Biofunctional electrospun nanofibrous scaffolds in tissue engineering and the potential application of siRNA-delivery for nerve regeneration

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**NANYANG
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SCAFFOLDS IN TISSUE ENGINEERING AND THE
POTENTIAL APPLICATION OF SIRNA-DELIVERY FOR
NERVE REGENERATION**

**CAO HAOQING
SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING
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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
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2011

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by

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

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ABSTRACT

The application of tissue engineering has been extensively explored for decades to improve biological functions. New strategies and technologies have been continuously developed in various fields over time. Among these developments, the use of tissue engineering scaffolds for nerve regeneration has important implications in understanding neural cell biology and recovering nerve functions. In this thesis, the potential application of electrospun nanofibrous scaffolds endowed with chemical and topographical signals for nerve regeneration was investigated. The versatility of electrospinning technique was first explored by producing diverse and functional fibrous scaffolds using biocompatible polymer polycaprolactone (PCL). Fiber properties can be tailored for specific purposes by altering operational parameters during electrospinning process and/or modification of poly properties. Core-shell structured fibers were introduced by co-axial electrospinning method, where scaffolds can be functionalized with desired characteristics. A scaffold-based drug delivery system was developed by incorporating small interfering RNA (siRNA) into PCL nanofibers for long-term RNA interference (RNAi) therapy. Intact siRNA was gradually released from nanofibers in a sustained manner for at least 49 days under physiological conditions. Compared to conventional bolus delivery, the scaffold-medicated delivery system is able to enhance cellular uptake and may decrease non-specific targeting to adjacent cells when applied *in vivo*. Meanwhile, topographical cues presented by the electrospun fibers also have profound impact on modulating cellular behaviors such as attachment, proliferation and

differentiation. Therefore, nanofiber topographical effects on astrocyte phenotypic changes, including the uptake of siRNA and the corresponding gene-silencing efficiency, were investigated to gain insights to construct a novel scaffold to deliver siRNA for CNS regeneration. Suppressed astrocytic growth, elevated apoptosis and enhanced gene silencing efficiency were observed on nanofibers in comparison with two-dimensional flat surfaces. These results suggested that the electrospun nanofibrous scaffolds may serve as a potential therapeutic treatment for nerve regeneration. We also investigated the biocompatibility with regard to scaffold architecture and topographical effect of nanofibrous scaffolds on the *in vivo* and *in vitro* foreign body reaction. PCL electrospun nanofibers with random and aligned fiber orientations yielded significantly thinner fibrous capsules compared to solid surfaces when implanted subcutaneously in rat. Furthermore, cell infiltration and cell-scaffold interaction were observed on the aligned fibrous scaffolds. Altogether, these results demonstrated the potential application of the bio-functionalized electrospun nanofibers for general tissue engineering applications and neural regenerative medicine.

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CHAPTER 1

INTRODUCTION

1.1. STATEMENT OF PROBLEM

The ability of neuroregeneration in human species is extremely limited and injuries in the nervous system usually cause irreversible damages, especially in the central nervous system (CNS). These injuries often result in permanent function loss and may greatly reduce the quality of the patients' lives. In the United States alone, over 90,000 people are suffering from traumatic brain injury (TBI) and spinal cord injury (SCI) each year, and 12,000 new SCI cases are added annually [1-4]. Although millions of dollars have been spent on the clinical treatment every year, the goals for functional are still facing great challenges. Only 80% of the functional recovery rate is approached for regenerations in the peripheral nervous system (PNS) using autologous nerve grafts, and worst still, no successful recovery has yet been reported in the treatment of CNS injuries [5]. With regard to the high incidence of neural injuries and low recovery rate, neural tissue engineering is becoming a fast growing field with new approaches being continuously developed to achieve desirable recovery after neural injuries.

Neural tissue engineering presents novel and exciting alternative strategies to supplement the conventional treatment. In the PNS, bioengineered scaffolds serve as substitutional nerve guidance to autologous nerve grafts, eliminating the need for second surgery and functional loss at donor site [6]. In terms of CNS regeneration, researches have been focused on creating permissive environment for growing axons [7-8]. The therapeutic strategies

include delivering biochemical cues to promote axonal growth and/or remove inhibitory components in the environment, and introducing physical cues to provide contact guidance. An extraordinary role of the electrospun nanofibrous scaffolds for tissue engineering and regenerative medicine has been widely recognized in a few decades [9], including the potential contributions to neuroregeneration (reviewed in Chapter 2).

Electrospinning is a simple and versatile technique to fabricate fibers ranging from micro- or nano-scales, using a repertoire of source materials. Improved axonal growth, which is supported by the physical and mechanical guidance presented by the electrospun fibrous scaffolds, has been observed in PNS [10-11], and in CNS to a less extent [12-13]. Combining physical and biochemical cues through the drug-loaded electