration site to the tissue of therapeutic interest. For systemically administered siRNAs, this implies crossing the capillary endothelium within the target tissue [89]. This process, called extravasation, is facilitated by the presence of a fenestrated endothelium, such as the one present in the liver and spleen; thus, systemically administered siRNAs are more prone to accumulate in these organs [101]. By contrast, the microvasculature in other sites is much less permeable. For instance, in the CNS, the endothelial cells are strongly linked to each other by tight junctions, forming functional barriers that prevent oligonucleotides (as well as the majority of other drug molecules) from entering the nervous tissue parenchyma [104].

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Ensuring adequate bioavailability of siRNAs in target tissues is also hindered by the fact that these molecules have very short circulatory half-lives (< 5 min) [105, 106]. Indeed, because their molecular weight (~14 kDa) is significantly below the glomerular filtration limit (30-50 kDa), circulating siRNAs are rapidly filtered by the kidneys and excreted in the urine [89, 105, 106]. Moreover, they do not seem to bind to serum proteins such as albumin [106], which could otherwise increase their circulatory half-life. Finally, unmodified siRNAs are also susceptible to rapid degradation by serum RNases [107].

Of course, local administration can be used to boost siRNA concentration in the tissue of interest. In fact, several siRNA candidates currently undergoing clinical trials are administered non-systemically, including through the intravitreal, intradermal, or intratumoral routes [65]. For bypassing the CNS barriers, routes such as intracerebral, intracerebroventricular, and intrathecal might be used [104]. Nevertheless, some of these local administration routes also pose challenges, such as higher invasiveness, difficulty in controlling the release profile, and limited drug diffusion within the tissue parenchyma [104, 108].

Upon reaching the tissue of interest, siRNAs must be taken up into the target cells. However, they are too large and negatively charged to cross the lipid bilayer through simple diffusion [102]. Instead, they are internalized by one of several endocytic pathways [101, 109]. These pathways lead to a complex intracellular trafficking network composed of several endomembrane vesicular compartments including early and recycling endosomes, late endosomes, lysosomes, and the Golgi complex [101, 109]. Due to their incapacity to translocate across lipid bilayers, most siRNAs become entrapped in these compartments, and can either be degraded as endosomes mature or be exocytosed back to the extracellular milieu [67, 110]. Escape from endosomal compartments to the cytosol is required for RISC assembly and it constitutes a key rate-limiting step in siRNA delivery [83, 110, 111]. Imaging studies indicate that only a tiny fraction of internalized siRNA molecules (as low as 1-2%) is released into the cytosol [83, 110, 111].

In sum, the 500 to 4000 cytosolic siRNA molecules per cell that are required for effective gene knockdown [83, 111, 112] are almost impossible to achieve with naked siRNAs. Most administered molecules will eventually hit one of several delivery barriers before they reach their intended target. These include the vascular-endothelial barrier, the cellular membrane, and the endosome escape barrier, as well as renal excretion and RNase degradation.

Innate immune activation

As previously mentioned, the early realization that the introduction of long dsRNAs (>30 bp) into mammalian cells triggers an interferon response led to the development of smaller, less immunogenic, synthetic siRNAs [57, 58]. However, it soon came to light that some of these siRNAs could also activate innate immunity [64, 113, 114].

Innate immunity activation can be due to the recognition of siRNA molecules by Toll-like receptors 3, 7, and 8, which are located in endosomal compartments [64, 113, 115]. Additionally, siRNAs can also be recognized by cytoplasmatic RNA sensors, including the dsRNA-binding protein kinase and the RIG1 protein [115]. Depending on the receptor(s) involved, different signaling

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pathways can be triggered; nevertheless, the end result is typically the induction of cytokine release [115]. Importantly, recognition can be sequence-dependent, and some immunostimulatory motifs have been identified [113, 114].

Immune activation was a serious hurdle for first-generation siRNA therapeutics [64]. However, this problem has been addressed in later-generation siRNAs, mostly through chemical modifications, as explained below.

1.2.5 Chemical modifications

Chemical modification of siRNAs has been pivotal to address many of the abovementioned challenges. While a wide range of chemistries has been developed [86, 116], the two most commonly present in siRNA drug candidates are ribose 2'-OH modifications and backbone modifications [65] (Figure 1.6). The former encompass modifications that replace the ribose 2'-OH group with other functional groups, typically 2'-fluoro (2'-F), 2'-O-methyl (2'-OMe), or 2'-O-methoxyethyl [91]. These substitutions tend to increase duplex stability, reduce siRNA degradation by RNases, and decrease immune activation [115, 117–119]. Backbone modifications are substitutions of the phosphodiester linkages by other linkage types. The most commonly used is the phosphorothioate (PS) modification, in which one non-bridging phosphate oxygen of a phosphodiester is replaced by a sulfur atom [86]. This increases resistance to nuclease activity and promotes nonspecific binding to plasma proteins, thereby retarding renal clearance and improving the overall pharmacokinetic profile [67, 86, 105, 120].

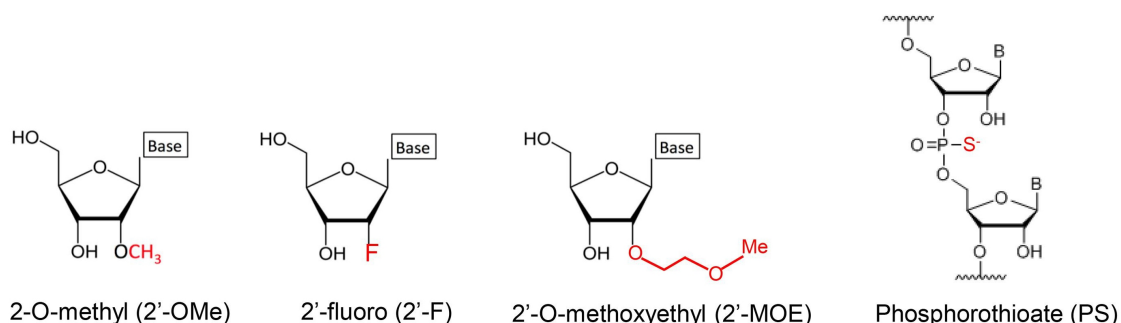


Figure 1.6 | Common chemical modifications of siRNAs. Adapted from Zhang et al. (2021) [67] with permission from Elsevier.

An important caveat is that the extensive use of the above-mentioned modifications can significantly reduce or even abolish RNAi activity [117, 119–121] and, in the case of the PS modification, can result in cytotoxicity [122, 123]. To avoid these detrimental effects, clinical-stage siRNA drugs adopt partial modification patterns, such as alternating 2'-F and 2'-OMe substitutions and partial PS modification at the end of the strands [67, 86].

1.2.6 Delivery systems

Chemically modified siRNAs, despite bearing improved pharmacokinetics in comparison to their unmodified counterparts, still retain a relatively high molecular weight and negative charge,

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which results in poor permeability across biological membranes [67, 91, 102]. As a result, combining these siRNAs with adequate delivery excipients is vital to facilitate cellular uptake in target sites [67, 91, 102]. A vast array of delivery systems has been proposed to improve siRNA delivery. The following sections will succinctly cover the most relevant classes, apart from dendrimers, which are discussed in more detail in section 1.3.

Lipid nanoparticles

Lipid nanoparticles (LNPs) were one of the first delivery technologies to be used in conjunction with siRNA [60, 112], and have benefited from continuous innovation over the course of several decades [124–126]. Patisiran, the first approved siRNA drug, employs this type of delivery technology [66].

Advanced LNP designs are multi-component formulations composed by ionizable cationic lipids in conjunction with several “helper lipids”, including cholesterol, phospholipids, and polyethylene glycol (PEG)-lipids [124, 125, 127]. Ionizable cationic lipids are positively charged under acid conditions [125], which is crucial for siRNA packing during manufacturing and to facilitate endosomal release [125, 127]. At physiological pH, the LNP surface charge is close to neutral, reducing nonspecific adsorption of serum proteins and, consequently, limiting nanoparticle clearance by phagocytosis [126]. Several helper lipids are incorporated to increase particle stability, improve interaction with cellular membranes, avoid immune activation, and increase circulation times [127].

LNPs offer several competitive advantages over other delivery systems. Optimized siRNA LNP formulations enable extremely potent silencing of hepatic genes, with doses lower than 0.03 mg kg^{-1} for non-human primates [128]. Moreover, years of technological maturation yielded improved formulation processes, which combine high-throughput capabilities with high siRNA entrapment efficiencies and rigorous control over particle size [125, 126]. However, LNPs also possess some drawbacks. Most LNP formulations have typical sizes around 100 nm [102]; consequently, intravenous administration results in preferential accumulation in fenestrated tissues, such as the liver, spleen, and kidney [86, 101]. Smaller LNPs (e.g. 30 nm) can be synthesized through microfluidic mixing techniques, but such formulations are less stable [129]. Given the natural predisposition of LNPs towards the liver, targeting delivery to other cell types or tissues is extremely difficult to achieve [101, 130]. Furthermore, the complex composition of existent LNPs makes the incorporation of targeting moieties particularly challenging, both in terms of manufacturing feasibility and from a cost-effectiveness perspective [101, 102]. Finally, the toxicity of some lipid excipients is problematic: administration of LNPs above certain doses can cause unwanted immune activation and inflammatory responses [91, 124, 130].

siRNA-ligand bioconjugates

Bioconjugate delivery consists of covalent binding of siRNAs to specific molecules that improve delivery and uptake into target cells [67]. The most prominent example is the conjugation to

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N-acetylgalactosamine (GalNAc), which allows for extremely efficient siRNA delivery to liver hepatocytes [67, 86, 101, 103, 116, 131]. This is unquestionably the most clinically successful delivery platform for siRNA, being employed in three out of four approved drugs and in up to two-thirds of all RNAi candidates currently undergoing clinical trials [67].

GalNAc is a ligand of the asialoglycoprotein receptor (ASGPR), which is highly abundant on the surface of hepatocytes (~500 000 receptors per cell) [86, 103, 116, 131]. The binding of GalNAc-siRNA to ASGPR leads to rapid endocytosis, upon which the conjugate is released and the receptor is rapidly recycled back to the cell surface (within 15 min) [86, 116]. To increase the binding affinity to ASGPR, trivalent or tetravalent GalNAc moieties are covalently attached to the siRNA molecule [67, 86, 103].

GalNAc-siRNA conjugates offer a simpler and chemically defined alternative to LNP formulations, alongside higher efficiency and specificity for liver delivery and an enhanced safety profile [86, 101, 103]. Additionally, they are therapeutically effective when administered subcutaneously in monthly or even quarterly dosing schemes, which reduces significantly the treatment burden [67, 116]. Nevertheless, while hepatic delivery can be efficiently achieved with the GalNAc platform, attempts at developing similar bioconjugate systems for extrahepatic delivery have so far resulted in limited success [65, 83]. This is in part because the tissue-specificity, high surface abundance, and rapid turnover of the ASGPR seem to be unparalleled [65, 83, 101]. Targeting other less-than-ideal receptors results in a much slower intracellular accumulation of the bioconjugate, which makes it challenging to achieve the therapeutic threshold before the drug is cleared from circulation. This consideration is of particular importance, given that bioconjugates offer less protection against nuclease degradation and renal clearance when compared with nanoparticle-based formulations [101].

Polymeric nanoparticles

Polymeric vectors have also been widely investigated as siRNA carriers. Similar to cationic lipids, cationic polymers can bind negatively charged siRNA through electrostatic interactions, forming nanoparticles called polyplexes [91, 132]. Cationic complexation can protect siRNAs from degradation, reduce renal filtration and enhance cellular uptake [132–134]. However, polymers with high charge densities have a poor toxicity profile. One noteworthy example is poly(ethyleneimine): despite being highly effective as an *in vitro* transfection reagent, it is unsuitable for *in vivo* applications due to its cytotoxic properties and low hemocompatibility [133, 134].

To overcome the limitations of classic cationic polymers, several other polymer chemistries have been put forward in recent years [132, 134]. These new polymeric vectors aim to combine efficient siRNA condensation, low cytotoxicity, and sufficient cellular uptake and intracellular release [132]. Nevertheless, despite rapid progression in the field, polymeric-based siRNA delivery is not as clinically advanced as GalNAc bioconjugates or LNPs. While numerous studies involving polymeric carriers have focused on improving particular aspects of siRNA delivery, there is still a lack of in-depth knowledge on key topics, such as how polymeric carriers aid endosomal release

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[132]. In addition, many manufacturing methods for polymeric nanoparticles yield broad size distributions; developing scalable manufacturing processes that afford rigorous size control is crucial to ensure the clinical viability of these delivery platforms [135].

Other delivery systems

Aside from LNPs, bioconjugates, and polymeric nanoparticles, several other strategies have been envisaged to deliver siRNA. Genetically engineered viruses containing short hairpin RNA (shRNA) expression cassettes have been used as an indirect method to induce RNAi [136]. Upon delivery, promoter-driver expression of these cassettes generates shRNAs, which are processed by Dicer to yield siRNAs [136]. The advantages of this approach include high transfection efficiencies, long-term expression of the therapeutic siRNA, and the possibility to target specific cell types through genetic manipulation of the virus tropism [63, 91, 136]. However, the clinical application of viral vectors is limited by safety concerns (risk of insertional mutagenesis and immunogenicity) and high production costs [91, 131]. With the development of efficient non-viral vectors, the interest in viral-mediated siRNA delivery has declined significantly [131].

Inorganic nanoparticles (gold, magnetic, silica, quantum dots) have also been tested as siRNA delivery vehicles [137]. Their main advantages are their stability, high purity, well-defined and adjustable size and morphology, and ease of surface modification [137]. However, their clinical translation is hindered by safety concerns, especially long-term accumulation resulting from low biodegradability [137].

Exosomes are endogenous nanovesicles secreted by numerous cell types that can carry nucleic acids and proteins to other cells [138]. In recent years, they have received a lot of attention as potential siRNA carriers due to their blood stability, low immunogenicity, and intrinsic capacity to target the cell types from which they were isolated [138]. In addition, exosomes can be engineered to express specific targeting biomolecules onto their surface [139]. Although important advances in exosome technology have been achieved, the application of this delivery system is still highly experimental [126, 138].

At last, an unconventional approach to siRNA delivery is the use of nucleic acid nanostructures, which result from the bottom-up assembly of DNA or RNA [140, 141]. Taking advantage of the predictability of nucleic acid interactions, a variety of nanoparticles with defined sizes, shapes, and thermal stabilities can be constructed [141]. siRNAs, as well as other functional modules, can be incorporated during the assembly process [140, 141]. This approach has shown promising results *in vitro* and *in vivo* [140, 141]; however, just like exosomes, it is still in the early stages of development.

Summary

siRNAs are emerging as a powerful new drug class due to their capacity to mediate potent, specific, and durable silencing of target genes. However, translating siRNA drug candidates into the clinics still poses considerable challenges, especially when it comes to delivery and uptake into

target cells. These challenges have been tackled both through chemical modification of the siRNA itself and through its incorporation into a wide range of delivery systems. While most of these delivery systems were already mentioned, dendrimer-based carriers are covered in more detail in the following sections.

1.3 Dendrimers as versatile vehicles for siRNA delivery

In 1978, Vögtle et al. [142] synthesized polymers with a tree-like branching structure, calling them ‘cascade molecules’. Several years later, in 1985, two papers by Tomalia et al. [143] and Newkome et al. [144] reported the synthesis of hyperbranched polymers with higher complexity, and the term ‘dendrimer’ was introduced to describe them [143].

Dendrimers are highly branched macromolecules consisting of three main components: (a) a central core with at least two reactive groups, (b) interior layers of repeating units covalently attached to the central core (generations), and (c) terminal functional groups on their surface [143, 145, 146] (Figure 1.7a). Such architectures can be achieved through divergent or convergent synthesis strategies [147, 148]. The divergent approach starts with coupling repeating units to the polyfunctional core to form the first dendritic layer, resulting in a generation 1 (G1) dendrimer. For further growth, the end-groups of the first layer are deprotected or transformed, thereby creating new reactive sites for attachment of a second layer of repeating units (G2), and so on. Conversely, the convergent method starts at the periphery, with repeating units being covalently attached in a stepwise manner to form dendrons (dendrimer wedges), which are then finally linked to the core to form the dendrimer.

Several key properties of dendrimers make them promising carriers for a variety of bioactive molecules. Unlike linear polymers, dendritic structures exhibit a well-defined architecture with low polydispersity [145, 146, 148]. By modification of the generation number, the size and shape of these structures can be tuned [145]. Moreover, the existence of multiple functional groups on their surface (multivalency) allows for the functionalization with ligands to facilitate the interaction with biological targets [145, 146].

In the context of nucleic acid delivery, cationic dendrimers are particularly attractive carriers due to their capacity to condense nucleic acids into compact nanostructures (dendriplexes) through electrostatic interactions [82, 149]. For this purpose, poly(amido amine) (PAMAM) dendrimers [143] (Figure 1.7b) have been the most extensively investigated, including in the field of siRNA delivery [149–152]. Gene knockdown efficiency can be modulated through modification of the PAMAM structure, including the central core [149], the generation number [151], and the terminal functional groups [152]. Additionally, other dendritic families such as poly(propylene imine) (PPI) dendrimers [142, 153] (Figure 1.7c), poly(L-lysine) dendrimers [154], and others [82], have also shown potential as nonviral vectors for siRNA delivery.

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1.3.1 Toxicity of dendrimers

Despite their promise as siRNA carriers, common cationic dendrimers such as PAMAM and PPI have well-documented toxicity issues which seriously limit their clinical translation [155–162]. Cytotoxicity occurs mostly as a result of nonspecific interactions between the polycationic dendrimer surface and the negatively charged biological membrane, which induces the formation of nanoscale holes and ultimately leads to cell lysis [163]. For the same reason, some of these dendrimers are also hemotoxic, inducing changes in erythrocyte shape, hemolysis, and blood coagulation [158, 162]. In general, higher-generation dendrimers are associated with more pronounced toxicity, due to the higher number of positively charged terminal groups [155–159].

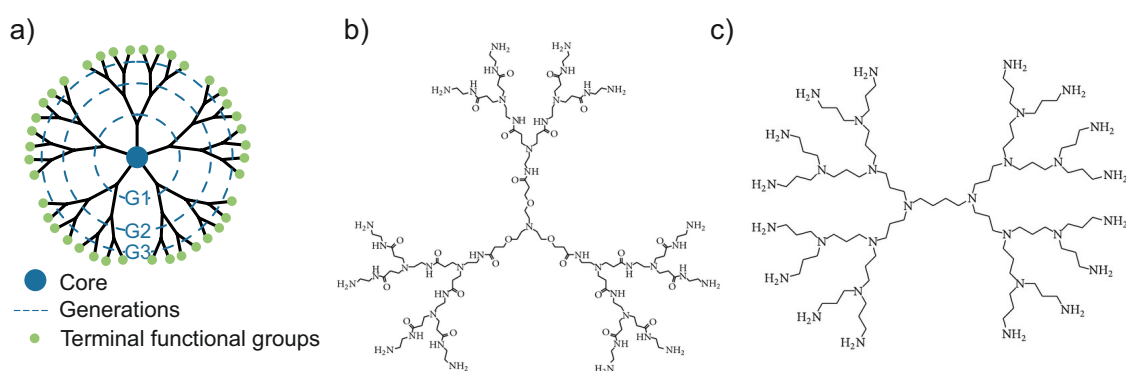


Figure 1.7 | Chemical structures of selected dendrimers used for siRNA delivery. a) Schematic illustration of a third-generation dendrimer, highlighting the core, the generations, and the terminal functional groups. b) Second generation poly(amido amine) (PAMAM) dendrimer with triethanolamine (TEA) core. c) Third-generation poly(propylene imine) (PPI) dendrimer. (b) and (c) were reprinted from Wu et al. (2013) [164] (Creative Commons License CC BY 3.0).

In order to circumvent these toxicity issues, the development of more biocompatible dendritic structures has long been a priority in the field [163, 165]. One commonly used approach has been PEGylation, i.e. the conjugation of dendrimers with PEG. This modification limits the toxicity by masking the peripheral cationic groups [157, 166–168]; additionally, it also provides other benefits, such as reduced immune recognition, increased plasma circulation times, and enhanced solubility [167, 168]. Efforts have also been made to develop completely new dendrimer chemistries that are less toxic than classic dendrimers [163]. One key aspect to consider in this case is biodegradability, as discussed in the following section.

1.3.2 Biodegradable dendrimers

To ensure an adequate safety profile for *in vivo* applications, any delivery carrier should be biodegradable under physiological conditions [146, 155]. This avoids the potentially toxic accumulation of nondegradable products, which is a particularly serious concern in scenarios where multiple administrations are required.

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In theory, biodegradable dendrimers can be achieved through the introduction of hydrolyzable bonds within the dendritic structure [146, 169]. This facilitates their breakdown into small fragments, which can be safely eliminated from the body [146, 169]. Additionally, carrier degradation can also enhance the intracellular release of the carried drug [170]. Nevertheless, the presence of labile bonds makes the already complex dendrimer synthesis process even more challenging because the dendritic structure is vulnerable to premature and undesired degradation [146, 169].

One important factor that determines the degradation rate is the type of biodegradable bond [146]. Most biodegradable dendrimers currently available contain ester linkages, which are biocompatible and represent a good compromise between degradability and ease of synthetic handling [146]. Many polyester dendrimers are based on the monomer 2,2-bis(hydroxymethyl)propionic acid (bis-HMPA) [146, 169, 171]. While these dendritic structures have a favorable safety profile, their high hydrolytic susceptibility commonly leads to undesired degradation during synthesis, purification, functionalization, or application [146]. Engineering biodegradable dendritic structures for nucleic acid delivery is even more challenging due to the need to conjugate in the same structure amine groups (which provide the positive charge needed for nucleic acid complexation) and electrophilic ester bonds [172]. So far, very few examples of biodegradable dendrimers for nucleic acid delivery have been reported [173–175].

To solve this unmet need, in 2017, our group proposed a new family of hybrid-biodegradable PEG-GATGE (PolyEthylene Glycol - Gallic Acid Triethylene Glycol Ester) dendritic block copolymers [170], composed of a nonbiodegradable core (GATG repeating unit [176], conjugated with PEG) and a biodegradable dendritic part. This biodegradable part incorporated a novel GATGE building unit (Figure 1.8a), which included hydrolyzable ester bonds at the periphery. These dendritic structures were grown until G2, followed by surface functionalization with alkynated amines, and assessment of their potential as siRNA carriers. They were shown to efficiently complex siRNA, forming supramolecular assemblies (dendriplexes) with hydrodynamic sizes of 140-180 nm. Despite their good cytotoxicity profile and ability to mediate cellular internalization of siRNA, the transfection efficiencies afforded by these dendriplexes were low due to poor endosomal escape.

In a recent follow-up work [172], we presented a fully biodegradable version of the PEG-GATGE dendritic block copolymers, which were synthesized until G3 (Figure 1.8b). While in the previous G2 dendrimers the ester bonds were only located in the peripheral dendritic arms, this new generation contains 39 hydrolyzable ester bonds throughout the entire dendritic structure, as well as an additional ester bond linking the PEG to the dendritic core. This allowed for an excellent biodegradability profile, which resulted in enhanced biological performance as vehicles for siRNA delivery. Indeed, compared to the hybrid G2 version, the dendriplexes formed by fully biodegradable G3 PEG-GATGE dendritic block copolymers were smaller in size (below 60 nm), were more efficiently internalized, and mediated potent silencing of the enhanced green fluorescence protein (eGFP) reporter in U2OS and ND7/23 cell lines expressing the eGFP-Luciferase fusion construct (U2OS/eGFPLuc and ND7/23 eGFPLuc, respectively) with no significant toxicity. Interestingly, the dendrimer functionalized with alkynated benzylamine moieties (fbB) (Figure 1.8b-c)

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performed better than the one functionalized with alkynated propylenediamine (fbD) in terms of siRNA complexation, siRNA protection capacity, and cellular internalization.

Summary

Cationic dendrimers are versatile carriers for siRNA delivery due to their well-defined architecture, low polydispersity, tunable size, and multivalency. However, the clinical translation of dendrimers is currently limited by their nonbiodegradability. To overcome this limitation, fully biodegradable PEG-GATGE dendritic block copolymers were proposed and have so far shown tremendous potential as biosafe delivery vectors for siRNA delivery.

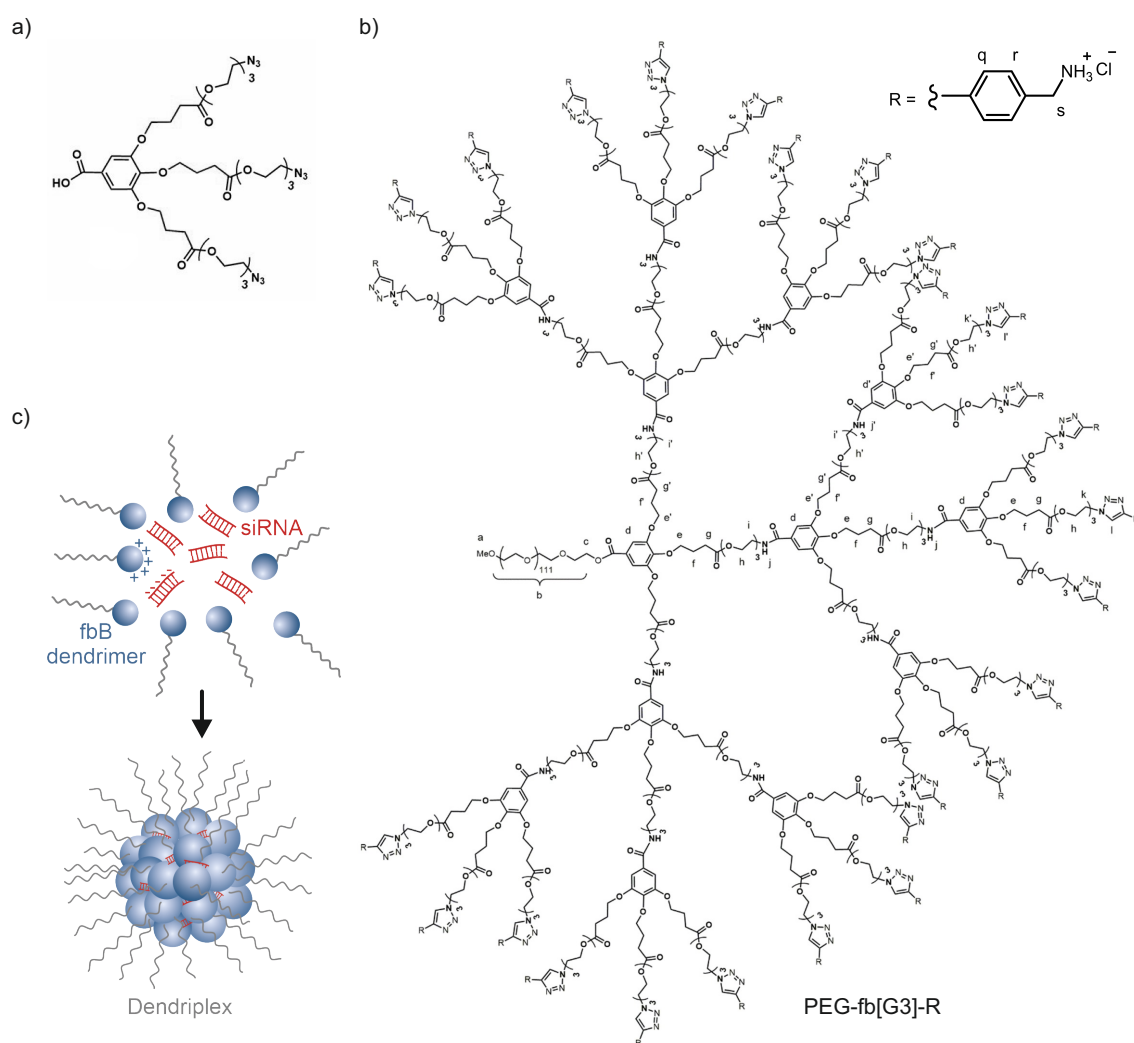


Figure 1.8 | Fully biodegradable PEG-GATGE dendritic block copolymers. **a)** Structure of the GATGE (Gallic Acid Triethylene Glycol Ester) building unit. **b)** Fully biodegradable third-generation PEG-GATGE dendritic block copolymer functionalized with benzylamine (fbB). **c)** Schematic representation of the preparation of fbB-siRNA dendriplexes.

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Chapter 2

Background and Aims

A major goal of our research group is to develop non-viral vectors that can efficiently deliver therapeutic nucleic acids to neurons following a minimally invasive peripheral administration. Previous work using neuron-targeted chitosan-based carriers demonstrated that this strategy can be successfully applied to the treatment of PNI [15].

Aiming to create nanoplateforms with even higher clinical translatability, we designed and synthesized novel PEG-GATGE dendritic block copolymers (see subsection 1.3.2). These combine the attractive features associated with common dendritic families (such as low polydispersity, nanosize, and multivalency) with full biodegradability, a trait critical for safety and biological performance [172]. Among the members of the PEG-GATGE family explored so far, the fbB dendrimer has demonstrated the greatest potential as siRNA carrier [172]. However, while fbB-based dendriplexes have been shown to mediate efficient silencing of the eGFP reporter in neuronal cell lines [172], their capacity to downregulate endogenous genes of therapeutic interest remains unexplored. In this regard, PTEN and Rac1 are two targets worth considering, both due to their relevance in the context of PNI (see subsections 1.1.5 and 1.1.6) and due to related expertise within our team [177, 178].

Additionally, while the endocytosis and intracellular trafficking of fbB-based dendriplexes in neuronal cells were the subject of a recent investigation [179], dendriplex exocytosis is yet to be studied. This is relevant for two reasons. On the one hand, endocytic recycling is generally undesirable in the context of siRNA delivery because it reduces the amount of siRNA reaching the cytosol [180]. Recent results suggested that this process might occur for fbB-based dendriplexes [179]. Identifying to what extent dendriplex exocytosis occurs would allow us to recognize whether it can negatively affect biological performance and, if appropriate, investigate new ways to improve siRNA delivery efficiency. On the other hand, endocytic recycling can, in certain contexts, be of benefit. In the framework of the ongoing FCT funded project JumpIN ("HiJacking the peripheral neurons retrograde trafficking machinery to deliver neuroprotective Molecules to sPinalcord InterNeurons", PTDC/BTM-MAT/4156/2021), we are designing a neurotargeted nucleic acid delivery nanosystem based on our PEG-GATGE dendrimers functionalized with a non-toxic fragment of the tetanus toxin. Envisioned for the treatment of spinal cord injury, this nanosystem will be

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designed to 1) being specifically internalized by peripheral neurons following intramuscular injection, 2) 'hijacking' the retrograde trafficking machinery of axons, and 3) reaching spinal cord interneurons. A fundamental aspect needed for the success of such strategy is the exocytosis of the dendriplexes by peripheral sensory neurons at the spinal cord level. Thus, investigating the process of dendriplex exocytosis is of fundamental interest.

Finally, since *in vitro* 2D culture models have well known limitations [181], it becomes essential to test the dendriplexes in biological models of higher complexity, which better mimic the *in vivo* environment. Preliminary internalization studies of fbB-based dendriplexes were already conducted using embryonic DRG explant cultures, which are an important 3D *ex vivo* model routinely used for the validation of new therapeutic compounds for the treatment of PNS diseases, including PNI [182]. However, the assessment of the biological performance of dendriplexes in this model warrants further investigation.

Taking all the above into account, the present work had four main aims:

- 1) Preparation and characterization of fbB-siPTEN and fbB-siRac1 dendriplexes;
- 2) Evaluation of dendriplex-mediated PTEN and Rac1 downregulation in a neuronal cell line (ND7/23);
- 3) Investigation of dendriplex exocytosis in a neuronal cell line (ND7/23);
- 4) Evaluation of biological performance of dendriplexes in an *ex vivo* model (DRG explant cultures).

Chapter 3

Materials and Methods

3.1 Materials

The synthesis and characterization of the third-generation PEG-GATGE dendritic block copolymer functionalized with alkynated benzylamine (fbB) were carried out following the procedure and techniques previously reported by our group [172]. Two different fbB batches were used in this study: PM33 (prepared by Pedro Mota) and SZ9 (prepared by Sara Zarzo).

For dendriplex characterization (section 3.3) and silencing studies (section 3.5), fbB-based dendriplexes were prepared using siRNAs targeting PTEN and Rac1 (designated as siPTEN and siRac1, respectively), whose sequences are listed in Table 3.1. For exocytosis (section 3.6) and internalization studies (section 3.7), where biological activity was not assessed, a double-stranded DNA labeled with the cyanine-5 (Cy5) fluorophore in the 5' end of one the strands was used as a mimetic siRNA (Cy5-siRNA_{mi}). The sequence of this siRNA_{mi}, also listed in Table 3.1, is the same as that of the anti-eGFP siRNA, which was used in our previous study [172]. All three nucleic acids (siPTEN, siRac1, and Cy5-siRNA_{mi}) were supplied by Integrated DNA Technologies (USA) and used as received.

Table 3.1 | Nucleic acid duplexes used in this work. 2'-O-methyl-modified nucleotides are underlined.

Nucleic acid	Sequence
siPTEN	sense: 5'-CGACUUAGACUUGACC <u>UAUAU</u> -3' antisense: 5'-AUAG <u>GUCAAGUCUAAGUCGAA</u> -3'
siRac1	sense: 5'-CGCACCUGUAACUUAUCAGTT-3' antisense: 5'-CUGAUAAAGUUACAGGUGCGTT-3'
Cy5-siRNA _{mi}	5'-GGTGCAGATGAACTTCAGGGTCAGCTT-3' 5'-/Cy5/GCTGACCCTGAAGTTCATCTGCACC-3'

Ammonium persulphate, bovine serum albumin, laminin L2020, magnesium chloride, poly(D-lysine) hydrobromide (PDL), potassium chloride, heparin sodium salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), IGEPAL[®] CA-630, and Triton[™] X-100 were purchased from Sigma-Aldrich (USA). Dipotassium hydrogen phosphate trihydrate, paraformaldehyde, and

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piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) were obtained from Merck Millipore (USA). D-(+)-Glucose, potassium phosphate monobasic, and tris-hydrochloride were supplied by Fisher Scientific (USA). Sodium dodecyl sulfate (SDS) was purchased from Fluka (USA). Sodium chloride and triethylene glycol diamine tetraacetic acid (EGTA) were obtained from VWR (USA). Ethylenediaminetetraacetic acid (EDTA) was acquired from LabChem (USA). Tris-borate-EDTA (TBE) buffer 10× and acrylamide/bisacrylamide 29:1 solution were obtained from GRiSP (Portugal). Tetramethylethylenediamine was supplied by AppliChem (USA). SYBR[®] Gold was obtained from Invitrogen (USA). DNase I and DNase I buffer 10× were purchased from Fermentas (USA). Dulbecco's Modified Eagle's Medium (DMEM) (1×) with GlutaMAX[™], fetal bovine serum (FBS), Hank's balanced salt solution, Opti-MEM[™], and penicillin-streptomycin solution were acquired from Gibco (USA). Cell culture plates were obtained from SPL Life Sciences (South Korea). Nuclease-free (NF) water was purchased from Qiagen (Germany). Milli-Q water was obtained from a Milli-Q water filtration system (Merck Millipore, USA). RNase-free microtubes were acquired from Axygen (Corning, USA).

3.2 Dendriplex preparation

A fbB solution (6 mg mL⁻¹) was prepared in NF water and filtered through a 0.45 µm polytetrafluoroethylene filter membrane (VWR). fbB-siRNA complexes were prepared at N/P ratios (where N is the number of positively-charged primary amines in the dendritic copolymer and P is the number of negatively-charged phosphate groups in the siRNA backbone) between 5 and 20 by addition of siRNA solution (20 µM) to different volumes of fbB solution in HEPES buffer (20 mM) with 5% (w/v) glucose (pH 7.4). The resulting mixture, where the siRNA concentration was 0.6 µM, was vortexed at medium speed for 10 s and incubated for 30 min at room temperature (RT) to allow for dendriplex stabilization prior to the experiments.

3.3 Dendriplex characterization

3.3.1 Hydrodynamic size, polydispersity index, and zeta potential

Hydrodynamic size, polydispersity index (PDI), and zeta potential (ZP) of dendriplexes were measured at 633 nm using a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments, UK). Measurements were performed at 25 °C with a detection angle of 173° using automatic attenuation selection. For each sample, three consecutive measurements were averaged to produce the final result.

For size and PDI, dendriplexes were prepared in a final volume of 80 µL and transferred to a ZEN0040 plastic cuvette (Malvern Instruments). For ZP, dendriplexes were initially prepared in a final volume of 250 µL, diluted to 750 µL in Milli-Q water, and transferred to a DTS1060 zeta plastic cuvette (Malvern Instruments). The Smoluchowski model was applied for ZP determination,

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and cumulant analysis (Z-average) was used for mean hydrodynamic diameter determination. Measurement data was extracted from the Zetasizer Software version 8.02 (Malvern Instruments).

3.3.2 SYBR[®] Gold intercalation assay

Dendriplexes were prepared as previously described and added (20 μ L) to a 96-well black plate containing 177 μ L of TBE buffer (1 $\times$) and 3 μ L of a SYBR[®] Gold solution (1:100 dilution in TBE buffer 1 $\times$). Following 10 min of incubation at RT, fluorescence was measured ($\lambda_{\text{exc}} = 485$ nm, $\lambda_{\text{em}} = 540$ nm) using a micro-plate reader (SynergyMx, Biotek, USA). Technical duplicates were performed. The amount of non-complexed siRNA in each sample was determined by interpolating its mean fluorescence reading to a standard curve. The results are given as percentage of complexation, where 100% represents complete siRNA complexation. Samples with the same fbB concentration but without siRNA were included as controls for fbB background fluorescence.

3.3.3 Transmission electron microscopy

siPTEN-dendriplexes were prepared at N/P 5 and 20 in a final volume of 20 μ L. Samples (5 μ L) were mounted on a 200-mesh Ni grid with Formvar and carbon supporting film (not glow discharged) and stained with 2% (w/v) uranyl acetate solution. The grid was dried at RT for 5 min prior to imaging. Samples were imaged using a transmission electron microscope JEM-1400 (JEOL, Japan). Images were processed using ImageJ software version 1.53k.

3.3.4 Heparin competition assay

Dendriplexes were prepared at N/P 5 and 20 and incubated with heparin (final heparin concentration ranging from 0 to 0.5 mg mL⁻¹) in HEPES buffer (20 mM HEPES, 5% (w/v) glucose, pH 7.4) for 2 h at 37 °C. The resulting siRNA concentration was 0.3 μ M, corresponding to a 2-fold dendriplex dilution.

For semi-quantitative assessment of siRNA release, samples (12 μ L, corresponding to 3 pmol siRNA) were loaded into the wells of 4-15% (w/v) polyacrylamide-TBE gels and run for 50 min at 90 V. After staining for 10 min with SYBR[®] Gold (1:10 000 dilution in TBE 1 $\times$), gels were imaged using the Gel Doc XR+ system (Bio-Rad, USA). Image Lab software version 6.1 (Bio-Rad) was used for image processing and quantification of band intensities.

For quantitative evaluation of siRNA release, the same samples (7 μ L, corresponding to 2.1 pmol siRNA) were added to a 96-well black plate containing 191 μ L of TBE buffer (1 $\times$) and 2 μ L of SYBR[®] Gold solution (1:100 in TBE buffer 1 $\times$). Technical duplicates were performed. Following 10 min of incubation at RT, fluorescence was measured as described in subsection 3.3.2. Samples with the same fbB concentration but without siRNA were included as controls for fbB background fluorescence. The results are given as percentage of release, where 100% represents complete siRNA release.

3.4 Cell culture

The mouse neuroblastoma × rat DRG neuron hybrid ND7/23 cell line (ECACC 92090903) was cultured in DMEM (1×) with GlutaMAX™, supplemented with 10% (v/v) heat-inactivated FBS (inactivation at 56 °C for 30 minutes) and 1% (v/v) antibiotic solution (10 000 U mL⁻¹ penicillin and 10 000 µg mL⁻¹ streptomycin) at 37 °C, 5% CO₂. Cells were passaged every 2 to 4 days, and were used between passages 3 and 15. Routine screening for mycoplasma contamination was carried out using polymerase chain reaction (PCR). Cell viability was always assessed before seeding using the Trypan blue exclusion test.

3.5 Silencing studies

3.5.1 Optimization of transfection conditions

The protocol for transfection of ND7/23 cells with fbB-siPTEN dendriplexes was subjected to iterative optimizations, varying conditions such as the culture plate format, the N/P ratios tested, the volume of dendriplexes added, the presence/absence of serum and antibiotic during transfection, and the time between transfection and mRNA quantification by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). This is summarized in Table 3.2, in which siPTEN transfection experiments are labelled #1 to #4. For siRac1, only one transfection was performed, which is labelled as #5 in Table 3.2.

Table 3.2 | Transfection conditions tested for PTEN and Rac1 silencing.

Parameter	Experiment				
	#1	#2	#3	#4	#5
siRNA	siPTEN	siPTEN	siPTEN	siPTEN	siRac1
Plate format	24-well	96-well	96-well	96-well	96-well
N/P ratio	20	5, 10, 20	5, 10, 20	5, 20, 40	20
Dendriplex volume (µL)	50	10	25	25	25
Final siRNA per well (pmol)	30	6	15	15	15
Serum/antibiotic during transfection	yes	no	no	no	no
Time of RT-qPCR evaluation (h)	96	48	48	72	48

In all transfections, cells were seeded at a density of 2.5×10^4 viable cells per cm² and incubated in supplemented medium¹ for 24 h up to a 70-80% confluence. For transfections #2 to #5, the medium was substituted with non-supplemented DMEM one hour before transfection. The volume of dendriplexes added during transfection always corresponded to 1/6 of the final transfection volume, in order to obtain a siRNA final concentration of 100 nM [172]. As a positive control, cells were transfected with Lipofectamine® 2000 (L2k, Invitrogen) lipoplexes, prepared

¹Unless otherwise specified, 'supplemented medium' or 'supplemented DMEM' always refer to DMEM with GlutaMAX™, supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic solution (see section 3.4), whereas 'non-supplemented medium' or 'non-supplemented DMEM' refer simply to DMEM with GlutaMAX™.

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in Opti-MEM™ according to manufacturer guidelines (final L2k concentration of 0.15% (v/v)). As negative controls, cells were transfected with lipoplexes or dendriplexes containing Silencer™ Select Negative Control No. 1 siRNA (siNC, Invitrogen) at a final concentration of 100 nM (according to the manufacturer, this siRNA does not target any gene product). Additional controls included non-treated cells (NT, all experiments) and mock-transfected cells (transfected only with L2k or fbB; only included in transfection #2). All conditions were tested in duplicate wells. Twenty-four hours after transfection, the medium was substituted by fresh supplemented DMEM and cells were further incubated for 24 h, 48 h, or 72 h, depending on the timing of RT-qPCR evaluation (see Table 3.2). Then, the medium was discarded and cells were washed twice with phosphate buffer saline (PBS, 1×, pH 7.4). Afterward, 30 µL (or 250 µL for 24-well plates) of qCell lysis buffer (composed of 10 mM tris-hydrochloride (pH 7.4), 0.25% (v/v) IGEPAL® CA-630, and 150 mM NaCl) was added to each well, and plates were incubated on ice for 10 min. The lysates were then transferred to RNase-free microtubes and centrifuged at 700 ×g at 4 °C for 5 min. The supernatants were transferred to new microtubes and either stored at –80 °C or used immediately for RT-qPCR. In transfections #3 to #5, lysates were diluted 10× before being used for RT-qPCR, in order to guarantee optimal amplification efficiencies (see Appendix A for more details).

3.5.2 Reverse-transcription quantitative polymerase chain reaction

RT-qPCR reactions were set-up in a 384-well PCR plate (Bio-Rad) using the One Step SYBR® PrimeScript™ RT-PCR Kit II (Perfect Real Time) (Takara Bio, USA) or the One Step NZYSpeedy RT-qPCR Green kit (NZYTech, Portugal). For the Takara kit, 1 µL of each lysate was added to 9 µL of a master mix consisting of 5 µL of 2× One Step TB Green RT-PCR Buffer 4, 3 µL of NF water, 0.4 µL of PrimeScript 1 step Enzyme Mix 2, and 0.3 µL of each 10 µM primer dilution (final primer concentration of 300 nM). For the NZYTech kit, the master mix consisted of 5 µL of 2× One-step NZYSpeedy qPCR Green master mix, 3 µL of NF water, 0.4 µL of NZYRT mix, and 0.3 µL of each 10 µM primer dilution. The primer sequences used can be found in Table 3.3. The RT-qPCR was run on an CFX384 Touch Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions (Takara kit): (i) reverse transcription (1 cycle) - 10 min at 42 °C, followed by 10 s at 95 °C, (ii) PCR amplification (40 cycles) - 10 s at 95 °C (denaturation), followed by 30 s at 55 °C (annealing), and 45 s at 72 °C (extension). For the NZYTech kit, the reverse transcription step involved 20 min at 50 °C followed by 2 min at 95 °C, and the extension was performed at 72 °C for 30 s. Melt curves were always performed for checking primer specificity. Non-template controls were included for each gene assessed and technical triplicates were performed for each qPCR reaction. Bio-Rad CFX Maestro Software version 2.3 (Bio-Rad) and Microsoft Excel were used for data analysis. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method [183]. PTEN or Rac1 mRNA expression was normalized against the reference gene *Ywhaz* and calibrated to NT controls. The presented data are expressed as the mean of two biological replicates.

3.6 Exocytosis studies

3.6.1 Exocytosis evaluation by fluorometry

ND7/23 cells were seeded in 48-well plates at a density of 2.5×10^4 viable cells per cm^2 and incubated in supplemented DMEM for 24 h up to a 70-80% confluence prior to transfection. At the time of transfection, half of the volume of culture medium (125 μL) was removed from the wells and 25 μL of fbB-Cy5-siRNAmi dendriplexes (N/P 5, 10, or 20) were added (Cy5-siRNAmi final concentration of 100 nM in a final volume of 150 μL). Twenty-four hours after, the culture medium was removed, the wells were washed once with PBS 1 $\times$, and fresh supplemented DMEM was added. Then, cells were further incubated for 2 h, 6 h, 24 h, or 48 h (one plate per condition), after which the culture medium was collected for fluorometry measurements. Controls included cells treated with free Cy5-siRNAmi (100 nM), NT cells (incubated with 20 mM HEPES with 5% (w/v) glucose, pH 7.4), and supplemented DMEM. All conditions were tested in triplicate wells.

To prevent Cy5 fluorescence quenching within dendriplexes (see Figure B.2), collected samples (68.6 μL) were mixed with 1.4 μL of 5% (w/v) SDS (final concentration of 0.1% (w/v)) and incubated for 30 min at RT to induce dendriplex destabilization prior to the fluorometry measurements. Afterward, 60 μL of each SDS-treated sample was transferred to a quartz glass cuvette (Ultra-Micro Cell 105.251-QS, light path 3 $\times$ 3 mm, Hellma Analytics, Germany) and the Cy5 fluorescence emission spectrum was recorded in a FluoroMax[®]-4 Spectrofluorometer (HORIBA Scientific, Japan). Measurement settings were as follows: $\lambda_{\text{exc}} = 633$ nm (5 nm slit), $\lambda_{\text{em}} = 650$ -700 nm (5 nm slit, 1 nm increment), 0.2 s integration time, 3 detector accumulations (averaged scans). Emission spectra were extracted from the FluorEssence[™] spectroscopy software (HORIBA Scientific) and further analyzed in Microsoft Excel. Plotted values are fluorescence intensity (FI) at 662 nm (Cy5 emission peak).

Table 3.3 | Primers used for RT-qPCR.

Primer designation	Primer sequence
<i>PTEN</i> Forward	5'-CAAAGAGATCGTTAGCAGAAACA-3'
<i>PTEN</i> Reverse	5'-ATGCTTTGAATCCAAAAACCTTACT-3'
<i>Rac1</i> Forward	5'-ATGCAGGCCATCAAGTGTG-3'
<i>Rac1</i> Reverse	5'-TAGGAGAGGGGACGCAATCT-3'
<i>Ywhaz</i> Forward	5'-ACGACGTACTGTCTCTTTTGG-3'
<i>Ywhaz</i> Reverse	5'-GTATGCTTGCTGTGACTGGT-3'

3.6.2 Cy5-siRNAmi degradation study

Cy5-siRNAmi was incubated for 1 h with varying amounts of DNase I (0-0.8 U) in a reaction mixture containing HEPES buffer (20 mM HEPES with 5% (w/v) glucose, pH 7.4) and DNase I buffer 1 $\times$ in a final volume of 40 μL (final Cy5-siRNAmi concentration of 0.3 μM). The DNase I was then inactivated by adding EDTA solution (final concentration of 0.05 M) and incubating

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the samples at 65 °C for 10 min. After stabilizing at RT for 30 min, mixtures were treated with SDS (final concentration of 0.1% (w/v)) and incubated at RT for further 30 min. Finally, samples (15 µL, corresponding to 3 pmol Cy5-siRNAmi) were loaded into the wells of a 4-15% (w/v) polyacrylamide-TBE gel. Cy5-siRNAmi in NF water (3 pmol) was used as control. Moreover, the equivalent concentrations of salts, SDS and EDTA were loaded in empty wells to guarantee an uniform band migration. After running for 50 min at 90 V, the gel was stained for 20 min with SYBR[®] Gold (1:10 000 dilution in TBE 1×) and imaged using a ChemiDoc MP Imaging System (Bio-Rad). A multichannel acquisition was performed in order to simultaneously detect the fluorescence signal from SYBR[®] Gold (blue epi illumination and emission filter of 530/28 nm) and Cy5 (red epi illumination and emission filter of 695/55 nm). Images were extracted from the Image Lab software version 6.1 (Bio-Rad).

To evaluate whether the total Cy5 fluorescence signal was affected by Cy5-siRNAmi degradation, the same DNase-treated samples were also analyzed by fluorometry. For that, each sample (30 µL) was diluted 2-fold in Milli-Q water and its Cy5 fluorescence spectrum was obtained as previously described (see subsection 3.6.1). Plotted values are FI at 662 nm (Cy5 emission peak).

3.6.3 Exocytosis evaluation by polyacrylamide gel electrophoresis

ND7/23 cells were seeded in 48-well plates at a density of 2.5×10^4 viable cells per cm² and incubated in supplemented DMEM for 24 h up to a 70-80% confluence prior to transfection. At the time of transfection, half of the volume of culture medium (125 µL) was removed from the wells and 25 µL of fbB-Cy5-siRNAmi dendriplexes (N/P 5 or 20) were added (Cy5-siRNAmi final concentration of 100 nM in a final volume of 150 µL). Twenty-four hours after, the culture medium was removed, the wells were washed once with PBS 1×, and fresh supplemented DMEM was added. Then, cells were further incubated for 24 h, after which the culture medium was collected into microtubes. Controls included cells treated with free Cy5-siRNAmi (100 nM), NT cells (incubated with 20 mM HEPES with 5% (w/v) glucose, pH 7.4), and supplemented DMEM. All conditions were tested in triplicate wells. Empty wells were filled with sterile water to reduce evaporation during incubation periods.

To remove cellular debris, collected culture medium samples were centrifuged at 300 ×g for 5 min and a fixed volume of supernatant (120 µL) was transferred into new 0.6 mL RNase-free microtubes. Samples were concentrated 10-fold by freeze-drying (Labconco 7420031, -80 °C, overnight), followed by resuspension in 12 µL of 1% (w/v) SDS. Concentrated samples were then loaded into the wells of a 10-well 4-10% (w/v) polyacrylamide-TBE gel and run for 1 h at 90 V. After staining for 20 min with SYBR[®] Gold (1:10 000 dilution in TBE 1×), gels were imaged using a ChemiDoc MP Imaging System, as previously described (see subsection 3.6.2). For quantification of the Cy5-siRNAmi present, dilutions of Cy5-siRNAmi in supplemented DMEM (5, 2.5, and 1 nM in a final volume of 150 µL) were processed in the same way (centrifugation, lyophilization, and resuspension in SDS) and run alongside the transfection samples.

3.7 Transfection of *ex vivo* neuronal cultures

3.7.1 Dorsal root ganglion culture

All animal experiments were conducted with the permission of the local animal ethical committee in accordance with the European Union Directive (2010/63/EU) and Portuguese law (DL 113/2013).

DRGs were obtained from mouse embryos (E16.5) from pregnant 2-3 months old female C57BL/6 mice. The embryos were harvested and maintained in ice-cooled Hank's balanced salt solution until DRG removal. The DRG isolation procedure was performed under a stereomicroscope (model SZ61, Olympus, Japan, magnification range: 6.7-45 $\times$). Using a sterile scalpel and tweezers, the vertebral column and surrounding tissue were separated from the embryo's body (Figure 3.1a-b), after which the spinal cord was removed to provide dorsal access to the DRGs (Figure 3.1c-d).

After isolation, DRGs were seeded in 15-well μ -Slide Angiogenesis plates (#81506, ibidi, Germany) previously coated with PDL and laminin. For the PDL coating, the wells were covered with a PDL solution (50 $\mu\text{g mL}^{-1}$ in 0.1 M borate buffer, pH 8.5) and the plate was incubated overnight at 37 °C. Then, the wells were rinsed three times with PBS 1 $\times$ and the plate was dried at RT. For the laminin coating, the PDL-coated plate was incubated with a laminin solution (5 $\mu\text{g mL}^{-1}$ in PBS 1 $\times$) for 30 min at 37 °C, after which the wells were rinsed once with PBS 1 $\times$. DRGs were then immediately added, in a final volume of 50 μL of NeurobasalTM medium (Gibco) supplemented with serum-free B-27TM Supplement 1 $\times$ (Gibco), L-glutamine (2 mM, Gibco) and nerve growth factor (NGF, 25 ng mL⁻¹, Calbiochem, USA). The explant cultures were incubated at 37 °C, 5% CO₂ for 5 days prior to transfection. During this time, neurite outgrowth was observed (Figure 3.1e).

3.7.2 Transfection

Transfection was performed on day 6 by adding 8 μL of fbB-Cy5-siRNAmi dendriplexes (N/P 5, 10, or 20) to each DRG (Cy5-siRNAmi final concentration of 138 nM). Then, plates were incubated for an additional 72 h. NT DRGs were included as control. At least two DRGs were used per transfection condition.

On day 9, explant cultures were fixed for 15 min at RT after addition of 1-2 drops of microtubule protecting fixative buffer (MP-PFA, containing 4% (w/v) paraformaldehyde, 65 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 3 mM MgCl₂ in PBS 1 $\times$, pH 7.4) per well. The culture medium with MP-PFA was then discarded, and fixation was completed by adding an additional 1-2 drops of MP-PFA and incubating at RT for further 15 min, followed by rinsing three times with PBS 1 $\times$. For neurite immunostaining, fixed DRGs were permeabilized for 45 min with TritonTM X-100 (0.3% (v/v) in PBS 1 $\times$), blocked for 15 min with bovine serum albumin (0.5% (w/v) in PBS 1 $\times$) and incubated overnight at 4 °C with mouse anti- β -III-tubulin monoclonal antibody (clone TUBJ1, #801202, BioLegend, USA, 1:500 dilution in PBS 1 $\times$). Then, the wells were rinsed once with

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PBS 1× and incubated with AlexaFluor™ 488 donkey anti-mouse secondary antibody (#A-21202, Invitrogen, 1:1000 dilution in PBS 1×) for 1 h at RT. Finally, nuclei staining was achieved by incubation with Hoechst 33342 (Thermo Fisher Scientific, 1 $\mu\text{g mL}^{-1}$ in PBS 1×) for 10 min at RT. Immunostained samples were kept immersed in PBS 1× at 4 °C until imaging.

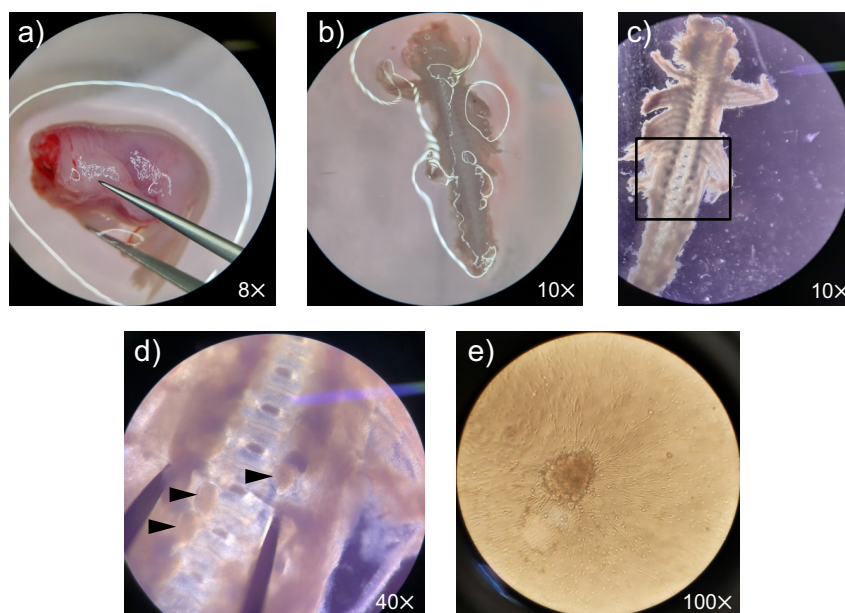


Figure 3.1 | Dorsal root ganglion (DRG) isolation and culture. **a)** Decapitated E16.5 mouse embryo (lateral view). **b)** Isolated spinal column (ventral view). **c)** Isolated spinal column with the spinal cord removed (dorsal view). **d)** Detail from (c), with the positions of some accessible DRGs being indicated by black arrowheads. **e)** DRG explant culture 5 days after isolation. Neurite outgrowth is visible. Images (a-d) were taken through the eyepiece of a stereomicroscope, whereas (e) was taken through the eyepiece of an inverted microscope. The approximate magnifications are indicated in the bottom-right corner of each image.

3.7.3 Image acquisition and analysis

Imaging was performed on a Leica SP8 Confocal Microscope (Leica Microsystems, Germany) using a 25× water-immersion objective (HC Fluotar L 25X/0.95 W VISIR, Leica Microsystems), three excitation laser lines (405, 488, and 633 nm), and three spectral detectors (2 PMT and 1 HyD). Three-dimensional Z-stacks with 2 μm Z-resolution and 300 nm pixel size were captured using the Leica LAS X software (Leica Microsystems). The full Z-stacks and their respective maximum intensity projection (MIP) images were extracted as .tif files and processed using MATLAB R2021a (The MathWorks, USA) with the Image Processing Toolbox installed.

Dendriplexes internalization/association

As a simple measure of the internalization and association of fbB-Cy5-siRNAmi dendriplexes to the DRGs, the total Cy5 FI within and outside the boundaries of each explant body was quantified. For this purpose, a custom MATLAB function was created, named *Cy5MIP.m* (Appendix C.1).

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This function loads a .tif MIP image and extracts the Cy5 and Hoechst channels. For segmentation of the explant body, the Hoechst channel is binarized by thresholding. Only the largest object is kept and potential holes within the explant's area are filled (Figure 3.2b). The perimeter of the explant body is then used to create a partition of the Cy5 image that only includes the Cy5 signal within the explant body (Figure 3.2c). The sum of all pixels in this image is computed, and then divided by the area of the explant body, as follows:

$$\text{Normalized Cy5 FI within explant body} = \frac{(\text{Sum of Cy5 pixel values within explant})/255}{\text{Area of the explant body [pixels]}} \quad (3.1)$$

This parameter has values between 0 and 1, where 0 corresponds to total absence of Cy5 signal (all pixel values within the explant body are 0) and value 1 corresponds to total saturation (all pixel values within the explant body are 255).

Additionally, *Cy5MIP.m* also outputs the fraction of the total Cy5 FI that lies within the explant body, calculated as:

$$\text{Fraction of Cy5 FI within explant body} = \frac{\text{Sum of Cy5 pixel values within explant}}{\text{Sum of all Cy5 pixel values}} \quad (3.2)$$

A batch-processing script (*batch_Cy5MIP.m*) was created in order to automatically call the *Cy5MIP.m* function for several images within a selected folder (Appendix C.1). All output values were stored in the same folder in an Excel file for further analysis.

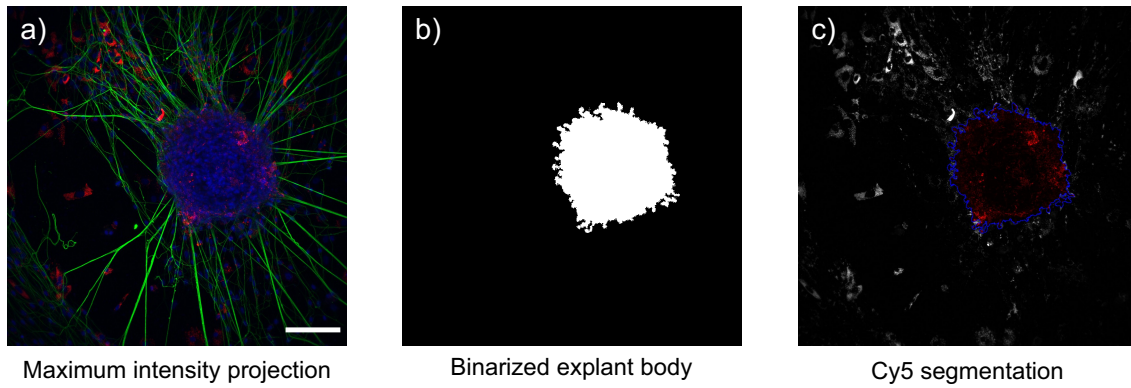


Figure 3.2 | Quantification of Cy5 fluorescence in dorsal root ganglion explants treated with fbB-Cy5-siRNAmi dendriplexes. **a)** Maximum intensity projection image of a dorsal root ganglion treated with fbB-Cy5-siRNAmi dendriplexes N/P 10 (red). Neurons were stained against β -III-tubulin (green) and nuclei with Hoechst (blue). **b)** The explant body was segmented by selecting the largest object after binarization. **c)** The boundary of the explant body (blue line) was used to segment the Cy5 channel into two parts, containing the Cy5 fluorescence signal inside and outside the explant body (red and white, respectively). Scale bar: 100 μ m.

Dendriplexes Z-distribution

To analyze the dendriplexes' Z-distribution, a custom MATLAB function was created, named *Cy5Zstack.m* (Appendix C.1). This function loads a multi-image .tif file and extracts the Hoechst,

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β -III-tubulin, and Cy5 channels. For each channel, it computes a vector containing the sum of all XY pixel values per Z-slice. An additional vector containing the corresponding Z-positions (in μm) is also calculated. Finally, it also finds the slice with the highest total FI for each channel.

The batch-processing script *batch_Cy5Zstack.m* (Appendix C.1) was used to automatically call the *Cy5Zstack.m* function for all multi-image .tif files within the selected folder. All output values were stored in the same folder in an Excel file for further analysis.

Neurite outgrowth analysis

Neurite outgrowth analysis was performed using *ExplantAnalyzer*, an open-source MATLAB routine recently proposed by Schmidbauer et al. [184]. This application includes several scripts for high-throughput quantification of organotypic explant outgrowth. The main script is the *ExplantAnalyzer.m* function, which takes as input a RGB image containing the β -III-tubulin staining in one channel and the nuclei staining (DAPI or Hoechst) in another. Several image processing steps are performed in order to obtain a precise skeleton of the neurite network. A schematic overview of these steps applied to one of our MIP images is included in Figure 3.3.

The first step is loading the image and separating it into two channels. The Hoechst channel is used to determine the size and boundary of the explant body without outgrown neurites, as described previously. The binarized explant is then dilated two times by a constant value. The 1x dilated area will be subtracted from the β -III-tubulin channel in order to improve adaptive thresholding near the explant body, whereas the 2x dilated area is used to determine the start-points of the neurite network.

For segmentation of the neurite network, the β -III-tubulin channel is first subjected to basic filter operations. These include background removal by subtraction of a fixed value from the whole image, high boost filtering to enhance the signal-to-background ratio, and a median filter to remove pixel noise. The filtered image is then binarized by adaptive thresholding and unconnected segments are removed. The area of all fibers is computed at this stage as one of the outgrowth measures. Afterward, the binarized fibers are smoothed (dilation followed by erosion) and reduced to their skeleton. The points where the skeletonized network intercepts with the 2x dilated explant perimeter are extracted as start-points. Additional downstream processing steps can yield more metrics describing neurite outgrowth but these were not applied in the present study.

A total of eight image processing parameters are required in order to perform the abovementioned operations. These were optimized iteratively using a subset of five MIP images, according to the developers' recommendations [184]. A list of all optimized parameters is provided in Appendix C.2. After this initial setup, the analysis was run automatically for all MIP images at once, using the *batch.m* script [184]. For each image within the selected folder, a .mat file was created containing the output from *ExplantAnalyzer.m*, as well as a .jpg image that provided an overview of the segmentation (Figure 3.3c). All individual .mat files were then batch-processed using the *simple_statistics.m* script [184], which computes several statistical values for each image and saves them to an Excel file.

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Two neurite outgrowth metrics were used for comparison among different treatment groups. The first was the area occupied by all fibers, computed from the binarized fiber image (Figure 3.3b, after processing step 4) as follows:

$$\text{Area occupied by neurites (\%)} = \frac{(\text{Number of "true" pixels in the binarized fiber image}) \times (\text{Pixel area})}{(\text{Image area}) - (1\text{x dilated explant area})} \quad (3.3)$$

The denominator of equation 3.3 corresponds to the area within each image where the presence of fibers is evaluated. It was introduced to compensate for the fact that, for larger DRGs, most of the area of the MIP image is occupied by the explant body, leaving less area for detection of the outgrown fibers.

The second metric used was the number of start-points normalized by the perimeter of the 2x dilated explant:

$$\text{Number of start-points per mm} = \frac{\text{Number of start-points}}{2\text{x dilated explant perimeter}} \quad (3.4)$$

Some minor modifications were made to the original MATLAB scripts provided by Schmidbauer et al. [184]. Most were related to 16-bit to 8-bit conversion (the *ExplantAnalyzer.m* was set for 16-bit images). Additionally, the *simple_statistics.m* was extended in order to compute the metrics described by equations 3.3 and 3.4. Finally, for cases where the automatic segmentation of the explant body was unsatisfactory, the boundary was manually added to the .tif image as an extra (alpha) channel using Adobe Photoshop CC 2019 (Adobe, USA) (see example in Appendix C.4). An if-statement was included in the *ExplantAnalyzer.m* script in order to use this extra channel instead of the Hoechst channel in images where it was present. A list of all modifications to the original scripts is included in Appendix C.3.

3.8 Statistical analysis

GraphPad Prism version 9.3 for Windows (GraphPad Software, USA) was used for graphical representation of results and statistical analysis. Normal distribution of data was previously verified using the Shapiro-Wilk test. Tests used for calculation of statistical significance are described in the corresponding figures. Results with $p < 0.05$ were considered statistically significant.

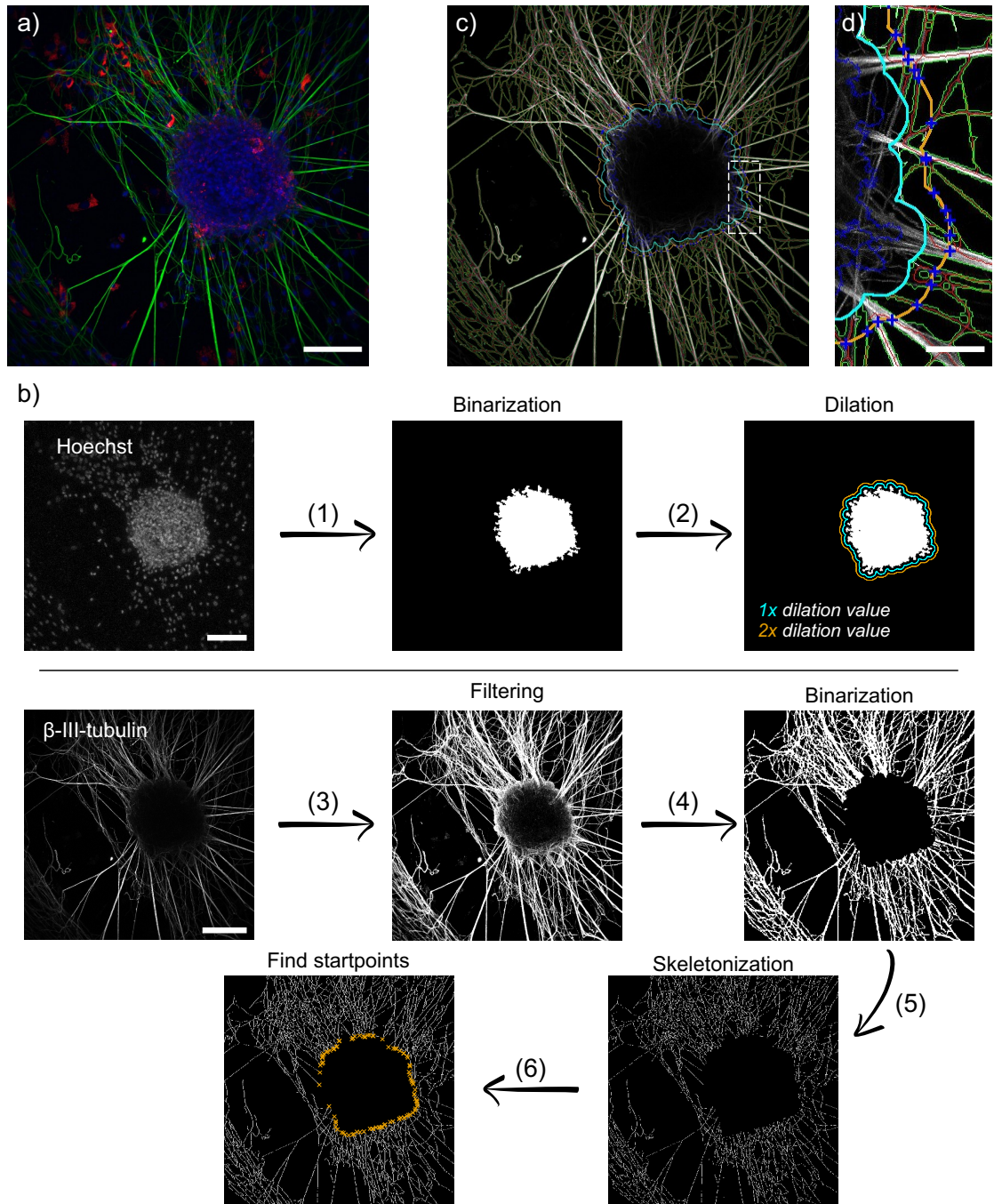


Figure 3.3 | Schematic overview of the main image processing steps for neurite outgrowth analysis. **a)** Maximum intensity projection image of a dorsal root ganglion treated with fbB-Cy5-siRNAmi dendriplexes N/P 10 (red). Neurons were stained against β -III-tubulin (green) and nuclei with Hoechst (blue). **b)** Processing of the Hoechst-stained explant body: (1) binarization; (2) dilation. Processing of the β -III-tubulin-stained fibers: (3) filter operations (background removal, high boost filter, median filter); (4) subtraction of the 1x dilated explant area, binarization by adaptive thresholding, keep the largest area; (5) smoothing; (6) skeletonization and subtraction of the 2x dilated explant area; (7) detection of start-points (orange +). **c)** Result of the image processing steps overlaid on the β -III-tubulin channel. Dark blue line: explant body segmentation. Cyan and orange lines: 1x and 2x dilated explant body (respectively). Green lines: neurite segmentation. Red lines: neurite skeleton. Blue crosses: start-points. **d)** Detailed view of (c). Scale bar: 100 μ m (a,b), 20 μ m (d). Based on a similar schematic from Schmidbauer et al. (2021) [184].

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Chapter 4

Results and Discussion

4.1 Dendriplex characterization

The capacity of any non-viral vector to bind, compact, and protect nucleic acids is of the utmost importance for its delivery efficacy. In a previous study [172], we characterized the interaction between the fbB dendritic block copolymer and a small DNA duplex (25-bp). This siRNA_{mi} was chosen because it is structurally similar to biologically active siRNA whilst being cheaper to acquire and having a longer shelf-life than the latter. The complexation capacity and the physicochemical properties of the resulting dendriplexes were assessed for N/P ratios ranging from 5 to 80, where N represents the total number of positively-charged primary amines in the dendritic copolymer and P represents the number of negatively-charged phosphate groups in the siRNA_{mi} backbone. An excellent capacity of fbB to bind and complex siRNA_{mi} was demonstrated, with the formation of small (52-56 nm) dendriplexes with narrow size distributions (PDI between 0.23 and 0.29).

In the present work, we aimed to assess the biological performance of dendriplexes formed by the fbB dendritic block copolymer and two different siRNAs with therapeutic interest: siPTEN and siRac1. Since internal data suggested that the same dendrimer can interact differently (more or less stably) with different siRNA sequences, it was important to thoroughly characterize the physicochemical properties of fbB-siPTEN and fbB-siRac1 dendriplexes prior to assessing their biological activity. Nevertheless, while in our previous study N/P ratios up to 80 were included, here the highest N/P ratio tested was 20. The reason for this is two-fold. Firstly, our data indicated that the capacity of fbB-based dendriplexes to mediate silencing of the eGFP reporter gene is not very dependent on the N/P ratio [172]. Thus, formulations with lower N/P ratios (i.e., lower amount of the fbB dendritic block copolymer) have higher cost-effectiveness. Secondly, while dendriplexes at N/P 40 and 80 are well-tolerated by the ND7/23 neuronal cell line [172], they cause significant cytotoxicity in DRG explant cultures [unpublished data], a more complex and relevant *ex vivo* model explored in the present study.

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4.1.1 Complexation capacity

The efficiency of complexation between the fbB dendritic block copolymer and both siPTEN and siRac1 was evaluated by the SYBR[®] Gold intercalation assay. SYBR[®] Gold is a cationic molecule which emits strong fluorescence upon binding to nucleic acids. In this assay, this property is employed to estimate the amount of free siRNA following dendriplex formation, since the nucleic acid becomes inaccessible to the dye when complexed with the fbB dendritic block copolymer.

As depicted in Figure 4.1a, fbB showed a high capacity to complex both siRNAs, with complexation efficiencies of $68 \pm 11\%$ and $55 \pm 13\%$ at N/P 5, increasing up to $89 \pm 2\%$ and $86 \pm 5\%$ at N/P 20, for siPTEN and siRac1, respectively. This increase in complexation efficiency with the increase in N/P ratio reached statistical significance for fbB-siRac1 dendriplexes ($p < 0.05$, N/P 5 vs. N/P 20). These results were expected since higher N/P ratios correspond to a higher amount of dendritic block copolymer available to interact with the same amount of siRNA. Nevertheless, no significant differences were detected between the two siRNAs when comparing formulations of identical N/P ratios ($p > 0.05$), even though the mean complexation efficiency for fbB-siPTEN dendriplexes was higher than that of fbB-siRac1 dendriplexes at all three N/P ratios tested.

4.1.2 Hydrodynamic size, zeta potential, and morphology

The dendriplexes' hydrodynamic size and PDI were determined by DLS. As shown in Figure 4.1d, a narrow particle size distribution in the nanometer scale was obtained for all formulations tested. Hydrodynamic sizes (Figure 4.1b) varied between 44 and 54 nm, with no significant differences between N/P ratios or siRNAs ($p > 0.05$). This range is very similar to the one obtained previously for fbB-siRNAmi dendriplexes [172]. Nanoparticles of this size range are ideal for nervous system applications, since they can penetrate through poorly permeable tissue (such as that of the PNS and CNS) and be efficiently uptaken by cells, whilst still remaining above the threshold for renal filtration [104, 185, 186].

PDI values varied between 0.24 and 0.36 (Figure 4.1c), reflecting a moderately homogeneous population of dendriplexes, albeit less homogeneous than the one reported for fbB-siRNAmi dendriplexes (PDI between 0.23 and 0.29) [172]. Although no statistically significant differences among PDI values were observed when comparing different N/P ratios or siRNAs ($p > 0.05$), the formulations at N/P 20 presented the lowest mean PDI (0.30 and 0.24 for siPTEN and siRac1, respectively). This might reflect a higher stabilization due to the excess charge.

The ZP was determined by laser Doppler electrophoresis. As presented in Figure 4.1e, all dendriplexes reported positive ZP values, except fbB-siRac1 dendriplexes at N/P 5, whose mean ZP was close to neutral (-0.3 ± 0.8 V). As expected, ZP values increased with increasing N/P ratio for both fbB-siPTEN and fbB-siRac1 dendriplexes ($p < 0.0001$, fbB-siPTEN N/P 5 vs. N/P 20; $p < 0.001$, fbB-siRac1 N/P 5 vs. N/P 20). Interestingly, fbB-siRac1 dendriplexes showed lower ZP values than fbB-siPTEN when comparing formulations at identical N/P ratios (statistically significant at N/P 20, $p < 0.05$), which suggests different dendriplex condensation. This is unlikely

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to result from differences in the size of the siRNA strands (both siRNAs are 21-bp duplexes with two-nucleotide overhangs at the 3' ends) or in nucleotide compositions (GC contents are 38.1% and 42.9% for siPTEN and siRac1, respectively). The most likely explanation is the fact that, while siRac1 is not chemically modified, siPTEN has 10 nucleotides in the antisense strand that possess the 2'-OMe modification (see Table 3.1). This ribose modification increases the overall hydrophobicity of siPTEN, probably affecting the interactions with the fbB dendritic block copolymer.

The positive ZP values displayed by fbB-siRac1 and fbB-siPTEN dendriplexes are advantageous to facilitate adhesion to the negatively-charged cellular membrane and subsequent cellular uptake [186, 187]. Nevertheless, it is relevant to note that the maximal ZP value reported herein (7.6 ± 1.5 mV, for fbB-siPTEN dendriplexes at N/P 20) is still relatively low comparing to other reported dendrimer-siRNA formulations (e.g. dendriplexes prepared with fourth-generation PAMAM were reported to have ZP values of around 40 mV [151]). This is important to avoid the negative effects associated with carriers bearing strong cationic charge, such as hematotoxicity, cytotoxicity, and enhanced clearance by the reticuloendothelial system [133, 163, 186]. On the other hand, too low ZP values are traditionally linked to low colloidal stability due to the higher tendency of neutral nanoparticles to aggregate [188]. However, in the case of the fbB dendritic block copolymer, additional factors other than electrostatic repulsion contribute to promote the colloidal stability of the dendriplexes. PEGylation, commonly used in nanoparticle formulations, is one such factor. Additionally, the hydrophobic interactions potentiated by the structure of the GATGE repeating unit and by the benzylamine moieties are a decisive force in assuring the formation of stable dendriplexes [170, 172].

Finally, the morphology of fbB-siPTEN dendriplexes at N/P 5 and 20 was assessed by transmission electron microscopy (TEM). As seen in Figure 4.1f, the dendriplexes present spherical morphologies at both tested N/P ratios. At N/P 5, TEM images show some signs of nanoparticle aggregation, which are not seen at N/P 20. This is in conformity with the close to neutral ZP values observed at the lowest N/P ratio. The particle sizes obtained at N/P 5 seem to be similar to the hydrodynamic diameters obtained by DLS analysis. As for the N/P 20 dendriplexes, TEM sizes tend to be slightly lower than the hydrodynamic sizes (most nanoparticles shown in Figure 4.1f have diameters under 35 nm). This can be explained by the different information provided by both techniques: DLS measures the hydrodynamic size of particles dispersed in a solvent (intensity-weighted), whereas TEM works on dry samples under ultrahigh vacuum conditions [188].

Overall, the physicochemical properties (size, surface charge, and morphology) of the fbB-siPTEN and fbB-siRac1 dendriplexes are in agreement with our previous characterization for fbB-siRNAmi dendriplexes, and highlight the potential of the fbB dendritic block copolymer as carrier for different siRNAs (including chemically modified ones such as siPTEN). It is also relevant to note that two different fbB batches were used in physicochemical characterization assays (represented by ● and ★ in the bar graphs of Figure 4.1). The fact that both batches yielded similar results highlights the robustness of the dendritic synthesis process.

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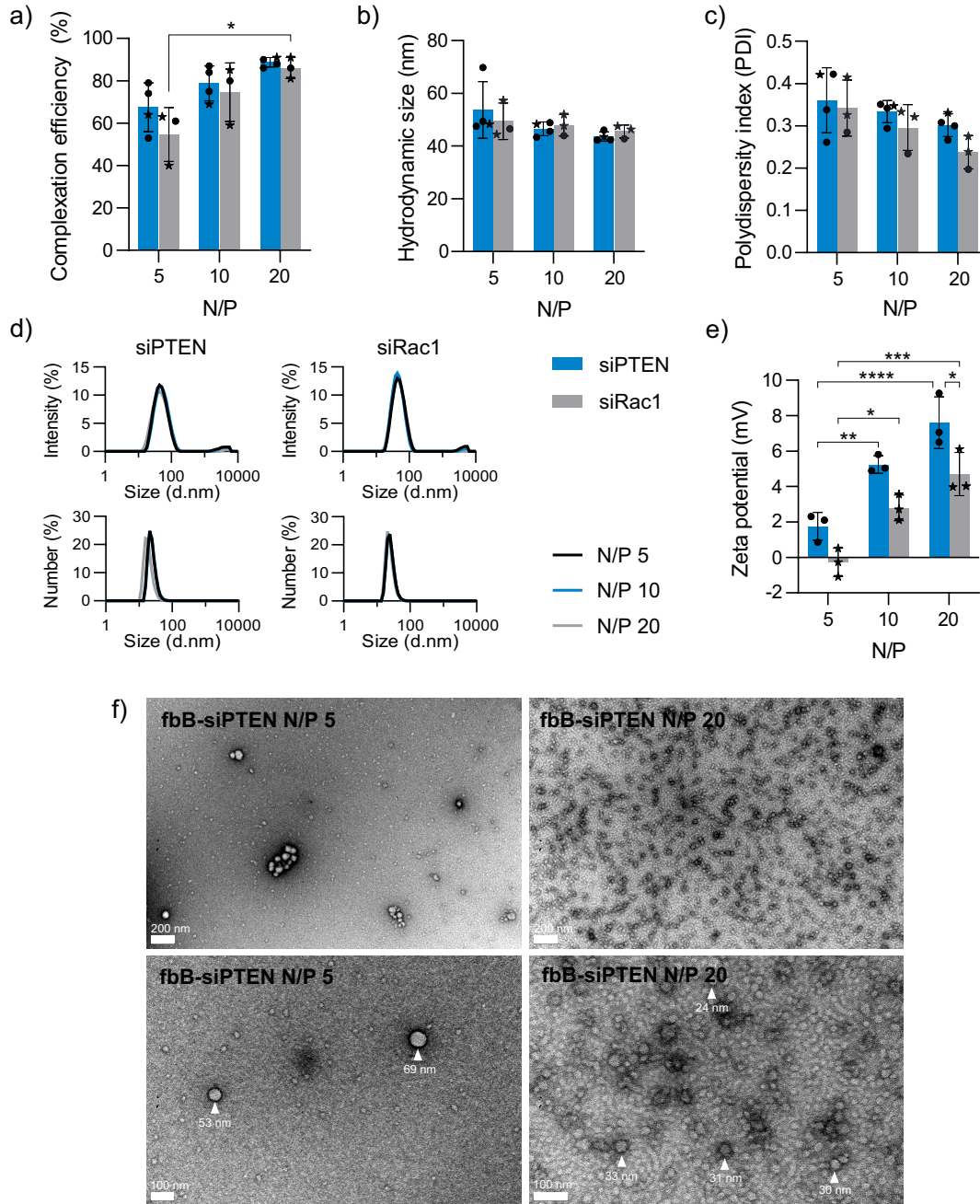


Figure 4.1 | Physicochemical characterization of fbB-siPTEN and fbB-siRac1 dendriplexes as a function of the N/P ratio. **a)** siRNA complexation efficiency (SYBR[®] Gold intercalation assay). **b)** Hydrodynamic size (Dynamic Light Scattering - DLS). **c)** Polydispersity index (PDI) (DLS). **d)** Representative intensity-weighted (top) and number-weighted (bottom) DLS size distributions. **e)** Zeta potential (Laser Doppler electrophoresis). **f)** Transmission-electron microscopy (TEM) images of fbB-siPTEN dendriplexes at N/P ratios 5 (left) and 20 (right). Scale bars: 200 nm (top) and 100 nm (bottom). Results in graphs (a), (b), (c) and (e) are shown as mean $\pm$ SD of three or four independent experiments. Individual data points for each independent experiment are represented by $\bullet$ or $\star$, depending on which fbB batch was used (PM33 or SZ9, respectively). Two-way ANOVA, followed by Tukey's multiple comparison test, was used for statistical analysis. Significant differences: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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4.1.3 Release capacity

The capacity of the fbB dendritic block copolymer to complex siRNA into compact nanoparticles is critical to ensure protection from premature degradation and efficient cellular uptake. However, once inside cells, the siRNA needs to be released from the dendriplexes in order to exert its biological activity. Thus, it is important to guarantee the reversibility of the siRNA-fbB interaction. This was evaluated by incubating fbB-siPTEN and fbB-siRac1 dendriplexes (N/P 5 and 20) at 37 °C for 2 h in the presence of heparin, a polyanionic molecule that acts as a competitor for complexation to cationic molecules and that is commonly used to test the stability and release of nucleic acids from nanoformulations [152, 189, 190]. The amount of siRNA released was assessed by two different methods: polyacrylamide gel electrophoresis (PAGE) (Figure 4.2a,b,d,e) and SYBR[®] Gold intercalation (Figure 4.2c,f).

All tested formulations showed extensive release of siRNA, especially at the highest heparin concentrations ($\geq 0.025 \text{ mg mL}^{-1}$). Both PAGE analysis and SYBR[®] Gold intercalation indicated that dendriplexes at N/P 20 have a higher siRNA retention capacity than at N/P 5. It is reasonable to assume that the higher N/P ratio also confers a higher protection against nuclease-mediated siRNA degradation, as previously reported by us [172]. However, even dendriplexes at N/P 20 not treated with heparin (first lane in the right PAGE gels) showed some siRNA release during the 2 h incubation period. Moreover, it is interesting to note that, in general, the quantification of PAGE band intensities (Figure 4.2b,e) seems to indicate greater release than the SYBR[®] Gold quantification (Figure 4.2c,f), when comparing dendriplexes at identical heparin concentrations. It is possible that some of the release detected by PAGE is due to the electrostatic forces present during the PAGE run rather than from the incubation period with heparin.

Finally, because the release profiles shown in Figures 4.2c and 4.2f were the result of a single experiment each, it is not possible to establish whether there are significant differences between fbB-siPTEN and fbB-siRac1 dendriplexes in terms of retention capacity. Nonetheless, the results in Figure 4.2 are consistent with the ones previously obtained for fbB-siRNA_{mi} dendriplexes [172], where an extensive release of siRNA was observed at N/P 5 even in the absence of heparin, but where complete release from N/P 80 dendriplexes was only reached for heparin concentrations above 0.050 mg mL^{-1} . This observation, together with the other results from the physicochemical characterization (Figure 4.1), supports the thesis that the fbB-siPTEN and fbB-siRac1 dendriplexes used in the present work should have an internalization profile similar to the one observed for fbB-siRNA_{mi} dendriplexes.

4.2 Silencing studies

Next, the dendriplexes' ability to mediate PTEN and Rac1 silencing was tested on the ND7/23 cell line. This hybrid cell line results from the fusion of rat DRG neurons with mouse neuroblastoma cells and it is frequently used as an *in vitro* model system of peripheral sensory neurons [191].

The transfection efficiency was first assessed for fbB-siPTEN dendriplexes. Briefly, ND7/23 cells were seeded into 24-well plates and incubated with fbB-siPTEN dendriplexes in supplemented

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DMEM for 24 h (final siPTEN concentration per well of 100 nM), followed by assessment of transfection efficiency at 96 h post-transfection. These transfection conditions were set based on our previous work [172], which tested the capacity of fbB dendriplexes carrying anti-eGFP siRNA (siGFP) to downregulate eGFP expression in ND7/23 eGFP^{Luc} cells. An important difference is that, while in that work silencing was assessed at the protein level (by measuring the eGFP FI), here silencing was evaluated by quantification of the levels of PTEN mRNA by RT-qPCR.

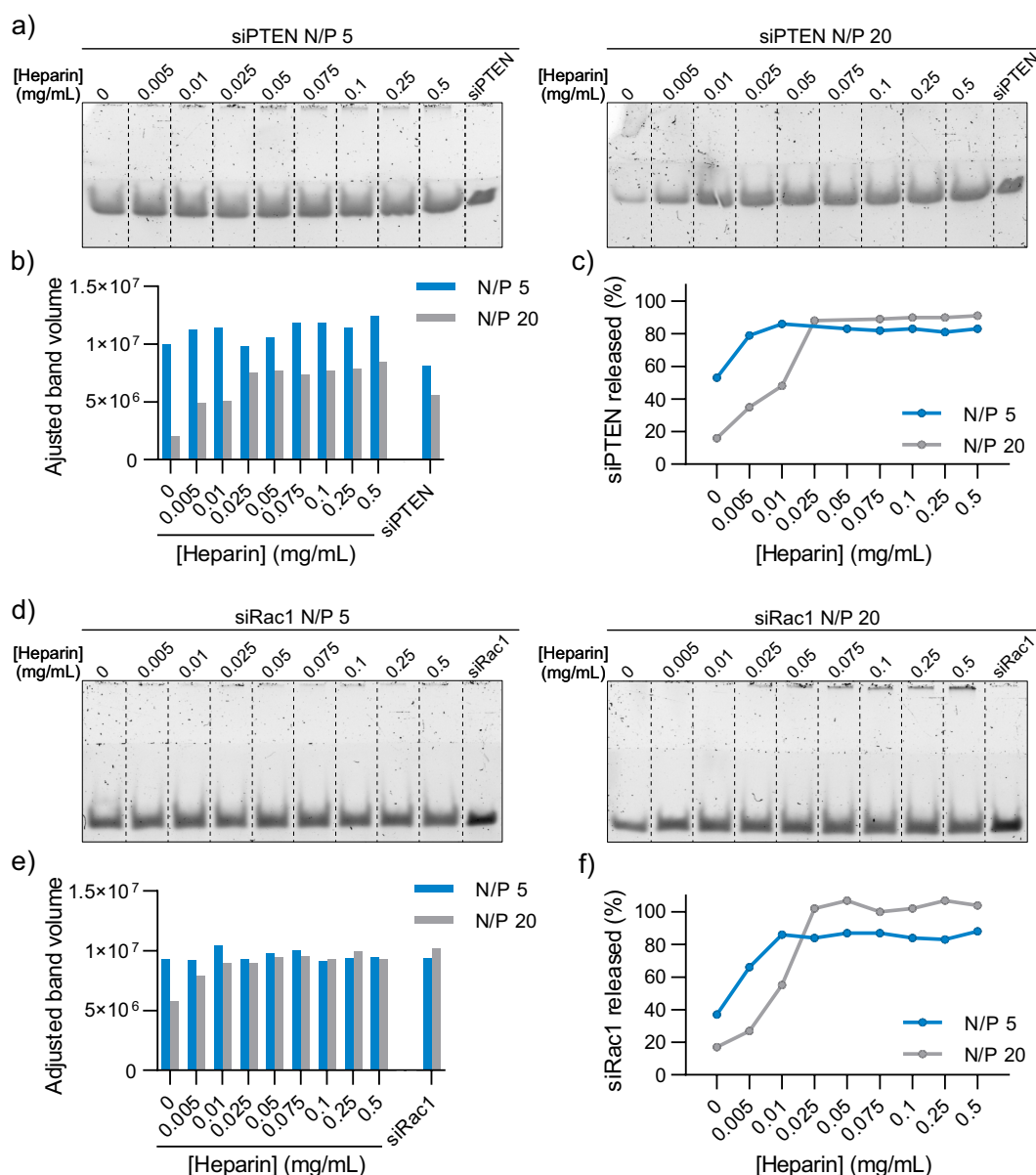


Figure 4.2 | Heparin competition assay for fbB-siPTEN and fbB-siRac1 dendriplexes. **a)** PAGE gels showing the extent of siPTEN dissociated from N/P 5 and N/P 20 dendriplexes after incubation with increasing concentrations of heparin at 37 °C for 2 h. In the siPTEN lane, the same amount of free siPTEN as used for the preparation of the dendriplexes was loaded. Images are representative from two independent experiments. **b)** Quantification of band intensities from the PAGE gels shown in (a). **c)** SYBR[®] Gold quantification of siPTEN released from N/P 5 and N/P 20 dendriplexes at increasing concentrations of heparin. **d-f)** Same as (a-c), but for fbB-siRac1 dendriplexes.

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As shown in Figure 4.3a, no reduction of the PTEN mRNA level was observed in cells transfected with fbB-siPTEN dendriplexes at N/P 20 compared to the NT controls. However, the siPTEN used was biologically active, as demonstrated by the 56% silencing observed in cells transfected with L2k, which is a commonly used commercially available *in vitro* transfection reagent. Nevertheless, this suggested that an optimization of the transfection conditions was needed. In a second attempt, some protocol modifications were introduced. Firstly, the plate format was changed from 24- to 96-well, enabling the inclusion of more transfection conditions (N/P 5, 10, and 20) whilst reducing the amount of siPTEN required per well (6 pmol). Secondly, since protein adsorption to the surface of nanoparticles can lead to reduced cellular uptake [187], non-supplemented DMEM was used during incubation with the dendriplexes. Thirdly, while protein-level eGFP knockdown was observed at 96 h post-transfection in our previous study [172], a downregulation at the mRNA level (upstream event) is likely to be more pronounced at earlier time-points [192]. Therefore, the timing of RT-qPCR evaluation was reduced to 48 h post-transfection, a time-point also used by other authors investigating dendrimer-based siRNA delivery [154, 190, 193, 194]. Despite these changes, no dendriplex-mediated PTEN silencing was observed at any of the N/P ratios tested (Figure 4.3b). By contrast, the PTEN mRNA downregulation in cells transfected with L2k-siPTEN lipoplexes was 94% compared to NT cells.

Both of the aforementioned experiments yielded seemingly good quality RT-qPCR data: melting curve analysis showed a single, well-defined melting temperature for both target and reference genes and a good consistency across technical and biological replicates was verified. However, given the underwhelming results, a more powerful method was used to assess whether the RT-qPCR reactions were fully optimized. This analysis, detailed in Appendix A, indicated that the cell lysates should be diluted 10-fold to guarantee optimal qPCR amplification efficiencies. This optimization was included in a third transfection experiment, which also introduced a 2.5-fold increase in the volume of fbB-siPTEN dendriplexes added per well (15 pmol siPTEN per well), in an attempt to increase the silencing effect. This experiment also did not reveal any dendriplex-mediated PTEN downregulation relative to NT controls (Figure 4.3c). Similar results were obtained in a fourth transfection experiment, where RT-qPCR evaluation was performed at 72 h post-transfection and N/P 40 dendriplexes were included as a new transfection condition (Figure 4.3d).

Regarding fbB-siRac1 dendriplexes, only one transfection experiment was conducted, using the same transfection conditions as those used in the third siPTEN transfection. As shown in Figure 4.3e, no reduction of the Rac1 mRNA level was observed in cells transfected with dendriplexes at N/P 20 compared to NT cells. Conversely, transfection with L2k-siRac1 lipoplexes resulted in 55% downregulation, thus proving that the siRac1 used is biologically active (although less potent than siPTEN, which yielded 95% of silencing under the same conditions - Figure 4.3c).

Taking all the above into account, it seemed clear that, at least under the conditions tested, fbB-based dendriplexes were not capable of delivering enough siPTEN or siRac1 to the cytosol of ND7/23 cells to elicit a detectable downregulation of the respective mRNAs. This result was unexpected, given that a similar nanocarrier system resulted in potent downregulation of the eGFP reporter protein in ND7/23 eGFPLuc cells [172].

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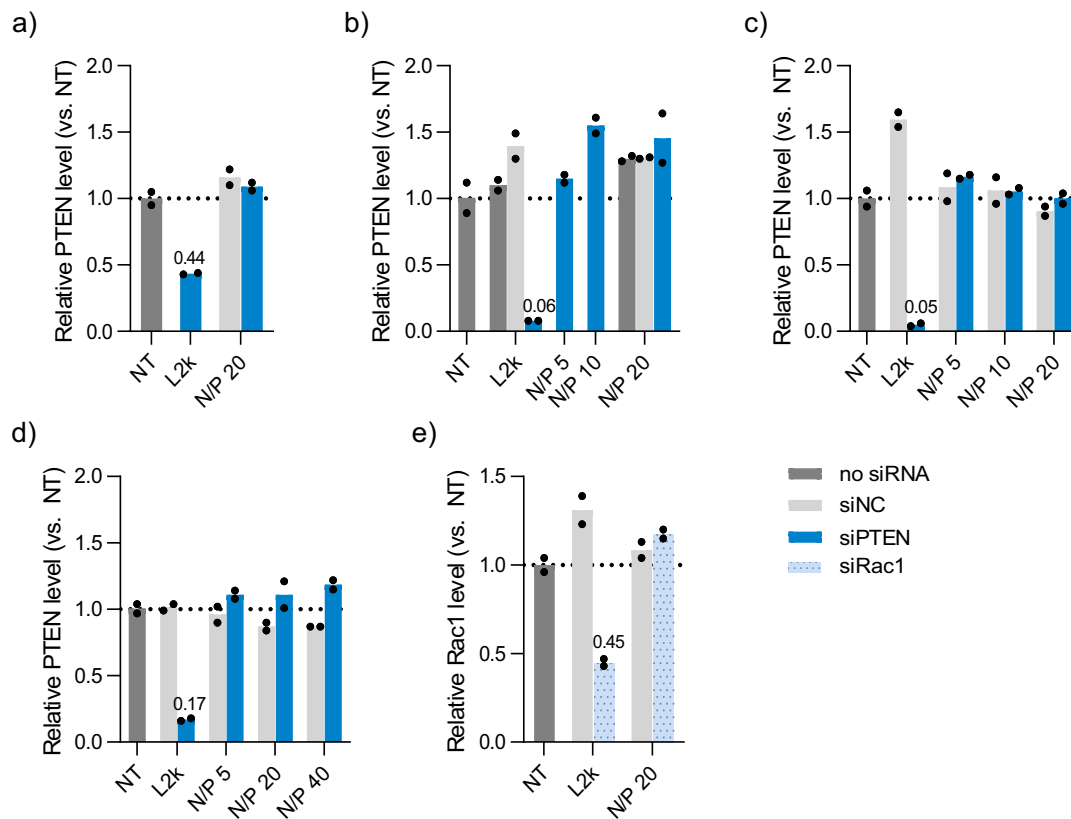


Figure 4.3 | Silencing of PTEN and Rac1 mediated by fbB-siPTEN and fbB-siRac1 dendriplexes. PTEN and Rac1 mRNA expression levels were determined by RT-qPCR and are relative to those of non-treated (NT) controls. Each graph corresponds to one independent experiment (transfections #1 to #5, whose conditions are indicated in Table 3.2). Individual data points (two biological replicates) are represented by dots. L2k: Lipofectamine® 2000. siNC: negative control siRNA. **a)** Transfection #1 (siPTEN). ND7/23 cells were plated in 24-well plates and transfected with 30 pmol siRNA in the presence of serum and antibiotic in the culture medium. RT-qPCR was performed at 96 h post-transfection. **b)** Transfection #2 (siPTEN). To test more conditions, the plate format was changed to 96-wells and the amount of siRNA used in transfection was scaled down appropriately (6 pmol). Serum and antibiotic were removed from the culture medium during internalization. RT-qPCR was performed at 48 h post-transfection. **c)** Transfection #3 (siPTEN). The amount of siRNA used for transfection was increased to 15 pmol relative to transfection #2. **d)** Transfection #4 (siPTEN). N/P 40 was included as a new transfection condition, and RT-qPCR was performed at 72 h. **e)** Transfection #5 (siRac1). Transfection conditions were the same as those of transfection #3.

At first sight, differences in the target protein (PTEN/Rac1 vs. eGFP) or in the intrinsic potency of the siRNAs could be put forward to explain this discrepancy. Indeed, it is well-known that the formation and degradation kinetics of the target mRNA, as well as its normal expression levels within cells, can significantly impact siRNA-mediated knockdown [195]. Moreover, depending on their intrinsic potency, different siRNAs can produce a wide range of knockdown efficiencies, even when present in the cytosol at similar amounts [88]. However, the fact that both siPTEN and siRac1 were capable of producing a significant downregulation of their target mRNAs when delivered with L2k suggests that the problem lies in the delivery vector rather than in the siRNA itself.

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Three main events could have constituted putative bottlenecks that hampered dendriplex-mediated siPTEN and siRac1 delivery: cellular uptake, siRNA release, and endosomal escape. In our previous work [172], efficient internalization was observed on the ND7/23 cell line for fbB-Cy5-siRNAmi dendriplexes, where siRNAmi was a double-stranded DNA bearing the same sequence as siGFP. Directly studying cellular uptake of fbB-siPTEN or fbB-siRac1 dendriplexes is not possible using the current sequences, given that they are not conjugated with a fluorescent label. Furthermore, since the physicochemical characterization of these dendriplexes (Figure 4.1) revealed a high degree of similarity with the previously characterized fbB-siRNAmi dendriplexes, it was reasonable to assume that similar cellular uptake kinetics would be attained.

Likewise, reduced siRNA release due to strong siRNA-carrier interactions is unlikely to be the main impediment to efficient delivery of siPTEN or siRac1, given that the heparin competition assay (Figure 4.2) showed extensive siRNA release from both fbB-siPTEN and fbB-siRac1 dendriplexes.

Thirdly, a common bottleneck in non-viral siRNA delivery is the entrapment of siRNA molecules in the endosomal compartment following cellular uptake [83, 110, 111]. This was a problem for the second-generation hybrid-biodegradable PEG-GATGE dendritic block copolymers [170], but less so for the fbB vector (at least judging by its capacity to mediate eGFP silencing [172]). Nonetheless, the results obtained herein indicated that the endosomal escape of fbB-based dendriplexes warranted further investigation. Thus, after the 24 h incubation period with fbB-siPTEN or fbB-siRac1 dendriplexes, ND7/23 cells were further incubated with chloroquine (100 μ M) for 8 h, and the PTEN/Rac1 mRNA levels were assessed by RT-qPCR at 48 h post-transfection. Since chloroquine acts as an endosomal disrupting agent, any improvement of dendriplex-mediated silencing could be interpreted as a result of improved endosomal escape [170, 196, 197]. Unfortunately, the chloroquine treatment resulted in evident cytotoxic effects on ND7/23 cells, including significant alterations in the expression of the housekeeping gene *Ywhaz* and a considerable degree of cell death [data not shown]. As a result, this experiment failed to provide any reliable conclusions on the impact of endosomal trapping. In a future work, the experimental conditions involving chloroquine treatment (namely the concentration and duration of exposure) will have to be optimized to minimize these adverse effects.

Looking ahead, it is crucial to bridge the gap between the results obtained in the present work and those reported previously for the fbB dendritic block copolymer [172]. To achieve this, two important sets of experiments should be conducted. The first will be to assess whether the observed dendriplex-mediated eGFP downregulation at the protein level can be reproduced at the mRNA level. While RT-qPCR is frequently used to detect mRNA knockdown, disparities between analyzing the effects of siRNA treatment by measuring mRNA levels or protein levels have been described [198]. If knockdown of eGFP at the mRNA level is observed, then it becomes important to consider cell line-specific effects. Indeed, while in this work we attempted to silence PTEN and Rac1 in ND7/23 cells (wild-type), eGFP silencing was reported in ND7/23 eGFPLuc cells. It is possible that this sub-lineage is more susceptible to transfection than wild-type cells. A simple way to test this would be to assess whether fbB-siPTEN and fbB-siRac1 dendriplexes can mediate knockdown of their respective mRNAs in ND7/23 eGFPLuc cells.

4.3 Exocytosis studies

4.3.1 Exocytosis evaluation by fluorometry

The uptake kinetics of fbB-based dendriplexes by neuronal cells (including the ND7/23 cell line) were the subject of a recent investigation by our group [179]. In that study, siRNA_{mi} labeled with the Cy5 fluorophore (Cy5-siRNA_{mi}) was used to enable fluorescence-based dendriplex tracking by flow cytometry and confocal microscopy. During incubation with fbB-Cy5-siRNA_{mi} dendriplexes, the cell-associated fluorescence signal increased gradually over time (due to the accumulation of dendriplexes inside cells) until it reached a maximum. Curiously, after this maximum, there was a reduction in the signal intensity value, potentially a result of Cy5-siRNA_{mi} degradation and/or exocytosis. For the reasons mentioned previously (see Chapter 2), investigating the process of dendriplex exocytosis by the ND7/23 cell line is an important goal of the present work.

The methodology initially employed for this purpose involved monitoring the Cy5 FI in the extracellular medium (Figure 4.4a). Firstly, ND7/23 cells were incubated for 24 h with fbB-Cy5-siRNA_{mi} dendriplexes. As expected, the extracellular FI values decreased during this period due to the cellular uptake of dendriplexes (Figure B.3). Afterward, the dendriplex-containing culture medium was removed, cells were rinsed with PBS, and fresh medium was added. This time-point was set as the starting point for exocytosis detection ($t = 0$ h). After different time intervals ($t = \Delta t$), the medium was collected and its FI was measured.

Within the first 2-6 h of incubation, no notable increase in the extracellular FI values was detected in cells treated with fbB-Cy5-siRNA_{mi} dendriplexes compared to NT cells (Figure 4.4b.1). Conversely, after 24 h, differences relative to NT cells were evident, both in cells transfected with dendriplexes (N/P 5, 10, or 20) and in cells transfected with 'free' (non-complexed) Cy5-siRNA_{mi}. It should be noted that the extracellular FI values measured for NT cells result from the autofluorescence of the culture medium (supplemented DMEM - DMEM_{sup}) rather than from the Cy5 fluorophore (see Figure B.4 for clarification).

Since this initial experiment showed more pronounced differences at the 24 h time-point, a second experiment was conducted where the extracellular FI was measured at 24 h and 48 h. As shown in Figure 4.4b.2, the measurements at 24 h were in agreement with the first experiment, with extracellular FI values for cells transfected with dendriplexes or with free Cy5-siRNA_{mi} being notably higher than the background FI detected for NT cells. After 48 h, this difference was slightly more pronounced, particularly for cells treated with free Cy5-siRNA_{mi}.

Overall, the abovementioned experiments seemed to indicate that at least some of the nucleic acid that is internalized in the form of dendriplexes is then excreted by ND7/23 cells. However, the results obtained for cells treated with free Cy5-siRNA_{mi} were unexpected. During the "internalization phase", these cells exhibited a higher decrease in the extracellular FI value than those transfected with fbB-Cy5-siRNA_{mi} dendriplexes (Figure B.3). However, previous results from confocal microscopy and flow cytometry had shown that internalization of free Cy5-siRNA_{mi} is much less efficient than dendriplex-mediated internalization [unpublished data]. Moreover, during

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the "exocytosis phase", cells treated with free Cy5-siRNAmi were also the ones associated with the highest increase in the extracellular FI value (Figure 4.4b). One hypothesis to explain these observations is that non-complexed Cy5-siRNAmi is more prone to nuclease degradation. If extensive degradation occurred over the course of the experiments, then small Cy5-labeled DNA fragments or even free dye could have originated and could be more easily internalized and released by cells.

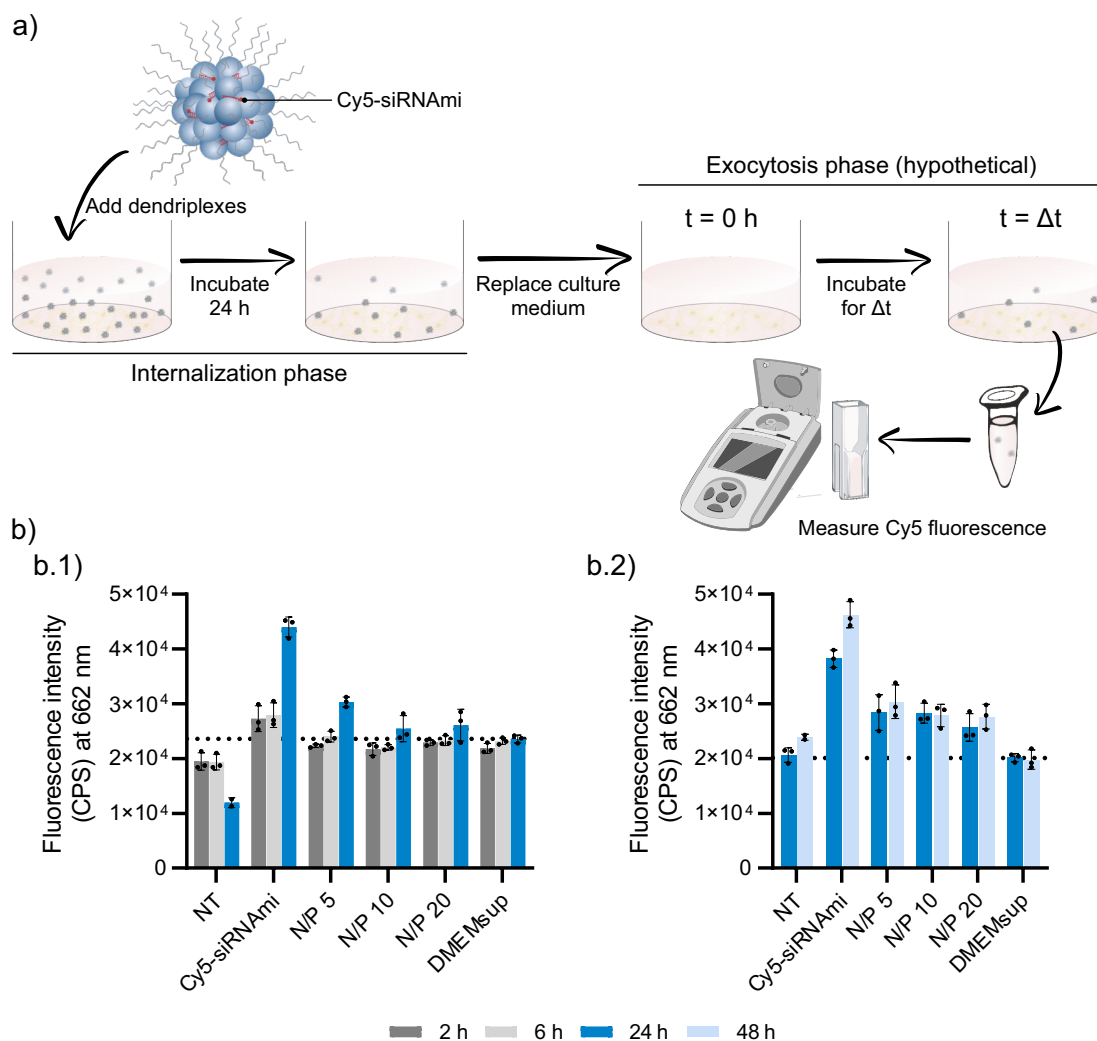


Figure 4.4 | Exocytosis evaluation by fluorometry. a) Schematic representation of the experimental design. ND7/23 cells were put in contact with fbB-Cy5-siRNAmi dendriplexes (N/P 5, 10, or 20) or with free Cy5-siRNAmi for 24 h. The culture medium was then replaced ($t = 0$ h) and the plates were incubated up to $t = \Delta t$. Finally, the culture medium was collected for quantification of the Cy5 fluorescence intensity. b) Cy5 fluorescence intensities (expressed in counts per second - CPS) measured in two independent experiments: (b.1) $\Delta t = 2$ h, 6 h, and 24 h; (b.2) $\Delta t = 24$ h, and 48 h. Data are expressed as mean $\pm$ SD of three biological replicates (dots). The dashed lines indicate the fluorescence intensity value (autofluorescence) obtained for supplemented DMEM (DMEMsup). NT: non-treated controls.

In order to test this hypothesis, a Cy5-siRNAmi degradation study was conducted. Cy5-siRNAmi was incubated for 1 h with increasing amounts of DNase I and then analyzed by two different techniques: native PAGE (to visualize the extent of degradation) and fluorometry (to assess whether

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degradation affects the Cy5 FI). As seen in Figure 4.5a, Cy5-siRNA_{mi} incubated without DNase I (leftmost lane) resulted in a single band appearing in the same position in the SYBR[®] Gold and Cy5 fluorescence images. This indicates that its integrity was preserved. Conversely, samples treated with DNase I resulted in smeared bands in the fluorescence image of SYBR[®] Gold, indicating siRNA_{mi} degradation. As expected, this degradation increased with increasing DNase I concentrations. Interestingly, bands originating from these samples did not exhibit full colocalization between the SYBR[®] Gold and Cy5 fluorescence signals. This can be explained by the fact that, as each Cy5-siRNA_{mi} molecule only includes one Cy5 moiety (at the 5' end of one of the strands), endonuclease-mediated degradation inevitably originated some siRNA_{mi} fragments labeled with Cy5 and some unlabeled fragments (Figure 4.5c). The latter were detected in the SYBR[®] Gold image but not in the Cy5 image.

Notably, when analyzed by fluorometry (Figure 4.5b), all samples yielded approximately the same Cy5 FI values, irrespective of the degradation state of Cy5-siRNA_{mi}. Thus, a Cy5 moiety conjugated with an intact siRNA_{mi} molecule yields the same FI as a Cy5 moiety conjugated to a fragment of siRNA_{mi}. This is particularly relevant for the interpretation of Figure 4.4b. It suggests that the FI increase observed in the culture medium of cells treated with free Cy5-siRNA_{mi} does not necessarily represent excretion of the intact nucleic acid; mostly likely, at least part of it is due to the excretion of products of Cy5-siRNA_{mi} degradation.

Emphasis should be given to the fact that this degradation study was not (and was not intended to be) an exact reproduction of the biological conditions of the experiments in Figure 4.4. In fact, the concentration of nucleases in the presence of ND7/23 cells and supplemented medium is unknown and there are certainly other components that may influence the kinetics of Cy5-siRNA_{mi} degradation. For dendriplexes, an additional variable is the protection against endonucleases conferred by the formulation, which increases with the N/P ratio [172]. Nevertheless, this simple degradation study yielded two main conclusions: (i) the integrity of the exocytosed products should be investigated and (ii) fluorometry is not the best-suited technique to make that evaluation.

These conclusions are also relevant in the broader context of intracellular dynamics studies. Conjugation of carriers or cargo with fluorescent labels is a widely used method to study the distribution of nanoparticles *in vitro* and *in vivo* [180, 199, 200]. While these methods are attractive due to their experimental amenability and high sensitivity, the interpretation of the results often relies on the assumption that the fluorophore remains bound to the nanoparticle over the course of the experiment. However, this assumption is rarely corroborated through biostability testing [199, 200]. As our experiment highlighted, when using a fluorescence-based approach, one should be remain cautions in the interpretation of the data and, whenever possible, verify the findings using complementary methodologies.

4.3.2 Exocytosis evaluation by polyacrylamide gel electrophoresis

In order to assess the integrity of the exocytosed Cy5-siRNA_{mi}, we opted to analyze the extracellular medium samples by native PAGE, since it allows for a direct visualization of both the nucleic acid (by SYBR[®] Gold staining) and its conjugated fluorophore. However, it was clear that

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the samples had to be concentrated in order to have a nucleic acid concentration within the range of detection. We decided to address this issue using lyophilization.

As a preliminary test, dilutions of Cy5-siRNAmi in supplemented DMEM were lyophilized, resuspended in a 10× smaller volume and subjected to PAGE. The high protein content of these samples did not prevent the electrophoretic mobility of Cy5-siRNAmi, which produced a visible band up to the second-lowest concentration tested (0.12 pmol) (see Appendix B.4). However, when the same workflow was applied to culture medium samples collected from cells at the $t = 24$ h exocytosis time-point, a sufficient level of electrophoretic separation could not be achieved (Figure 4.6a). In addition to a high concentration of FBS-derived proteins (present in both samples and standards - Figure 4.6b), the cellular-derived samples could also contain cellular debris that probably clogged the pores of the polyacrylamide-TBE gel. Presumably, the centrifugation step used to clear out cellular debris ($300 \times g$ for 5 min) was not fully effective. Because of this, no conclusions regarding Cy5-siRNAmi exocytosis could be taken from this experiment.

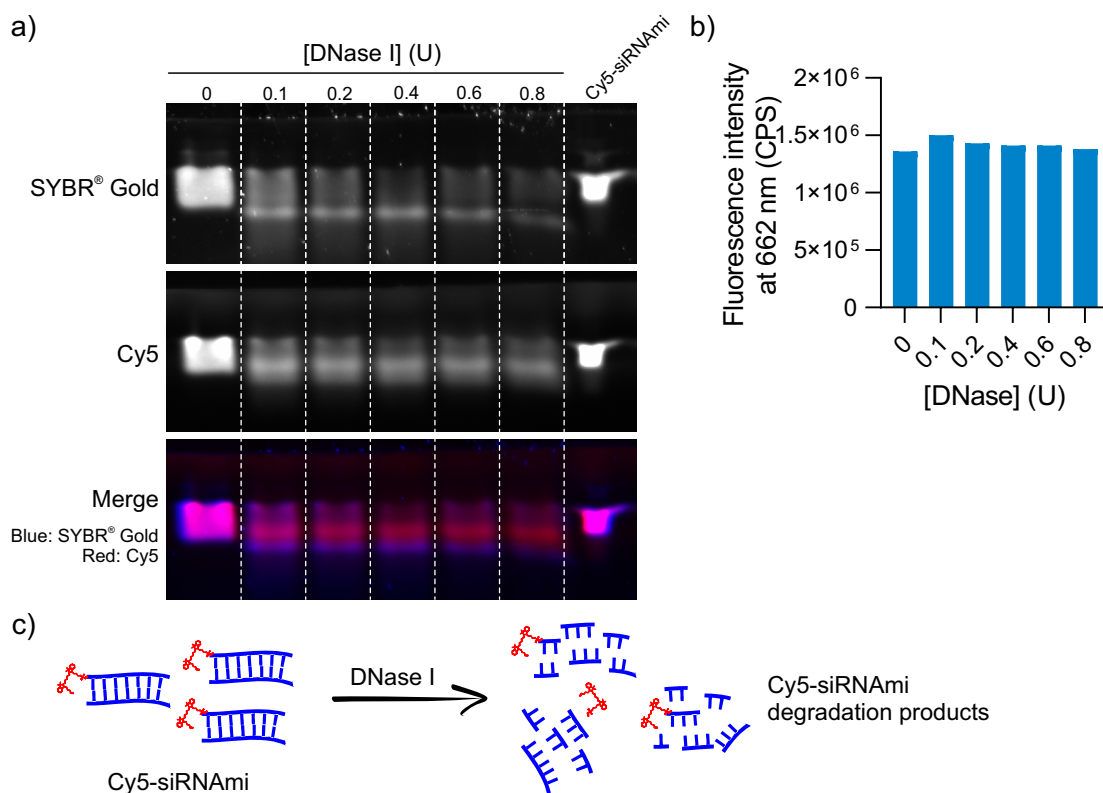


Figure 4.5 | Cy5-siRNAmi degradation study. **a)** Cy5-siRNAmi was incubated for 1 h with increasing amounts of DNase I and then analyzed by polyacrylamide gel electrophoresis (PAGE). Top: fluorescence image from SYBR[®] Gold; middle: fluorescence image from Cy5; bottom: merged image (blue: SYBR[®] Gold, red: Cy5). In the Cy5-siRNAmi lane, the same amount of intact Cy5-siRNAmi in NF water (3 pmol) was loaded as control. **b)** The same DNase-treated samples loaded into the polyacrylamide-TBE gel on (a) were also analyzed by fluorometry. Fluorescence intensities at 662 nm are expressed in counts per second (CPS). **c)** Schematic representation of the DNase-mediated degradation of Cy5-siRNAmi into small fragments. Note that degradation originates some siRNAmi fragments labeled with Cy5 and some unlabeled fragments.

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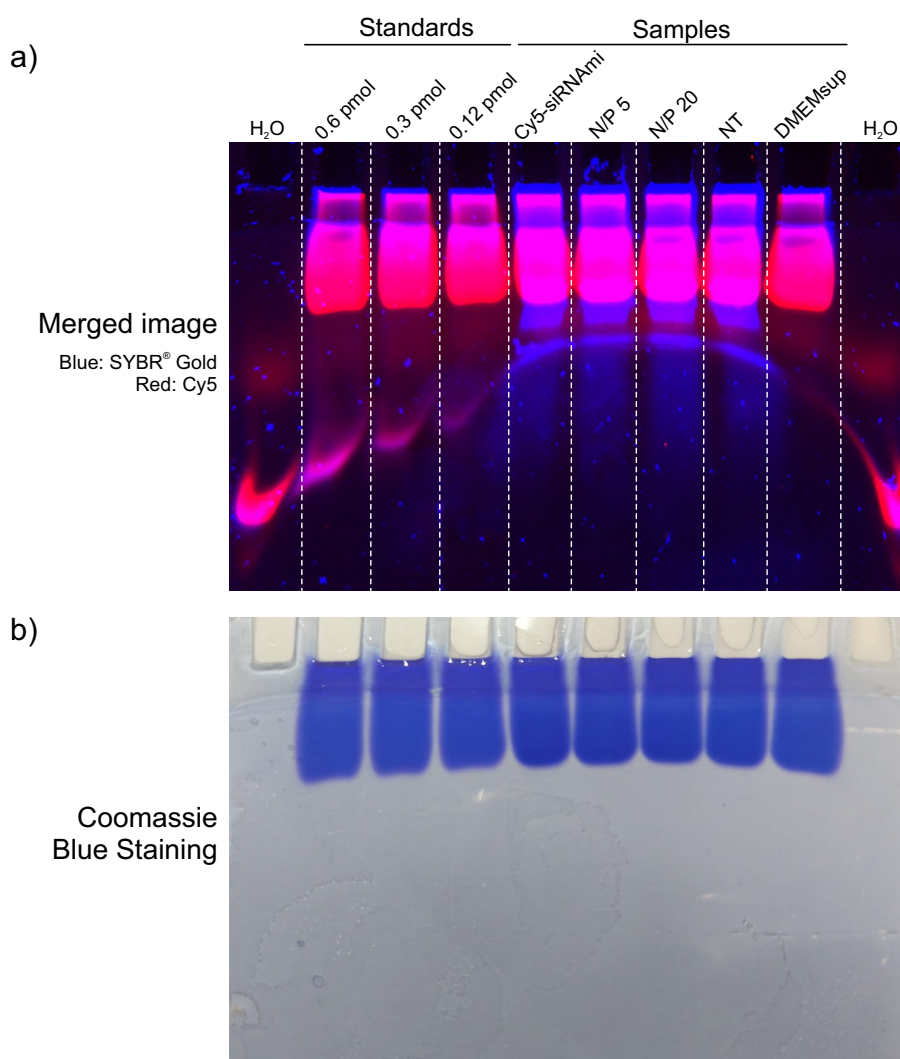


Figure 4.6 | Exocytosis evaluation by polyacrylamide gel electrophoresis. **a)** Fluorescence image of the polyacrylamide-TBE gel. Blue: SYBR® Gold fluorescence signal (blue epi illumination and emission filter of 530/28 nm). Red: Cy5 fluorescence signal (red epi illumination and emission filter of 695/55 nm). Culture medium samples were collected from cells transfected with free Cy5-siRNAi, fbB-Cy5-siRNAi dendriplexes (N/P 5, N/P 20), as well as from two transfection controls: non-treated cells (NT) and a non-cell control (supplemented DMEM - DMEMsup). Standards correspond to dilutions of known amounts of Cy5-siRNAi in supplemented DMEM, which were subjected to the same processing steps as the samples (centrifugation, lyophilization, and resuspension in SDS 1% (w/v)). Controls corresponding to 0.6 pmol Cy5-siRNAi in a NaCl aqueous solution (50 mM) were also included (marked as H₂O). There was an evident distortion of the gel front due to poor electrophoretic separation of the samples. **b)** The same gel visualized in (a) was stained with the protein dye Coomassie blue. Both standards and samples show intense Coomassie staining near the top of the gel.

Alternative approaches to study exocytosis

Although the prospect of detecting exocytosed Cy5-siRNAi with minimal sample processing using PAGE analysis initially seemed within reach, it is now clear that nucleic acid purification steps will have to be implemented in future experiments to reduce the sample complexity prior to PAGE analysis. In this discussion, four different approaches are considered: ultrafiltration,

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protease treatment, phenol/chloroform extraction, and ultracentrifugation.

The principle behind ultrafiltration is the separation of sample components by size exclusion or particle capture. Albumin and globulins, which are by far the most abundant proteins in serum, have molecular weights above 60 kDa, whereas Cy5-siRNA_{mi} has a molecular weight of 16.5 kDa. Thus, using a centrifugal filter with the appropriate molecular weight cutoff, these two components can potentially be separated. However, when this approach was tested, efficient separation was not attained (Figure B.6).

A different approach is pre-treatment of the extracellular medium samples with a protease, such as proteinase K. The goal is to convert high molecular weight proteins into small peptides, which will have a much higher electrophoretic mobility and, as such, will not clog the pores of the resolving gel. Additionally, a DNA extraction protocol could be implemented in order to obtain purified Cy5-siRNA_{mi}. For example, in the classic phenol/chloroform DNA extraction, the lipids and cellular debris are partitioned into the organic phase, whereas DNA is retained in the aqueous phase. The latter could then be analyzed by PAGE.

Finally, using ultracentrifugation protocols similar to those currently applied for exosome isolation [201], it might be possible to isolate fbB-Cy5-siRNA_{mi} dendriplexes present in the culture medium. This would offer the possibility of assessing whether Cy5-siRNA_{mi} is exocytosed as free nucleic acid or in nanoparticle form, which is relevant to determine the possibility of cellular re-uptake.

Evidently, compared to the simple concentration by lyophilization explored in the present work, the abovementioned alternatives have several disadvantages: they are more time-consuming, require higher sample volumes, and result in lower recoveries.

Other methodologies employed in studies on nanoparticle intracellular pathways may also be useful to study dendriplex exocytosis. A common strategy involves the use of drugs that inhibit or stimulate exocytosis pathways. Exocytosis inhibitors include 2-(4-fluorobenzoylamino)-benzoic acid methyl ester (Exo1), vacuolin-1, and wortmannin [199, 202, 203]. Monensin and methyl- β -cyclodextrin are examples of exocytosis stimulators [203]. However, to the best of our knowledge, no data is available in the literature regarding the application of these drugs on neuronal cell lines. Alternatively, dendriplex exocytosis can be inferred by the colocalization between exocytosis markers and dendriplexes, which can be evaluated by confocal microscopy. Several Rab proteins (Rab8, Rab10, Rab3, Rab26, etc.) were used as exocytosis markers by other authors [202–204].

Ultimately, the main reason why dendriplex exocytosis is of interest to us is the possibility of exploiting this process to promote the transcytosis of nanoparticles from peripheral sensory neurons to spinal cord interneurons (see Chapter 2). We are currently developing a microfluidic coculture system that will allow us to study this process more directly.

4.4 Transfection of *ex vivo* neuronal cultures

Building on previously obtained results [172], we aimed to evaluate the biological performance of our fbB-dendriplexes in embryonic DRG explants (organotypic cultures). This is a model

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of higher biological complexity particularly relevant in the field of PNS regeneration. Indeed, DRG isolation involves axon damage, which is then followed by regrowth under favorable culture conditions. Different therapeutic agents can be added at this stage and their effect on neurite formation can be conveniently monitored within an *in vivo*-like 3D microenvironment [181]. Furthermore, DRGs constitute an important communication point between the PNS and CNS, which makes them relevant therapeutic targets for the treatment of PNI-derived neuropathic pain [205].

For our study, DRGs were isolated from mouse embryos (E16.5) and cultured in PDL/laminin-coated plates for 5 days to allow adhesion to the plate and neurite outgrowth initiation. Afterward, they were further incubated for 72 h with fbB-Cy5-siRNAmi dendriplexes at N/P 5, 10, or 20. This period was longer than the one used for ND7/23 cells (24 h) to provide more time for the penetration of dendriplexes into the dense extracellular matrix of the DRGs and for potential internalization-related toxic effects to become visible. At the end of the incubation period, explants were fixed and their nuclei and neurites stained. Using confocal microscopy, whole Z-stacks for a total of 21 DRGs were captured. Representative MIP images for each transfection condition are shown in Figure 4.7 (all other images are included in Appendix C.5). All treated DRGs showed Cy5 fluorescence signal superimposed to the cluster of neuronal cell nuclei (explant body), as well as in the vicinity of the nuclei of surrounding cells. This points to the dendriplexes' ability to mediate the association and/or internalization of siRNAmi. Unfortunately, the large size of the explant bodies (100-350 μm in diameter) led to poor penetration of the antibodies used for β -III-tubulin staining in some explants. The near absence of this marker's signal in the center of the DRGs undermines the distinction between membrane association and cellular uptake of dendriplexes. Additionally, Hoechst staining was also suboptimal for some DRGs (Figure C.2), giving the false impression that they were hollow (this was not the case, as assessed by brightfield microscopy).

To gain further insight into the dendriplexes' distribution along the Z-axis of the explants, the total Cy5, β -III-tubulin, and Hoechst FIs were calculated for each Z-slice (Figures 4.8 and C.3). The peak for the Hoechst FI (which occurred at a Z-distance of 24 μm for the DRG in Figure 4.8) corresponds to the Z-plane which goes through the highest number of cell nuclei. A considerable number of nuclei that were captured in this plane belong to individual cells that were co-isolated with the DRG (Figure 4.8c). At higher Z-positions, only the cell nuclei belonging to the explant body were detected, which explains the drop in total Hoechst FI. Interestingly, the Cy5 FI profiles indicate that dendriplexes were also mostly present in the lowest Z-slices (under 25 μm), with the maximum total Cy5 FI occurring, on average, 3.4 ± 3.2 μm below the Z-slice corresponding to the highest total Hoechst FI. These observations suggest that the isolated cells scattered around the explant body are more prone to associate/internalize dendriplexes. This might be because their isolated position and inexistence of a dense extracellular matrix increases their exposure to the dendriplexes, or their cell type is more prone to allow dendriplexes' internalization. Nonetheless, it is worth mentioning that some Cy5 signal could still be detected at higher Z-positions, were only cells within the DRG cluster are located.

RESULTS AND DISCUSSION

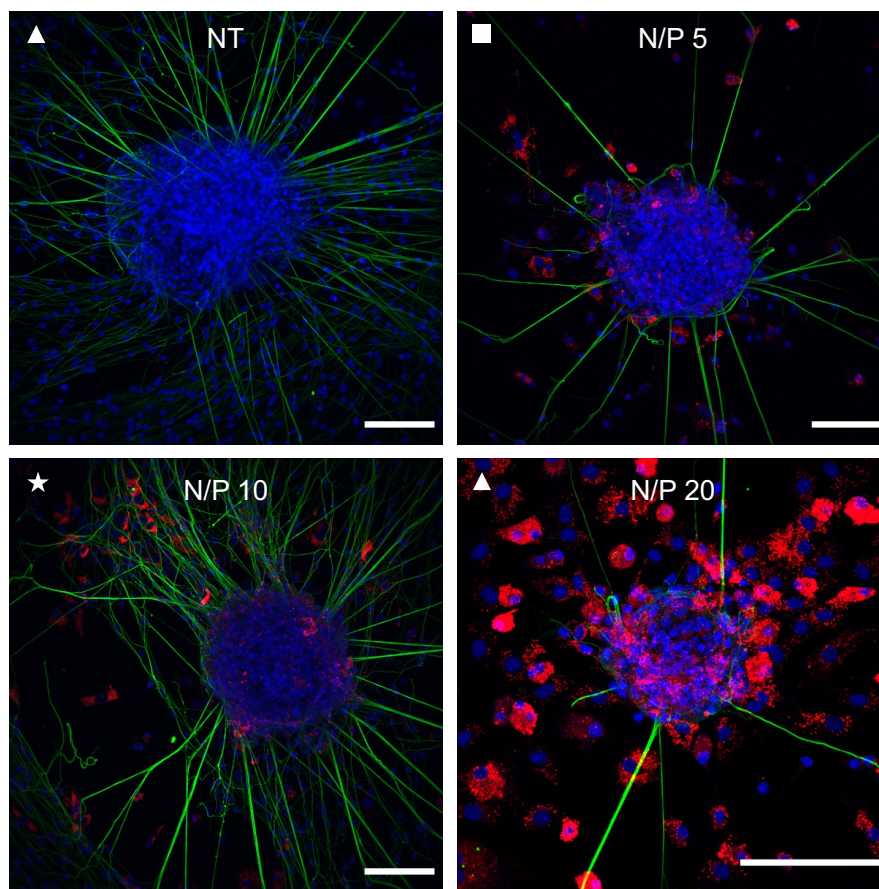


Figure 4.7 | Representative maximum projection images of DRG explants. DRGs were incubated with fbB-Cy5-siRNAmi dendriplexes (red) at N/P ratios 5, 10, or 20 for 72 h, starting at day 6 post-isolation. Neurons were stained against β -III-tubulin (green) and nuclei with Hoechst (blue). NT: non-treated. Scale bars: 100 μ m. The symbol in the top-left corner of each image allows the tracking of each individual DRG in the following analysis (Figures 4.9, C.2, C.3, and C.4).

The intensity and distribution of dendriplex-associated fluorescence were also evaluated by automated analysis of the MIP images represented in Figure C.2. In comparison with DRGs treated with N/P 5 or N/P 10 dendriplexes, DRGs treated with N/P 20 dendriplexes showed significantly higher values of normalized Cy5 FI within the explant body ($p < 0.05$, Figure 4.9a). This indicates their superior ability to mediate the association/internalization of siRNAmi.

The fraction of the total Cy5 FI detected within the perimeter of the explant body was approximately 30-40% for all three N/P ratios tested (Figure 4.9b), which reinforces the hypothesis that cells located outside the explant body are more susceptible to transfection.

In order to evaluate the biocompatibility of our nanosystem, the effect of the incubation with dendriplexes on the explants' neurite outgrowth was assessed. All MIP images from Figure C.2 were analyzed automatically using the *ExplantAnalyzer* MATLAB application recently developed by Schmidbauer et al. [184] (with minor modifications - Appendix C.3). The script applies several image processing steps in order to segment the explant body and the neurite network (Figure 3.3); the latter is then skeletonized in order to compute several metrics describing the outgrowth morphology.

RESULTS AND DISCUSSION

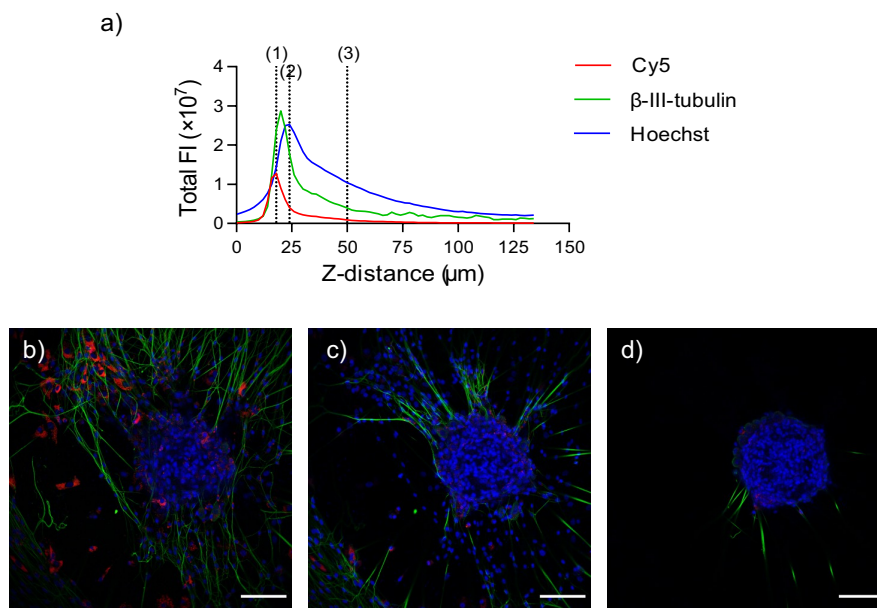


Figure 4.8 | Analysis of a Z-stack of a DRG explant treated with fbB-dendriplexes N/P 10. a) For each Z-slice, the total XY fluorescence intensity (FI) signal detected in each of the three channels (Cy5, β -III-tubulin, Hoechst) is plotted against the corresponding Z-distance. Note that the highest Cy5 FIs were detected at low Z-distances. b-d) Three confocal microscopy images taken at Z-positions 18 μm (b), 24 μm (c), and 50 μm (d) are shown. The brightness of the images was increased to aid visualization. Scale bars: 100 μm .

After an initial optimization of the image processing parameters (Appendix C.2), all images could be batch-processed in approximately five minutes. The vast majority of fibers was recognized correctly (Figure C.4), even though some segmentation flaws were detected. These happened mostly in regions with overlapping fibers or due to low or interrupted fluorescence signals (Figure C.5), and were deemed acceptable considering the high-throughput nature of the analysis. Two different neurite outgrowth metrics were selected for comparison between treatment groups: the area occupied by neurites (equation 3.3) and the number of start-points per mm of the explant perimeter (equation 3.4). The results are represented in Figures 4.9c and 4.9d, respectively.

None of the metrics assessed showed statistically significant differences between NT DRGs and DRGs treated with N/P 5, N/P 10, or N/P 20 dendriplexes. This suggests that interaction between the explant and the dendriplexes did not negatively affect neurite outgrowth. However, it should be noted that high variability was observed between individual DRGs within the same treatment group, particularly amongst dendriplex-treated explants. This might reflect diverse levels of sensitivity to transfection, which is unsurprising considering that different DRGs can differ significantly in size and neuronal composition [181]. Figure 4.9e indicates that DRGs which showed the highest dendriplex internalization/association (treated with N/P 20 dendriplexes) were associated with low levels of neurite outgrowth (area occupied by neurites under 5%). Besides this observation, no general correlation between these two parameters could be extracted: explants presenting a normalized Cy5 FI within the explant body under 0.1 displayed a wide range of areas occupied by neurites (2-34%).

RESULTS AND DISCUSSION

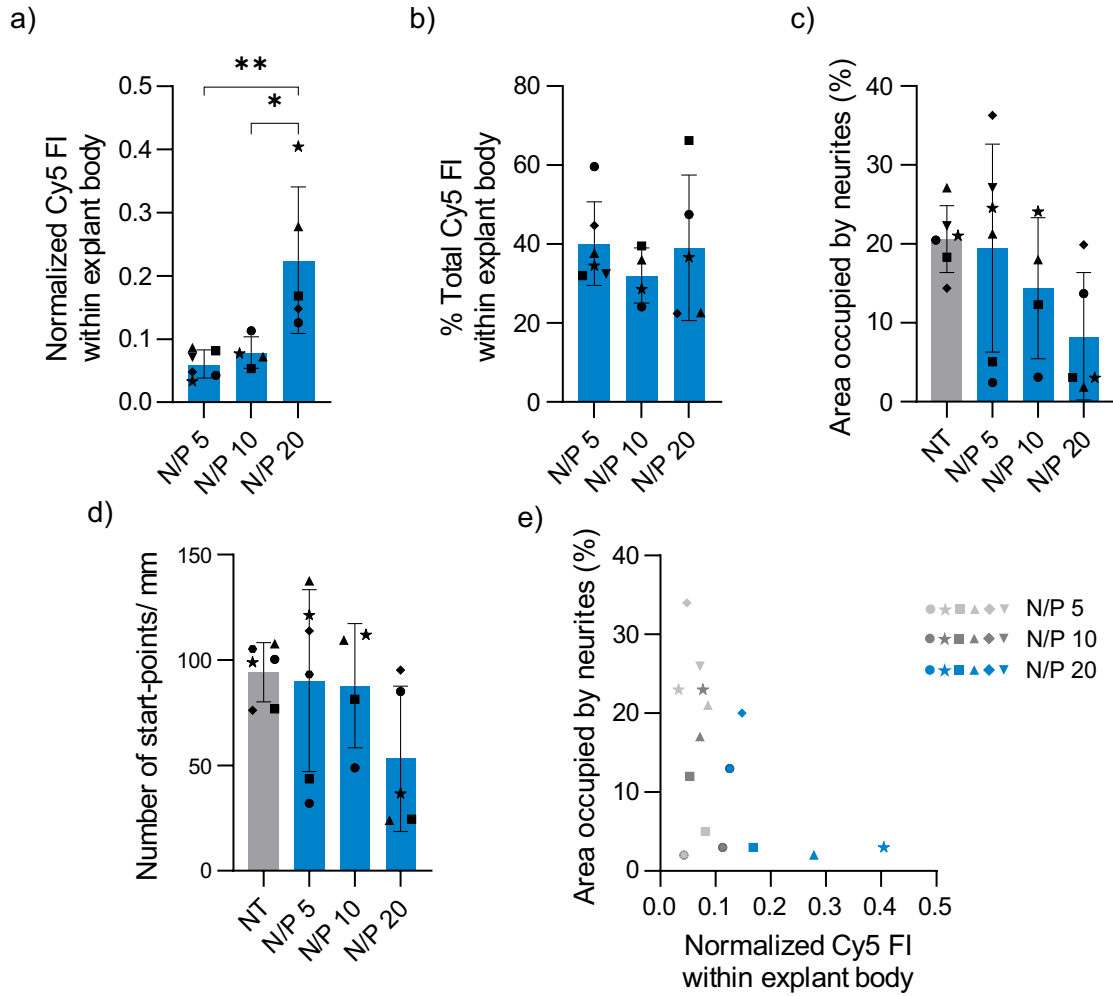


Figure 4.9 | Analysis of the maximum intensity projection images of DRG explants. a) Normalized Cy5 fluorescence intensity (FI) within the explant body, calculated by equation 3.1. b) Fraction (%) of the total Cy5 FI detected within the explant body, calculated by equation 3.2. c) Area occupied by neurites, calculated by equation 3.3. d) Number of start-points per mm, calculated by equation 3.4. e) Relation between the area occupied by neurites and the normalized Cy5 FI detected within the explant body. NT: non-treated. Each individual data-point within a given treatment group identifies the corresponding DRG in Figure C.2. Data from two independent experiments was plotted together. For graphs (a-d), statistical analysis was conducted using one-way ANOVA, followed by Tukey's multiple comparison test. Significant differences: *p < 0.05, **p < 0.01.

Based on the above, it must be highlighted that the conducted experiment has some limitations. Firstly, the low number of DRGs used per condition (four to six), combined with the high diversity of explant morphologies, resulted in relatively low statistical power. While inter-explant variability could have been reduced by isolating DRGs only from a specific anatomical location [181], we decided against this in order to minimize the required number of embryos. In the future, we plan to expand the sample size in order to confirm and extend our findings.

Secondly, the neurite network developed in the first days post-isolation (i.e. before incubation with the dendriplexes) varied significantly between DRGs. This variability is likely to stem both

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from their different spine locations and from varying levels of surgical damage inflicted during isolation. Before transfection, the explants were observed under a phase-contrast microscope, and only the ones that showed evidence of neurite formation were randomly assigned to each treatment group. Nevertheless, because a thorough neurite outgrowth assessment was not conducted at this stage, it is possible that some of the variation seen in the images acquired at the end of the experiment was not directly caused by the treatment.

Thirdly, the whole explants could not be captured in a single frame with the objective lenses available. For some larger DRGs, this meant that most of the image area was taken up by the explant body (see for example the third NT DRG in Figure C.2). This was a significant hurdle for neurite outgrowth analysis, since most of the metrics that were computed by the *ExplantAnalyzer* application required an image of the full neurite network. These include the total length of skeletonized neurites, the mean neurite length, the total number of branch-points, among others [184]. In a future study, tiled Z-stack acquisitions could be considered in order to capture the full extent of the outgrowth, although this would mean a significant increase in acquisition time (which was already approximately 20 min per Z-stack in the present study). An alternative approach is to obtain Z-stacks only for the explant body (for evaluation of internalization) and then use conventional epifluorescence microscopy with tiled acquisition in order to obtain a high-resolution, bidimensional image of the neurite network (as suggested by Schmidbauer et al. [184]).

The abovementioned consideration brings us to a fourth caveat, which is the fact that neurite quantification was performed on the 2D MIP images and, as such, could not capture tridimensional neurite outgrowth. Nonetheless, since the explants were cultured on a 2D substrate, most of the β -III-tubulin-stained fibers grew within the lowest Z-slices (see Figures 4.8 and C.3), in close contact with the PDL/laminin coating. Thus, most of them could be captured by a simple 2D analysis. In the future, we might consider encapsulating the explants in an hydrogel to promote true 3D neurite outgrowth [181]. In such circumstances, other quantification methods will become more suitable.

Finally, it is worth investigating how altering the treatment conditions (siRNA concentration and timing of exposure) might affect the internalization kinetics. In the present study, N/P 20 dendriplexes showed the highest internalization/association ability, but signs of toxicity in some explants were noted. Lowering the final siRNA concentration could be a way to avoid these effects.

Chapter 5

Conclusions and Future Perspectives

PNIs are tremendously impactful conditions for which new treatment options are urgently needed. Preclinical evidence suggests that targeted inhibition of PTEN and Rac1 through specific siRNAs are promising therapeutic strategies. However, ensuring efficient siRNA delivery to extra-hepatic tissues is tremendously challenging due to the lack of appropriate delivery technologies. Dendrimer-based vectors could fill this gap due to their tunable nanosize, low polydispersity, and multivalency, but their clinical translation is currently limited, in part by the available dendrimers' non-biodegradable character. To overcome this challenge, our group designed and synthesized a novel family of fully biodegradable PEG-GATGE dendritic block copolymers that is being explored for siRNA delivery. The arms of the proposed dendrimers have been functionalized by click chemistry with different amine containing moieties [172]. So far, fbB has demonstrated the greatest potential as siRNA carrier. Apart from a great capacity to bind, protect, and deliver siRNA, fbB-based dendriplexes mediated potent *in vitro* silencing of the eGFP reporter protein [172].

Following these promising results, the present work explored the use of novel fbB-siPTEN and fbB-siRac1 dendriplexes for PTEN and Rac1 downregulation in a neuronal cell line. The formulations were first prepared and characterized using well-established methods. Efficient complexation of both siRNAs was achieved, particularly at the highest N/P ratio tested (N/P 20, >85%). The resulting dendriplexes showed suitable physicochemical properties for efficient siRNA delivery, including small hydrodynamics sizes (44-54 nm), spherical morphologies, and positive ZPs. Furthermore, the siRNA release profiles asserted the reversibility of the siRNA-fbB interaction.

Next, the transfection efficiencies of these formulations were investigated in the ND7/23 cell line. Several experimental conditions were evaluated, including different N/P ratios (5 to 40), different quantities of siRNA added per well (6 or 15 pmol), and various timepoints for RT-qPCR evaluation of mRNA levels (48, 72, and 96 h). However, none of the conditions tested yielded a detectable downregulation of PTEN or Rac1 mRNA. This indicated that our fbB-dendriplexes were not capable of delivering enough siPTEN or siRac1 to the cytosol of ND7/23 cells. The reasons for this failure are yet to be completely unveiled, but poor endosomal escape is likely to be a contributing factor. A high priority for future works is bridging the gap between these results and the previously obtained downregulation of the eGFP reporter at the protein level. At

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first, this will involve reproducing at the mRNA level the silencing of the eGFP protein seen in ND7/23 eGFP/Luc cells, through RT-qPCR evaluation. Provided that positive results are obtained, then the downregulation of PTEN and Rac1 will be attempted using this cellular sublineage.

Another goal of this dissertation was to investigate dendriplex exocytosis. For this purpose, we employed dendriplexes carrying Cy5-labeled siRNAmi (Cy5-siRNAmi). Fluorometry measurements in the extracellular medium of ND7/23 cells previously incubated with these dendriplexes revealed evidence of exocytosis in the period between 24 and 48 h after culture medium renewal. Nevertheless, an additional study suggested that products of Cy5-siRNAmi degradation could also be contributing to the observed FI increase in the extracellular medium. Therefore, efforts were made to develop a complementary method to assess the integrity of the exocytosed nucleic acid, combining concentration by lyophilization with PAGE analysis. However, technical challenges prevented the successful implementation of this methodology. Future work will be directed at reducing the complexity (particularly the protein content) of the samples. Additionally, alternative approaches to study exocytosis such as the colocalization between dendriplexes and exocytosis markers will also be explored to strengthen our findings.

Finally, the present work also expanded significantly our preliminary evaluation of the internalization of fbB-Cy5-siRNAmi dendriplexes by DRG explant cultures, an *ex vivo* model of higher biological complexity. An image analysis workflow to quantify the level of internalization and its effect on neurite outgrowth was envisioned and implemented. An efficient uptake/association of dendriplexes by DRG explants was verified, particularly at the highest N/P ratio tested (N/P 20). Neurite outgrowth analysis did not show statistically significant detrimental effects resulting from the interaction with dendriplexes, thus attesting to their biocompatibility. Despite this, high inter-explant variability and low sample size might have masked relevant differences between treatment groups, which suggests that the sample size should be expanded in the future. After internalization conditions for DRG explants are optimized, then the biological performance of fbB-siPTEN and fbB-siRac1 on this *ex vivo* model will be assessed. At first, this will be done through quantification of PTEN or Rac1 downregulation at the mRNA level, by RT-qPCR. Later, an evaluation of the neurite outgrowth-promoting effect will be conducted. Therefore, the high-throughput image analysis workflow presented herein will certainly be of use in the future.

All these goals contribute to the ongoing project JumpIN, which aims to achieve intercellular translocation of dendriplexes from peripheral sensory neurons to spinal cord interneurons. By tethering a non-toxic fragment of the tetanus toxin at the PEG chain of our PEG-GATGE dendritic block copolymers, we aim to obtain a multifunctional nanovector that can simultaneously bind and protect therapeutic siRNA and be endowed with the retrograde transport and transcytosis properties associated with the tetanus toxin. Consequently, it will be interesting to study how the addition of active targeting will influence the transfection efficiency, as well as the exocytosis of the resulting dendriplexes. Efforts are already being made to develop a microfluidic system for the coculture of PNS neurons (dissociated DRGs) and CNS neurons, which will be used to test our nanosystem.

Concomitantly, we will continue to design and synthesize novel members of the PEG-GATGE family. While the functionalization of the peripheral groups with benzylamine has so far proven

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the most successful, we are investigating the use of other functionalizations that will introduce pH responsiveness, a feature that should boost endosomal escape. These new formulations must always represent a balance between the nucleic acid delivery capacity and the biocompatibility of the nanosystem, in line with our commitment to incrementally increase clinical translatability.

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Appendix A

RT-qPCR optimization

In order to test whether the RT-qPCR assay was fully optimized, 10-fold serial dilutions of three different lysates from transfection #3 (see Table 3.2) were used as templates for *PTEN* and *Ywhaz* amplification reactions. The results were used to generate standard curves, constructed by plotting the log of the starting quantity of template against the cycle threshold (C_T) value obtained during amplification of each dilution [206]. The results are shown in Figure A.1. The slopes of the standard curves were used to calculate the amplification efficiencies for each RT-qPCR reaction (Table A.1), using the following formula:

$$\% \text{Efficiency} = (10^{-1/\text{slope}} - 1) \times 100\% \quad (\text{A.1})$$

Table A.1 | RT-qPCR amplification efficiencies, calculated using the slopes of the standard curves presented in Figure A.1.

Cell lysate	1- to 1000-fold dilutions		10- to 1000-fold dilutions	
	PTEN	Ywhaz	PTEN	Ywhaz
NT_1	131.5%	128.7%	96.3%	95.4%
siPTEN-L2K_1	125.7%	123.3%	100.3%	96.9%
siPTEN-NP10_1	131.8%	133.8%	97.2%	97.9%

An amplification efficiency of 100% occurs when there is perfect doubling of the amount of PCR product during each cycle of exponential amplification. In practice, robust qPCR reactions have amplification efficiencies of 90-105% [206]. As shown Table A.1, the calculated amplification efficiencies for our qPCR reactions were in the range of 120-130%. This apparent increase in efficiency can occur in the presence of inhibitors. In such cases, the samples with the highest concentration of template also have the highest level of inhibitors, causing a delayed C_T , whereas samples with lower template concentrations have lower levels of inhibitors, leading to a minimally delayed C_T values. As a result, the absolute value of the slope decreases and the calculated amplification efficiency appears to increase [206].

APPENDIX A

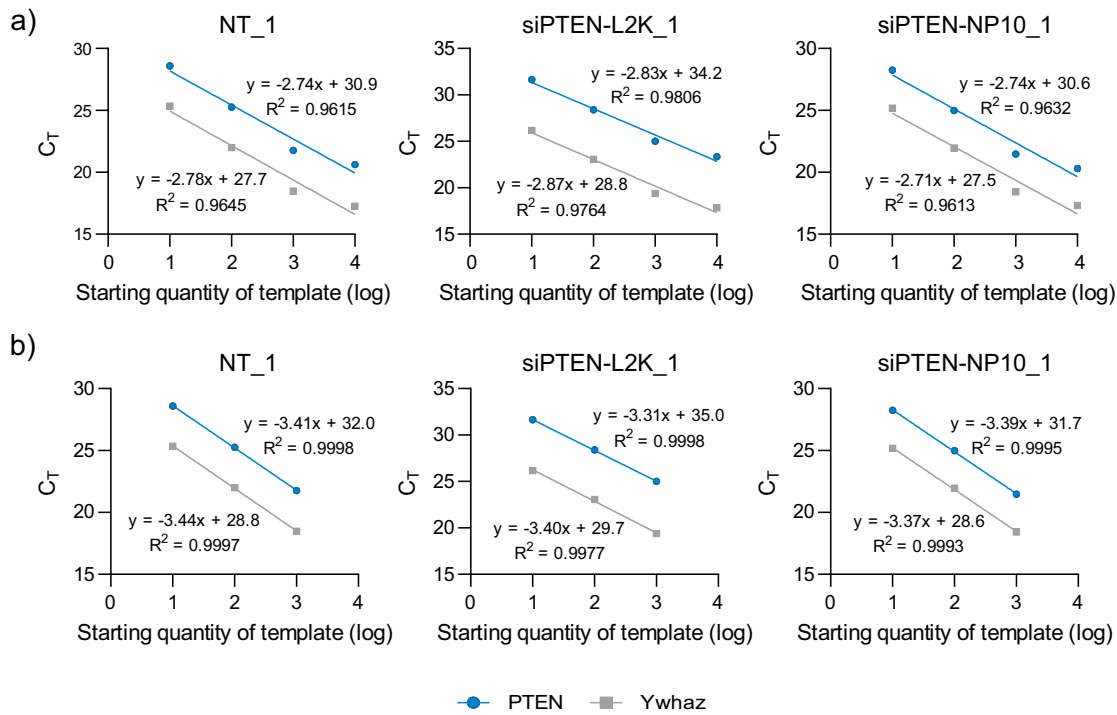


Figure A.1 | Standard curves used to determine the RT-qPCR amplification efficiencies. The curves were generated using a 10-fold dilution of three different cell lysates prepared in a transfection experiment (NT_1, siPTEN-L2K_1, and siPTEN-NP10_1). The cycle threshold values (C_T) are plotted against the log of the starting quantity of template for each dilution. Both *PTEN* (target gene) and *Ywhaz* (reference gene) were assayed for each cell lysate. The equation for the regression lines and the R^2 value are shown next to each line. Linear regression analysis was performed **a)** including all data points, and **b)** excluding the data point corresponding to the highest sample concentration (non-diluted lysate).

An inspection of the standard curves in Figure A.1a suggests that this occurred in our case. Indeed, the sample with the highest concentration of template (non-diluted lysate) deviated from the linear regression fitting in all six qPCR reactions assayed. Removing this data point led to a significant improvement of the R^2 value (> 0.997 for all standard curves - Figure A.1b) and, subsequently, of the calculated amplification efficiencies (95.4-100.3% range, as shown in Table A.1). This analysis indicated that, in order to guarantee optimal RT-qPCR reaction conditions, it was beneficial to dilute cell lysates at least 10-fold prior to adding them to the RT-qPCR plate.

Appendix B

Exocytosis studies

B.1 Optimization of fluorometry

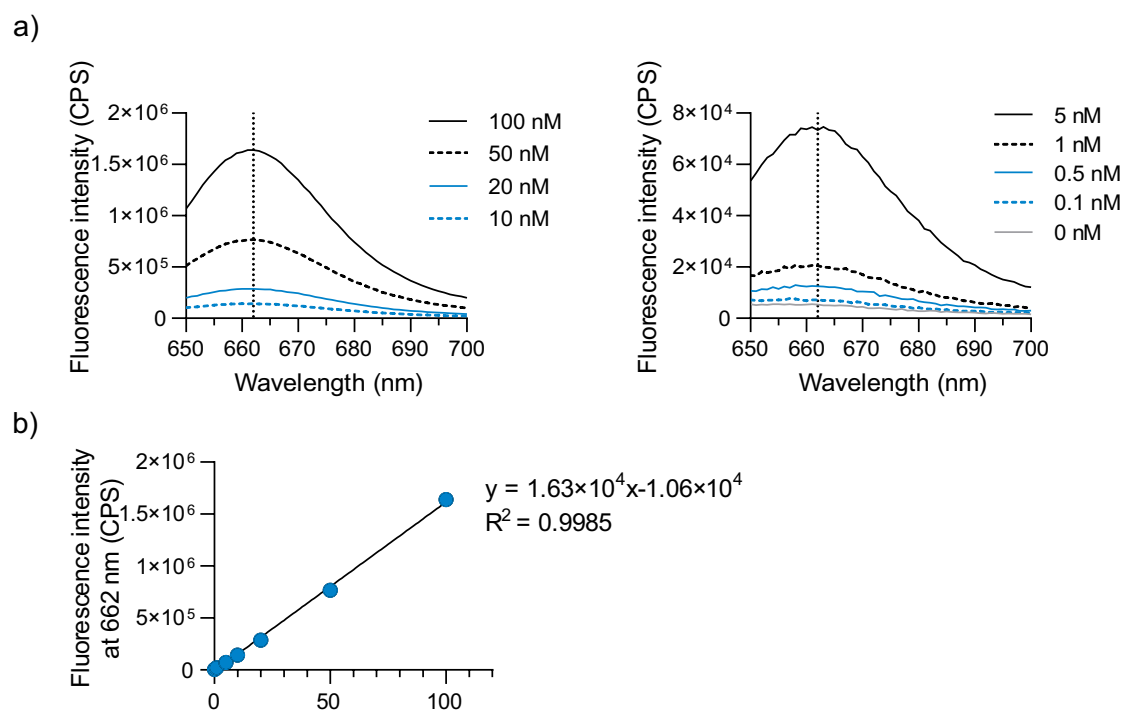


Figure B.1 | **a)** Fluorescence emission spectra of serial dilutions of Cy5-siRNAmi in supplemented DMEM (concentration range: 0 - 100 nM). Measurement settings: $\lambda_{\text{exc}} = 633$ nm (5 nm slit), $\lambda_{\text{em}} = 650$ -700 nm (5 nm slit, 1 nm increment), 0.2 s integration time, 3 detector accumulations (averaged scans). The vertical dashed line marks the Cy5 emission peak ($\lambda = 662$ nm). The limit of detection is 0.5 nM. **b)** Calibration curve of Cy5-siRNAmi in supplemented DMEM, constructed from the spectra represented in (a). An excellent linear fit was achieved ($R^2 = 0.9985$).

APPENDIX B

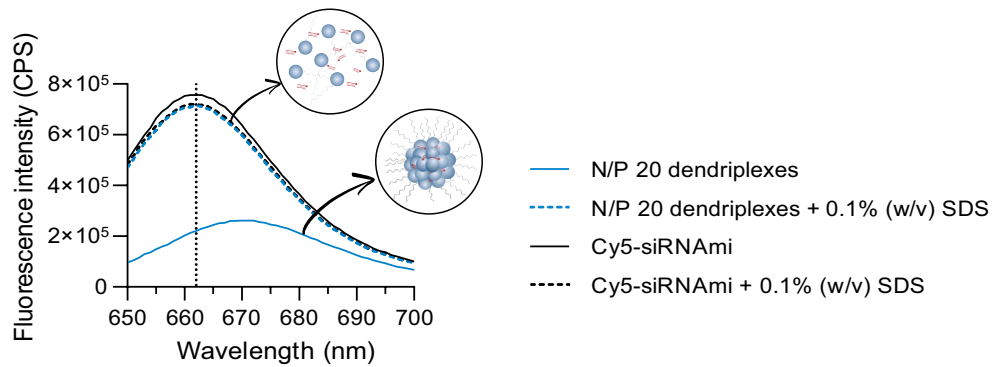


Figure B.2 | Quenching of Cy5 fluorescence within dendriplexes. fbB-Cy5-siRNAmi dendriplexes (N/P 20) or "free" Cy5-siRNAmi were diluted in HEPES buffer (20 mM HEPES, 5% (w/v) glucose, pH 7.4) and their fluorescence emission spectra were measured ($\lambda_{\text{exc}} = 633 \text{ nm}$). The fluorescence intensities obtained for the dendriplex sample (solid blue line) were remarkably lower compared to those obtained for the free nucleic acid (solid black line), even though both samples were prepared with the same concentration of Cy5-siRNAmi (90 nM). This suggested that the packaging of Cy5-siRNAmi within dendriplexes resulted in quenching of the Cy5 fluorescence. In order to prevent this from affecting the results of the exocytosis experiments, all samples were treated with sodium dodecyl sulfate (SDS) in order to destabilize the dendriplexes prior to the fluorometry measurements. As seen by the dashed lines, treatment with a final SDS concentration of 0.1% (w/v) equalizes the fluorescence spectrum of N/P 20 dendriplexes and free Cy5-siRNAmi.

B.2 Evaluation of dendriplex internalization by fluorometry

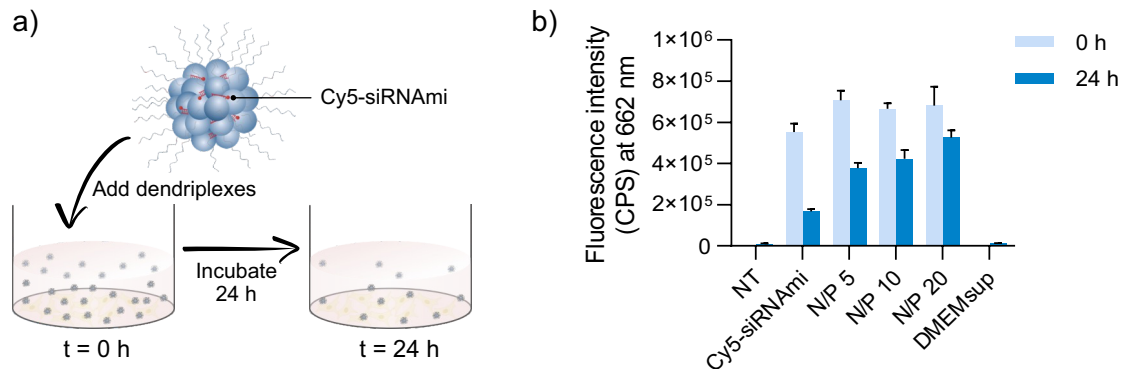


Figure B.3 | Evaluation of dendriplex internalization by fluorometry. a) Schematic representation of the experimental design. ND7/23 cells were put in contact with fbB-Cy5-siRNAmi dendriplexes (N/P 5, 10, or 20) or with free Cy5-siRNAmi for 24 h. Then, the culture medium was collected for quantification of the Cy5 fluorescence intensity. b) Cy5 fluorescence intensities (expressed in counts per second - CPS). The Cy5 fluorescence signal in the culture medium decreased during the incubation period due to the cellular uptake of dendriplexes. Interestingly, this decrease was also verified for cells treated with free Cy5-siRNAmi. Data are expressed as mean $\pm$ SD of three or six biological replicates (for the $t = 0 \text{ h}$ and $t = 24 \text{ h}$ time-points, respectively). NT: non-treated controls; DMEMsup: supplemented DMEM.

B.3 Autofluorescence from supplemented DMEM

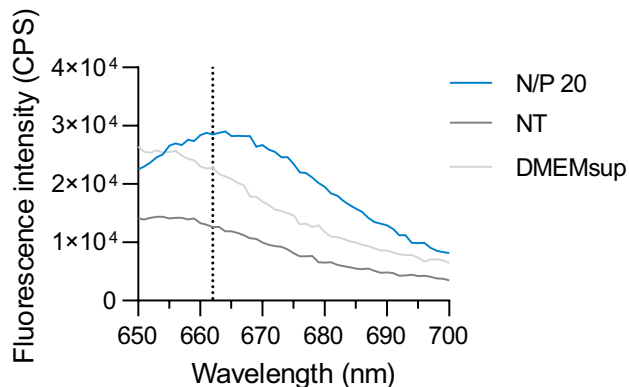


Figure B.4 | Autofluorescence from supplemented DMEM. Representative fluorescence emission spectra of culture medium samples collected at the 24 h time-point on Figure 4.4b.1. The emission spectrum of the culture medium of cells treated with N/P 20 dendriplexes shows the characteristic emission peak of Cy5 (at 662 nm, as marked by the dashed vertical line). Conversely, the emission spectra of the culture medium of non-treated (NT) cells and of supplemented DMEM (DMEMsup) do not exhibit a peak at this wavelength. The levels of fluorescence emission detected in these samples mostly likely arise from certain non-identified components of the fetal bovine serum (FBS) that is present in DMEMsup.

B.4 Cy5·siRNA_{mi} detection by polyacrylamide gel electrophoresis

To test the feasibility of using PAGE to detect small amounts of Cy5·siRNA_{mi} in extracellular medium, serial dilutions of Cy5·siRNA_{mi} (0-3 pmol) were prepared in supplemented DMEM, in a final volume of 120 μ L. These mixtures were then concentrated 10-fold through lyophilization followed by resuspension in 12 μ L of SDS 1% (w/v). Each concentrated sample was loaded into the wells of a 4-10% (w/v) polyacrylamide gel and run alongside a control sample containing the same amount of Cy5·siRNA_{mi} but in NF water. The results are shown in Figure B.5. In the control lanes (marked as H₂O), Cy5·siRNA_{mi} was detected as a single band exhibiting both SYBR[®] Gold and Cy5 fluorescence signals. The detection limit was 0.12 pmol for the SYBR[®] Gold channel (Figure B.5a) and 0.3 pmol for the Cy5 channel (Figure B.5b). In the lanes corresponding to Cy5·siRNA_{mi} dilutions in supplemented DMEM (marked as DMEMsup), the Cy5·siRNA_{mi} band was also detected, with detection limits appearing similar to the controls. Interestingly, additional bands not present in the control samples were also detected. These can be attributed to the autofluorescence of proteins contained in supplemented DMEM, since: (i) they were present irrespective of the Cy5·siRNA_{mi} concentration, including in the sample that did not contain the nucleic acid (last lane); (ii) contrary to the band corresponding to Cy5·siRNA_{mi}, they did not exhibit colocalization between the SYBR[®] Gold and Cy5 fluorescence signals; (iii) they have a higher molecular weight than Cy5·siRNA_{mi} (remained close to the top of the gel).

APPENDIX B

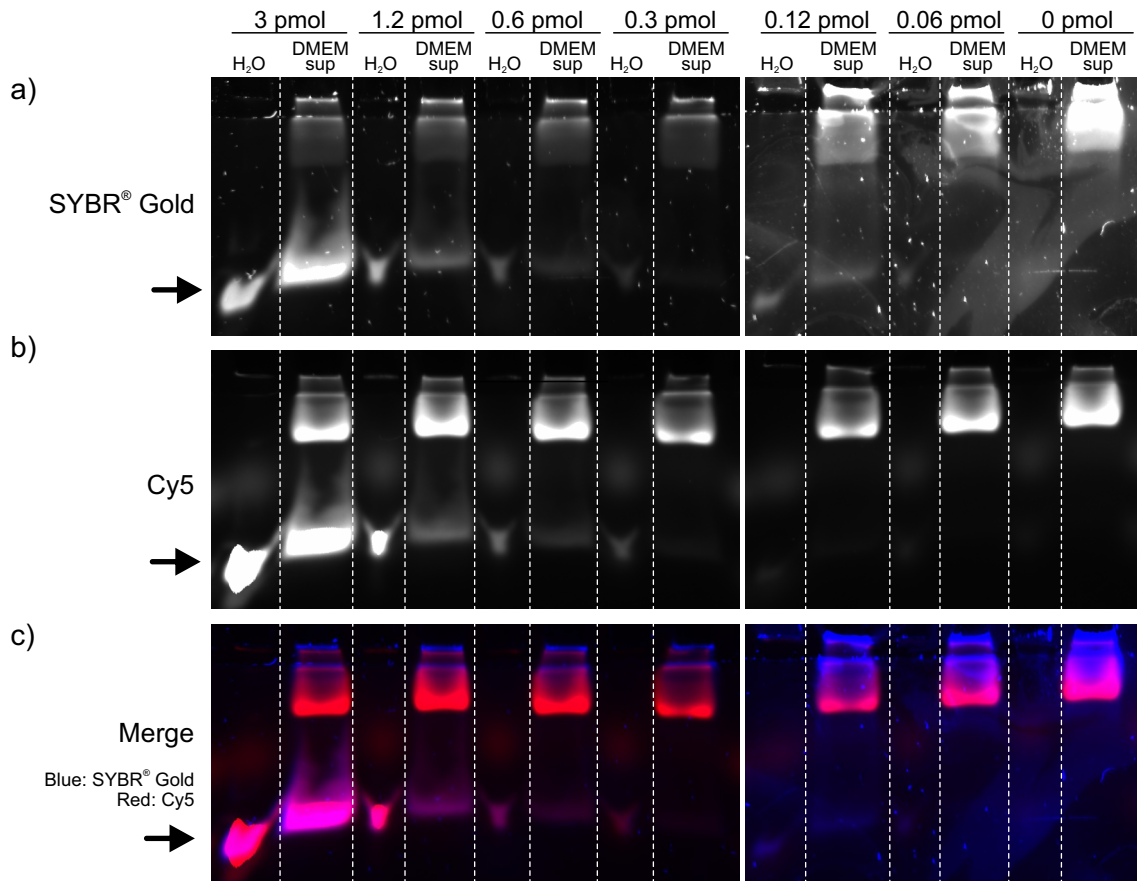


Figure B.5 | Detection of Cy5-siRNAi in supplemented DMEM by polyacrylamide gel electrophoresis (PAGE). Serial dilutions of Cy5-siRNAi (0-3 pmol) were prepared in supplemented DMEM. These mixtures were then concentrated by lyophilization and analyzed by PAGE (DMEMsup lanes). For each sample, a control corresponding to the same amount of Cy5-siRNAi in nuclease-free water was also loaded into the gel (H₂O lanes). **a)** Fluorescence image from SYBR[®] Gold (blue epi illumination and emission filter of 530/28 nm). **b)** Fluorescence image from Cy5 (red epi illumination and emission filter of 695/55 nm). **c)** Merged image. Note that images from two separate gels are displayed side-by-side and that the gel on the right was subjected to higher exposure times during imaging than the one on the left. The arrow indicates the approximate position of the Cy5-siRNAi band.

B.5 Purification of Cy5-siRNAmi by ultrafiltration

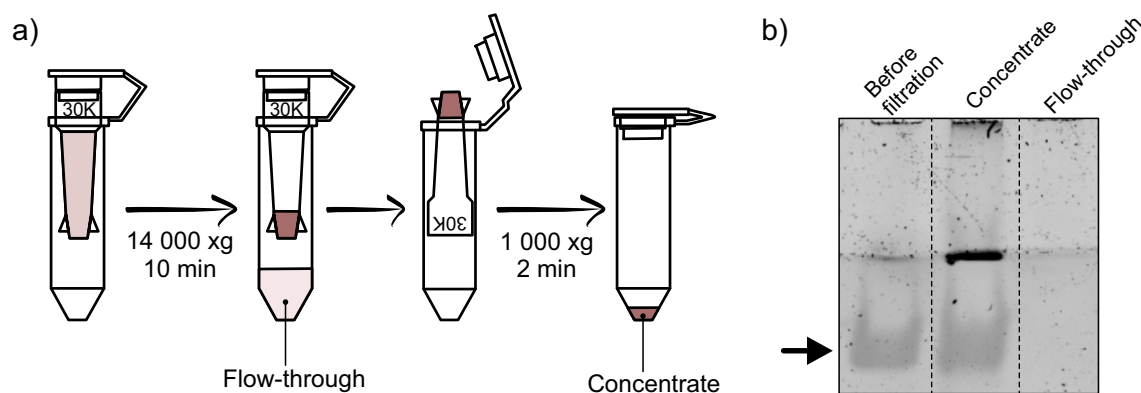


Figure B.6 | Purification of Cy5-siRNAmi from culture medium by ultrafiltration. **a)** Cy5-siRNAmi was diluted in supplemented DMEM at a final concentration of 100 nM and filtered using a Ambicon® Ultra 0.5 mL centrifugal filter (Merck, USA) with a molecular weight cutoff of 30 kDa. Given its molecular weight (16.5 kDa), Cy5-siRNAmi was expected to be recovered in the flow-through, whilst most serum proteins (> 60 kDa) should be retained in the concentrate. **b)** The initial mixture, the concentrate, and the flow-through were loaded into the wells of a 4-15% (w/v) polyacrylamide-TBE gel, which was run for 30 min at 100 V and then stained with SYBR® Gold. The band corresponding to Cy5-siRNAmi was visualized in the initial mixture and in the concentrate, but not in the flow-through, indicating that ultrafiltration did not work as intended. It is possible that Cy5-siRNAmi molecules can bind to serum proteins, thus reducing their ability to cross the membrane pores.

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Appendix C

Image analysis

C.1 MATLAB custom scripts

Disclaimer: some of the features provided by these scripts were inspired by similar features of the *ExplantAnalyzer* tool [184]. This includes the segmentation of the explant body and the general structure of the batch-processing scripts.

batch_Cy5MIP.m

```
1 %% batch_Cy5MIP
2
3 % This script processes all images in a folder by calling the Cy5MIP function.
4 % Choose whether or not to open an overview image, then run the script and select a folder containing
5 % all images to be analyzed.
6 % Values are calculated and stored in an excel file.
7
8 % Rafael Santos
9 % Version 1.0
10
11 %% Clear command window and variables
12 clear all
13 clc
14
15 %% Set values for image processing
16
17 global setup
18 % If setup == 1 then an overview image will be opened, containing an image of each major processing step
19 % to facilitate parameter optimization.
20 setup = 1;
21
22 %% Start batch
23
24 % Add current path.
25 addpath(pwd);
26
27 % Select the folder containing the images to be analyzed.
28 selpath = uigetdir;
29 cd(selpath);
30
31 % Find all .tif images in the selected folder and all subfolders.
32 listing = dir('**/*.tif');
33
34 % Initialize error counter.
35 err_count = 0;
36
37 % Start timer for the total time.
38 total = tic;
39
40 % Loop all images.
41 for i = 1:size(listing,1)
42     try
43         % Start timer for one explant.
```

APPENDIX C

```

43     tic
44     % Go to subfolder
45     cd(listing(i).folder);
46     % Run Cy5MIP.
47     [Total_cy5(i), Sum_cy5_explant(i), Explant_area(i), Image_area(i), Cy5_explant_normalized(i),
48         Cy5_explant_fraction(i)] = Cy5MIP(listing(i).name);
49     % Stop timer.
50     t = toc;
51     %Print current explant number and time.
52     fprintf(1, ['Finished explant ', num2str(i), ' of ', num2str(size(listing,1)), ' in ', num2str(t), '
53         s\n']);
54     catch E
55         % Print error message in case an error occurs.
56         fprintf(2, ['Processing explant ', num2str(listing(i).name), ' caused an error.\n\n']);
57         fprintf(2, '%s\n\n', getReport(E, 'extended'));
58         err_count = err_count + 1;
59     end
60 end
61 % Excel Export
62 Internalization = table({listing.name}.', {listing.folder}.', [Total_cy5].', [Sum_cy5_explant].', [
63     Explant_area].', [Image_area].', [Cy5_explant_normalized].', [Cy5_explant_fraction].', ...
64     'VariableNames', {'Name', 'Folder', 'Total_Cy5', 'Cy5_Explant', 'Explant_Area(pixels)', 'Image_Area(
65     pixels)', 'Cy5_Explant_normalized', 'Cy5_Explant_fraction'});
66 writetable(Internalization, 'Internalization.xlsx', 'FileType', 'spreadsheet', 'Sheet', 'Stats')
67 % Print final outcome.
68 fprintf(2, '\nBatch finished in %.0f s! %d error(s) occurred!\n', toc(total), err_count);
69 clearvars -except Internalization
70 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
71 %% Function Cy5MIP
72 %
73 % This function processes images of dorsal root ganglion (DRG) explants treated with Cy5-labeled
74 % nanoparticles and stained for DAPI/hoechst.
75 % The input image has to be a RGB image, containing the Cy5 fluorescence
76 % signal in channel 1 (red) and the DAPI/Hoechst staining in channel 3
77 % (blue). It can also contain a fourth channel, which could be added
78 % manually to the image in cases when the automatic segmentation of the explant body fails.
79 %
80 % Outputs:
81 % - total_cy5: sum of all pixel intensities from the cy5 channel
82 % - sum_cy5_explant: sum of all pixel intensities from the cy5 channel
83 %   that lie within the explant body
84 % - explant_area: total area of the explant body (in pixels)
85 % - image_area: total area of the image (in pixels)
86 % - cy5_explant_normalized: sum_cy5_explant normalized to the total area
87 %   of the explant body
88 % - cy5_explant_fraction: fraction of the total cy5 intensity that lies
89 %   within the explant body
90 %
91 % Rafael Santos
92 % Version 1.0
93 %% Function
94 function [total_cy5, sum_cy5_explant, explant_area, image_area, cy5_explant_normalized, cy5_explant_fraction]
95     = Cy5MIP (input_image)
96 %% Open image and extract channels
97 image = imread(input_image);
98 cy5(:, :, 1) = image(:, :, 1);
99 dapi(:, :, 1) = image(:, :, 3);
100
101 %% DAPI operations
102 % Binarize DAPI image and keep only biggest blob.
103 if size(image,3)==4 % manual segmentation (included in a 4th channel)
104     manual_dapi(:, :, 1) = image(:, :, 4);
105     dapi_bw = ~imbinarize(manual_dapi);
106 else % automatic segmentation
107     dapi_bw = imbinarize(dapi);
108 end
109 explant = bwareafilt(dapi_bw, 1);
110 explant = imfill(explant, 'holes');
111
112 % Calculate the size of explant (in pixels)
113 explant_area = sum(explant, 'all');
114
115 % Calculate total image size (in pixels)
116 image_area = size(explant,1)*size(explant,2);
117
118 %% Cy5 operations
119 % Sum of intensities of all pixels in Cy5 channel
120 total_cy5 = sum(cy5, 'all');
121

```

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```

122 % Sum of intensities of all pixels inside the explant
123 cy5_explant = cy5.*uint8(explant);
124 sum_cy5_explant = sum(cy5_explant,'all');
125
126 % Sum of intensities of inside the explant normalized by explant area
127 cy5_explant_normalized = (sum_cy5_explant/255)/explant_area;
128
129 % Fraction of the total cy5 intensity that lies within the explant body
130 cy5_explant_fraction = sum_cy5_explant/total_cy5;
131
132 %% Save an overlay image
133 inside = double(cy5_explant)/255; % Cy5 signal inside the explant body
134 outside = double(cy5).*double(~explant)/255; % Cy5 signal outside the explant body
135 P(:,1) = inside + outside; % red channel
136 P(:,2) = outside; % green channel
137 P(:,3) = outside; % blue channel
138 perim = imdilate(bwperim(explant),ones(2)); % explant perimeter
139 P = imoverlay(P,perim,[0 0 1]);
140 % Save image as JPEG.
141 imwrite(P,sprintf('%s.jpg',input_image),'jpg','Quality',100)
142
143 %% Show original and processed image side-by-side
144 if setup == 1
145
146     t = tiledlayout('flow');
147
148     ax1 = nexttile;
149     imshow(image(:,:,1:3));
150     title('Original')
151
152     ax2 = nexttile;
153     imshow(P)
154     title('Processed')
155
156     linkaxes([ax1 ax2],'xy')
157
158     input('') %wait for user input
159
160     close all
161 end
162 end

```

batch_Cy5Zstack.m

```

1 %% batch_Cy5Zstack
2
3 % This script processes all images in a folder by calling the Cy5Zstack function.
4 % Input Z-step size, then run the script and select a folder containing all images to be analyzed.
5 % Values are calculated and stored in an excel file.
6
7 % Rafael Santos
8 % Version 1.0
9
10 %% Clear command window and variables
11 clear all
12 clc
13
14 %% Variables
15 % Z-step in um
16 z_step_size = 2;
17
18 %% Start batch
19 % Add current path.
20 addpath(pwd);
21
22 % Select the folder containing the images to be analyzed.
23 selpath = uigetdir;
24 cd(selpath);
25
26 % Find all .tif images in the selected folder and all subfolders.
27 listing = dir('**/*.tif');
28
29 % Initialize error counter.
30 err_count = 0;
31
32 % Start timer for the total time.
33 total = tic;
34
35 % Loop all images.
36 for i = 1:size(listing,1)
37     try

```

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```

38     % Start timer for one explant.
39     tic
40     % Go to subfolder
41     cd(listing(i).folder);
42     % Run Cy5Zstack for explant i
43     [Sum_cy5,Sum_b3,Sum_dapi,Max_cy5,Pos_cy5,Max_b3,Pos_b3,Max_dapi,Pos_dapi,Number_of_slices] =
        Cy5Zstack(listing(i).name);
44     % Store output variables for explant i
45     Samples(i).Sum_cy5 = Sum_cy5;
46     Samples(i).Sum_b3 = Sum_b3;
47     Samples(i).Sum_dapi = Sum_dapi;
48     Samples(i).Max_cy5 = Max_cy5;
49     Samples(i).Pos_cy5 = (Pos_cy5-1)*z_step_size;
50     Samples(i).Max_b3 = Max_b3;
51     Samples(i).Pos_b3 = (Pos_b3-1)*z_step_size;
52     Samples(i).Max_dapi = Max_dapi;
53     Samples(i).Pos_dapi = (Pos_dapi-1)*z_step_size;
54     Samples(i).Number_of_slices = Number_of_slices;
55     % Stop timer.
56     t = toc;
57     %Print current explant number and time.
58     fprintf(1, ['Finished explant ', num2str(i), ' of ', num2str(size(listing,1)), ' in ', num2str(t), '
        s\n']);
59     catch E
60         % Print error message in case an error occurs.
61         fprintf(2, ['Processing explant ', num2str(listing(i).name), ' caused an error.\n\n']);
62         fprintf(2, '%s\n\n', getReport(E, 'extended'));
63         err_count = err_count + 1;
64     end
65 end
66 % Excel Export
67
68 Stacks = table({listing.name}.', {listing.folder}.', [Samples.Max_cy5].', [Samples.Pos_cy5].', [Samples.
        Max_b3].', [Samples.Pos_b3].', [Samples.Max_dapi].', [Samples.Pos_dapi].', [Samples.Number_of_slices
        ].', ...
69     'VariableNames', {'Name', 'Folder', 'Max_Cy5', 'Pos_Cy5(um)', 'Max_b3', 'Pos_b3(um)', 'Max_dapi', '
        Pos_dapi(um)', 'Number_of_slices'}});
70 writetable(Stacks, 'Stacks.xlsx', 'FileType', 'spreadsheet', 'Sheet', 'Stats')
71
72 for i = 1:size(listing,1)
73     z_step_vector = [0: (Samples(i).Number_of_slices - 1)] * z_step_size;
74     Per_stack_table = table([z_step_vector], [Samples(i).Sum_cy5], [Samples(i).Sum_b3], [Samples(i).
        Sum_dapi], 'VariableNames', {'Step_distance(um)', 'Sum_Cy5', 'Sum_b3', 'Sum_dapi'});
75     writetable(Per_stack_table, 'Stacks.xlsx', 'FileType', 'spreadsheet', 'Sheet', listing(i).name)
76 end
77
78 % Print final outcome.
79 fprintf(2, '\nBatch finished in %.0f s! %d error(s) occurred!\n', toc(total), err_count);
80
81 clearvars -except Samples
82
83 %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
84 %% Function Cy5Zstack
85
86 % This function processes images of dorsal root ganglion (DRG) explants treated with Cy5-labeled
87 % nanoparticles and stained for DAPI/Hoechst and beta-iii-tubulin.
88 % The input image has to be a multi-image RGB z-stack, containing the Cy5 fluorescence signal in
89 % channel 1 (red), the beta-iii-tubulin staining in channel 2 (green) and the DAPI/Hoechst staining
90 % in channel 3 (blue).
91
92 % Outputs:
93 % — sum_cy5: vector in which each value is the sum of all pixel intensities in the Cy5 channel for one
94 % xy frame
95 % — sum_b3: vector in which each value is the sum of all pixel intensities in the DAPI/Hoechst channel
96 % for one xy frame
97 % — sum_dapi: vector in which each value is the sum of all pixel intensities in the beta-iii-tubulin
98 % channel for one xy frame
99 % — max_cy5: max value of sum_cy5
100 % — pos_cy5: z-position where max_cy5 occurs
101 % — max_b3: max value of sum_b3
102 % — pos_b3: z-position where max_b3 occurs
103 % — max_dapi: max value of sum_dapi
104 % — pos_dapi: z-position where max_dapi occurs
105
106 % Rafael Santos
107 % Version 1.0
108
109 function [sum_cy5,sum_b3,sum_dapi,max_cy5,pos_cy5,max_b3,pos_b3,max_dapi,pos_dapi,number_of_slices] =
    stackquantifier(input_image)
110 % Read stack
111 tiffInfo = imfinfo(input_image); % get the TIFF file information
112 image_size = tiffInfo(1).Width; % get image size
113 number_of_slices = numel(tiffInfo); % get the number of slices in the file
114
115 % initialize channel variables

```

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```

110 cy5=zeros(image_size,image_size,number_of_slices);
111 b3=cy5;
112 dapi=cy5;
113
114 % loop through all slices and extract channels
115 for k = 1:number_of_slices
116     image = imread(input_image, 'Index',k, 'Info',tiffInfo);
117     cy5(:,:,k)=image(:,:,1);
118     b3(:,:,k)=image(:,:,2);
119     dapi(:,:,k)=image(:,:,3);
120 end
121
122 % Calculations
123 sum_cy5=sum(cy5,[1 2]);
124 sum_cy5=sum_cy5(:);
125 [max_cy5,pos_cy5]=max(sum_cy5);
126
127 sum_b3=sum(b3,[1 2]);
128 sum_b3=sum_b3(:);
129 [max_b3,pos_b3]=max(sum_b3);
130
131 sum_dapi=sum(dapi,[1 2]);
132 sum_dapi=sum_dapi(:);
133 [max_dapi,pos_dapi]=max(sum_dapi);
134 end

```

C.2 Optimized image processing parameters for neurite outgrowth analysis

Table C.1 | Optimized image processing parameters used by the *ExplantAnalyzer.m* function.

Parameter	Variable	Default value	Optimized value
Background subtraction value	<i>bg_sub</i>	1300 ^a	4
Explant dilation value	<i>explant_dil_value</i>	25	8
Center element of the high boost kernel	<i>high_boost</i>	20	20
Size of the median filter	<i>median_size</i>	[3 3]	[2 2]
Size of the window for adaptive thresholding	<i>neighborhood_size</i>	65	15
Sensitivity for adaptive thresholding	<i>sensitivity</i>	0.5	0.5
Neurite smooth size	<i>neurite_smooth_size</i>	1.5	1
Length of spurs removed during skeletonization	<i>spur_removal</i>	6	6

^a This default is set for 16-bit images.

C.3 Modifications made to *ExplantAnalyzer* MATLAB scripts

simple_statistics.m

added after line 66

```
Samples(i).Image_Size = size(explant,1)*size(explant,2)*voxel_size^2; %um^2
Samples(i).Explant_dil_1_Size = sum(explant_dil_1, 'all') * voxel_size^2; %um^2
Samples(i).Explant_dil_2_Perimeter = regionprops(explant_dil_2, 'Perimeter').Perimeter.*
    voxel_size; %um
Samples(i).Normalized_Neurites_Area = neurites_area/(Samples(i).Image_Size-Samples(i).
    Explant_dil_1_Size)*100; %
Samples(i).Number_of_Startpoints_G_per_mm = Samples(i).Number_of_Startpoints_G/Samples(i).
    Explant_dil_2_Perimeter*1000;
```

original (line 164)

```
Samples_export = table({Samples.
    Name}.', {Samples.Folder}.', [
    Samples.Number_of_Neurites].', [
    Samples.Mean_Length].', [
    Samples.Median_Length].', [
    Samples.Max_Length].', [
    Samples.
    Number_of_Startpoints_G].', [
    Samples.
    Number_of_Startpoints_TR].', [
    Samples.
    Number_of_Branchpoints_G].', [
    Samples.
    Number_of_Branchpoints_TR].',
    [Samples.Total_Length_G].', [
    Samples.Total_Length_TR].', [
    Samples.Sum_Lengths_TR].', [
    Samples.Explant_Size].', [
    Samples.Hull_Area].', [Samples.
    .Neurites_Area].', [Samples.
    Covered_Area].', [Samples.
    b3_dapi_multi_explant].', [
    Samples.b3_dapi_mean_explant
    ].', [Samples.b3_sum_explant
    ].', 'VariableNames', {'Name',
    'Folder', 'Number_of_Neurites',
    'Mean_Length', '
    Median_Length', 'Max_Length',
    'Number_of_Startpoints_G', '
    Number_of_Startpoints_TR', '
    Number_of_Branchpoints_G', '
    Number_of_Branchpoints_TR', '
    Total_Length_G', '
    Total_Length_TR', '
    Sum_Lengths_TR', 'Explant_Size',
    'Hull_Area', 'Neurites_Area',
    'Covered_Area', '
    b3_dapi_multi_explant', '
    b3_dapi_mean_explant', '
    b3_sum_explant'}});
```

adapted

```
Samples_export = table({Samples.
    Name}.', {Samples.Folder}.', [
    Samples.Number_of_Neurites
    ].', [Samples.Mean_Length].', [
    Samples.Median_Length].', [
    Samples.Max_Length].', [Samples.
    .Number_of_Startpoints_G].', [
    Samples.
    Number_of_Startpoints_TR].', [
    Samples.
    Number_of_Branchpoints_G].', [
    Samples.
    Number_of_Branchpoints_TR].',
    [Samples.Total_Length_G].', [
    Samples.Total_Length_TR].', [
    Samples.Sum_Lengths_TR].', [
    Samples.Image_Size].', [Samples.
    .Explant_Size].', [Samples.
    Explant_dil_1_Size].', [
    Samples.
    Explant_dil_2_Perimeter].', [
    Samples.Hull_Area].', [Samples.
    .Neurites_Area].', [Samples.
    Covered_Area].', [Samples.
    Normalized_Neurites_Area].', [
    Samples.
    Number_of_Startpoints_G_per_mm
    ].', 'VariableNames', {'Name',
    'Folder', 'Number_of_Neurites',
    'Mean_Length(um)', '
    Median_Length(um)', '
    Max_Length(um)', '
    Number_of_Startpoints_G', '
    Number_of_Startpoints_TR', '
    Number_of_Branchpoints_G', '
    Number_of_Branchpoints_TR', '
    Total_Length_G(mm)', '
    Total_Length_TR(mm)', '
    Sum_Lengths_TR(mm)', '
    Image_Size(um^2)', '
    Explant_Size(um^2)', '
    Explant_dil_1_Size(um^2)', '
    Explant_dil_2_Perimeter(um)',
    'Hull_Area(um^2)', '
    Neurites_Area(um^2)', '
    Covered_Area(um^2)', '
    Normalized_Neurites_Area(%)', '
    Number_of_Startpoints_G_per_mm
    '}});
```

ExplantAnalyzer.m

original (line 47)

```
dapi_bw = imbinarize(dapi);
```

adapted

```
if size(image,3)==4
    manual_dapi(:,:,1) = image(:,:,4);
    dapi_bw = ~imbinarize(manual_dapi);
else
    dapi_bw = imbinarize(dapi);
end
```

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original (lines 59-63)	adapted
<pre> if bg_sub == 0 b3_sub = imadjust(b3, [(double(median(b3(:)))/2^16) 1]); else b3_sub = imadjust(b3, [bg_sub/2^16 1]); end </pre>	<pre> if bg_sub == 0 b3_sub = imadjust(b3, [(double(median(b3(:)))/2^8) 1]); else b3_sub = imadjust(b3, [bg_sub/2^8 1]); end </pre>
original (lines 66-68)	adapted
<pre> b3_dapi_multi_explant = sum(im2uint16((double(dapi)./65535) .* (double(b3_sub) ./ 65535)) .* (im2uint16(explant) ./ 65535), 'all'); b3_dapi_mean_explant = sum(im2uint16(mean(cat (3,dapi,b3_sub),3) ./ 65535) .* (im2uint16(explant)./65535), 'all'); b3_sum_explant = sum(b3_sub .* (im2uint16(explant) ./ 65535), 'all'); </pre>	<pre> b3_dapi_multi_explant = sum(im2uint8((double(dapi)./255) .* (double(b3_sub) ./ 255)) .* (im2uint8(explant) ./ 255), 'all'); b3_dapi_mean_explant = sum(im2uint8(mean(cat (3,dapi,b3_sub),3) ./ 255) .* (im2uint8(explant)./255), 'all'); b3_sum_explant = sum(b3_sub .* (im2uint8 (explant) ./ 255), 'all'); </pre>
original (lines 89-90)	adapted
<pre> T = adaptthresh(b3_filt .* uint16(~ explant_dil_1), sensitivity,... 'NeighborhoodSize', neighborhood_size, 'Statistic', 'mean'); b3_bw = imbinarize(b3_filt .* uint16(~ explant_dil_1), T); </pre>	<pre> T = adaptthresh(b3_filt .* uint8(~ explant_dil_1), sensitivity,... 'NeighborhoodSize', neighborhood_size, 'Statistic', 'mean'); b3_bw = imbinarize(b3_filt .* uint8(~ explant_dil_1), T); </pre>

C.4 Manual segmentation of the explant body

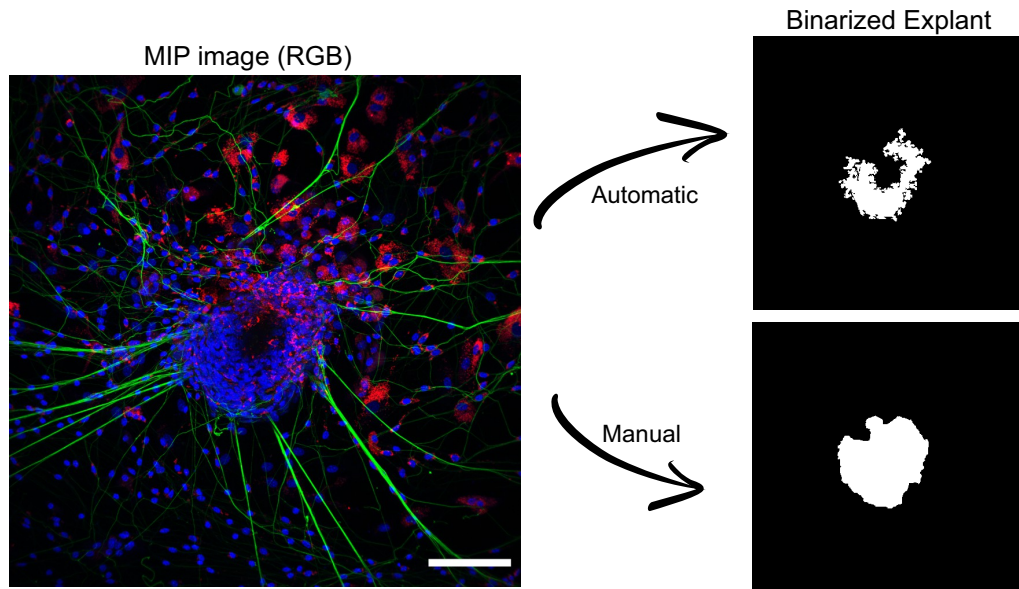


Figure C.1 | Manual segmentation of the explant body. The maximum intensity projection (MIP) image in the left is an example of incomplete Hoechst staining. It appears that the explant body is hollow, even though brightfield microscopy showed otherwise. In these cases, the automatic explant body segmentation performed by *ExplantAnalyzer* produced a donut-like shape. In order to ensure proper segmentation, the boundary of the explant body was manually added to the MIP image as an extra (alpha) channel using Adobe Photoshop. Scale bar: 100 μm .

C.5 Maximum intensity projection images

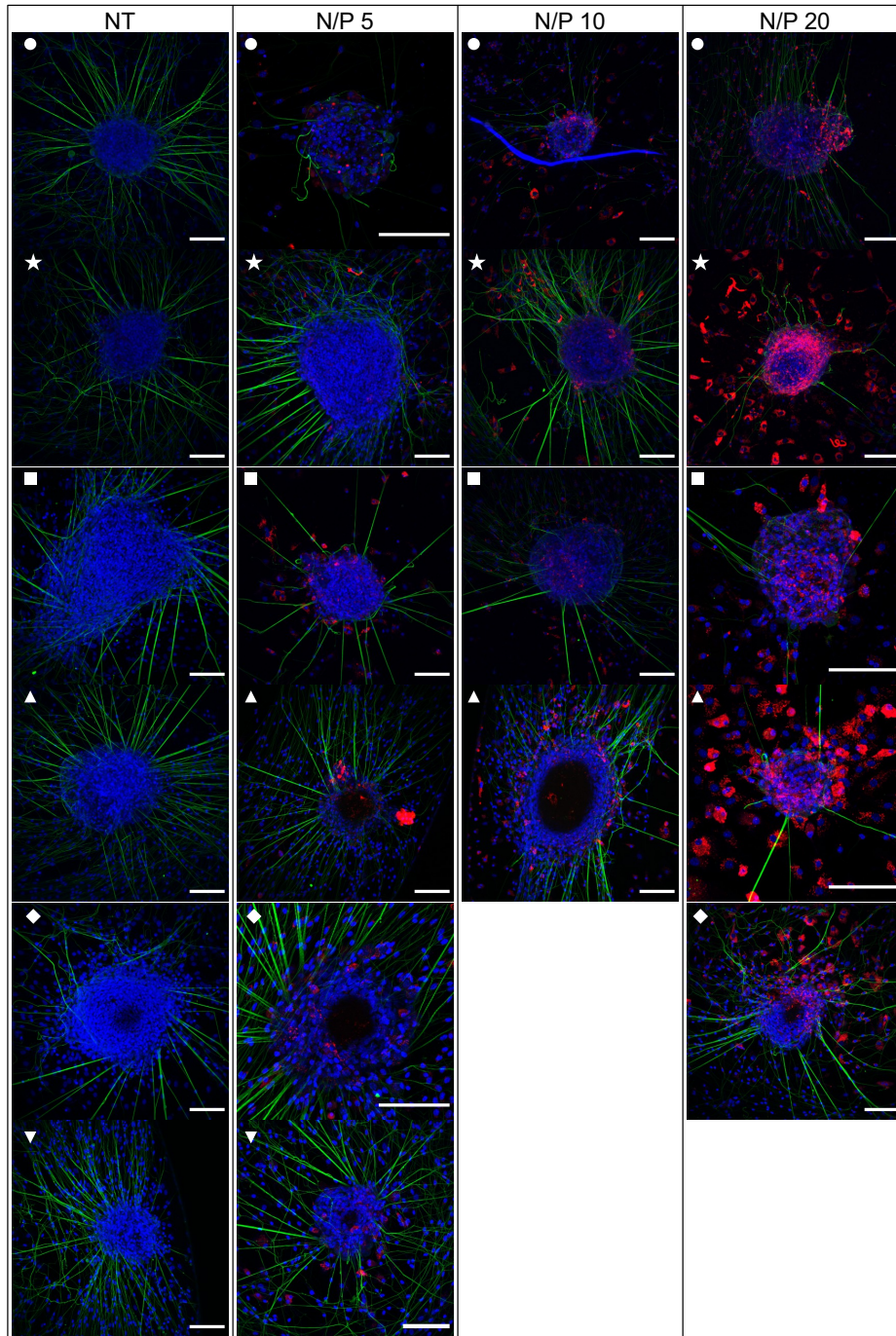


Figure C.2 | Maximum projection images of DRG explants. DRGs were incubated with fbB-Cy5-siRNAmi dendriplexes (red) at N/P ratios 5, 10, or 20 for 72 h, starting at day 6 post-isolation. Neurons were stained against β -III-tubulin (green) and nuclei with Hoechst (blue). NT: non-treated. Scale bars: 100 μ m. The symbol in the top-left corner of each image allows the tracking of each individual DRG in Figures 4.9, C.3, and C.4. The imaging data was the result of two independent experiments.

C.6 Z-distribution of fluorescence intensities

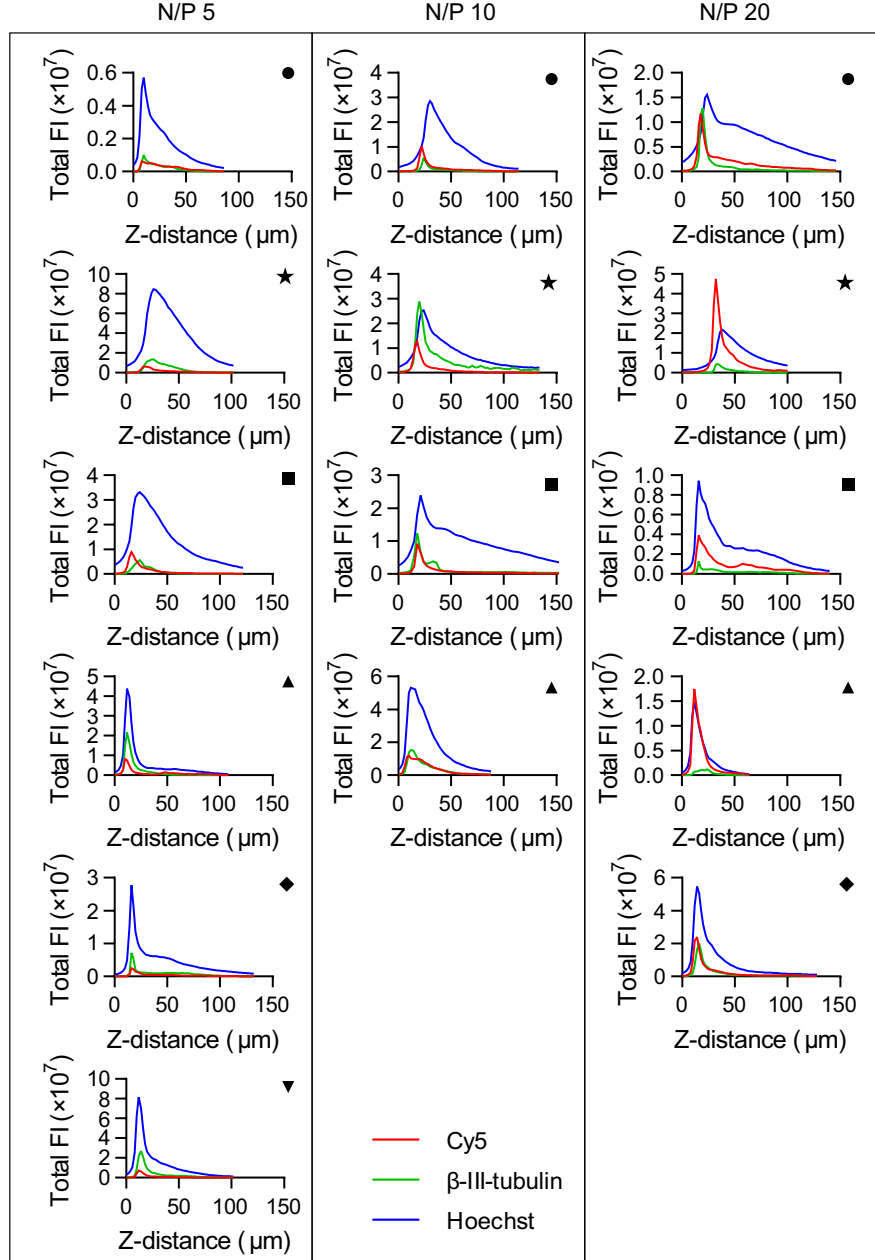


Figure C.3 | Z-distribution of fluorescence intensities in Z-stacks of dorsal root ganglion (DRG) explants. For each Z-slice, the total XY fluorescence intensity (FI) signal detected in each of the three channels (Cy5, β -III-tubulin, Hoechst) was plotted against the corresponding Z-distance. The symbol in the top-right corner of each graph identifies the corresponding DRG in Figure C.2.

C.7 Neurite segmentation by *ExplantAnalyzer*

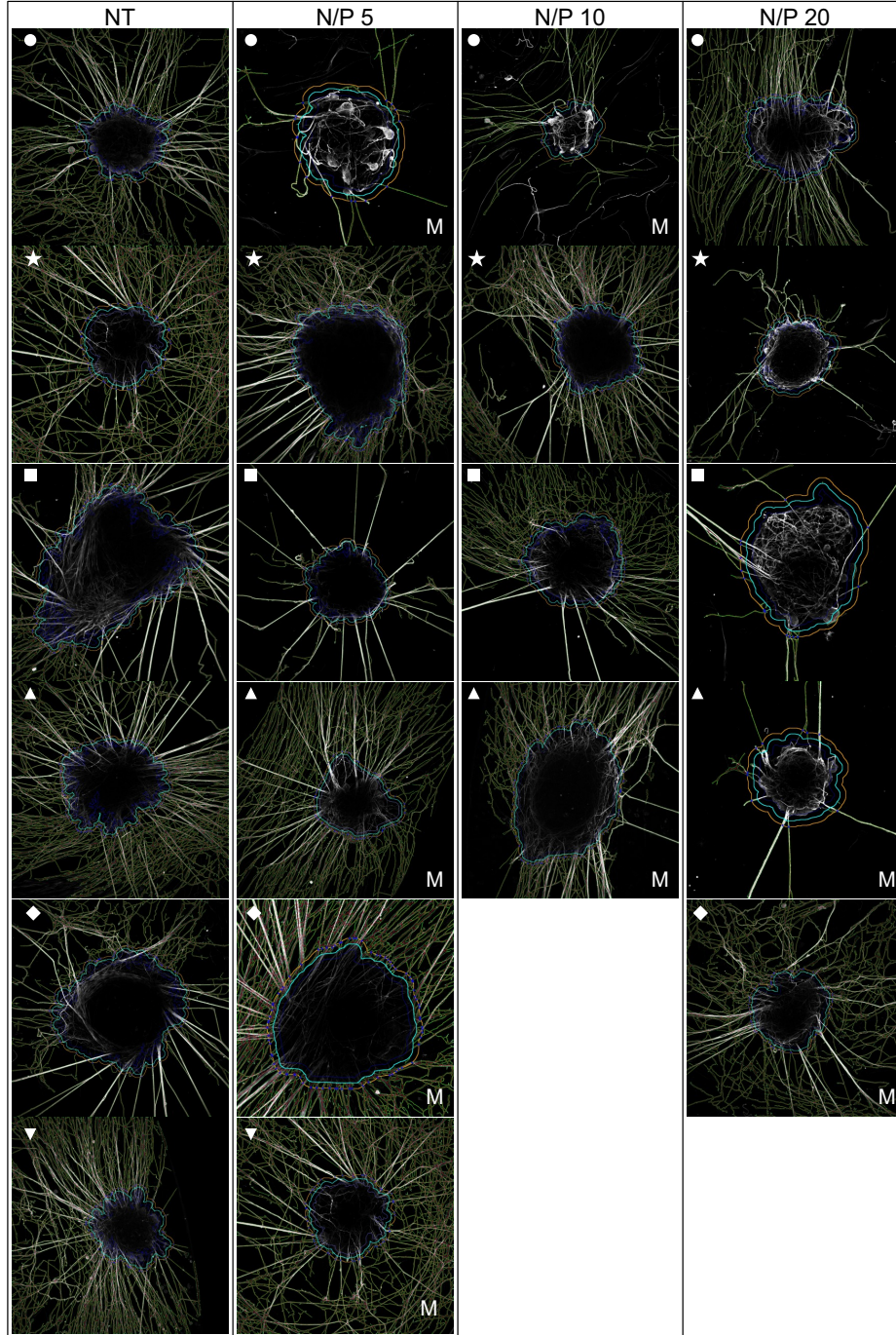


Figure C.4 | Overview images of neurite segmentation obtained using *ExplantAnalyzer*. The features detected are overlaid on top of the β -III-tubulin channel (white). Dark blue line: explant body segmentation. Cyan and orange lines: 1x and 2x dilated explant body (respectively). Green lines: neurite segmentation. Red lines: neurite skeleton. Blue crosses: start-points. The symbol in the top-left corner of each image identifies the corresponding DRG in Figure C.2. In the images marked with an 'M' in the bottom-right corner, manual explant body segmentation was carried out (see Figure C.1).

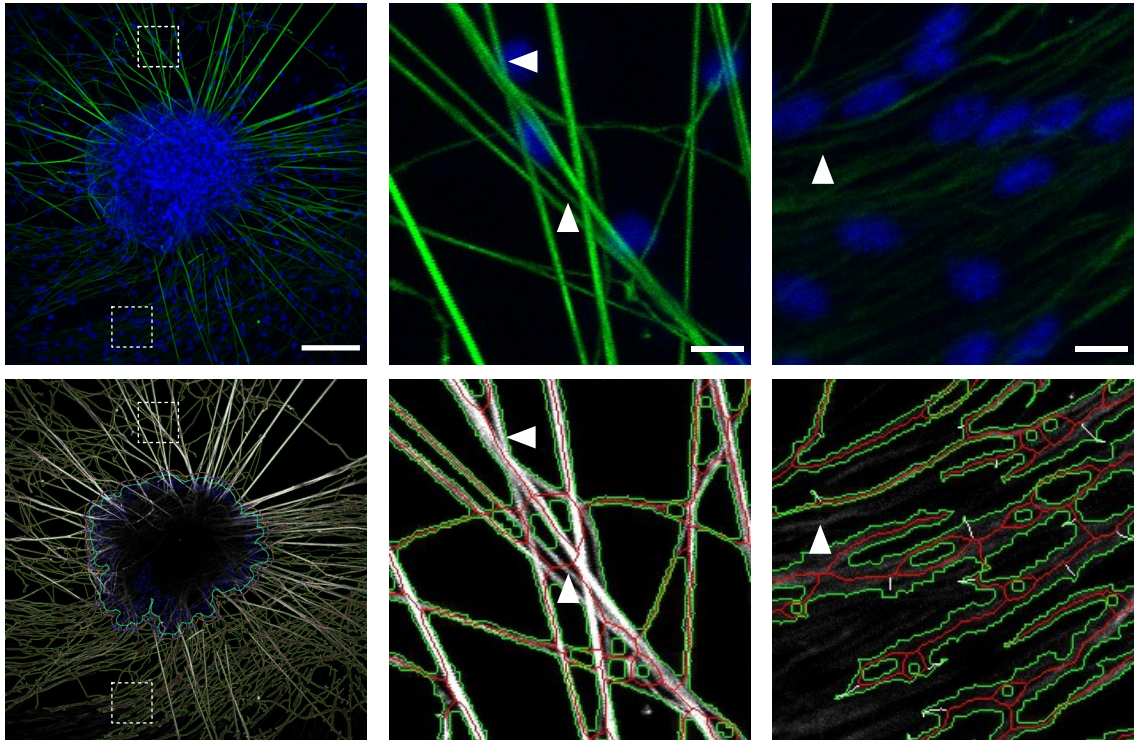


Figure C.5 | Details of neurite segmentation using *ExplantAnalyzer*. The maximum intensity projection image and exemplary detail views are shown on the top panels, whereas the result of the automatic segmentation is shown in the bottom panels. The green lines in the bottom panels identify the area occupied by fibers (used in equation 3.3), whereas the red lines indicate the result of the skeletonization. Most fibers are correctly segmented. Minor segmentation errors occurred mostly due to overlapping structures (middle panels) or to low or interrupted fluorescence signals (right panels). Scale bars: 100 μm (left), 10 μm (middle and right).

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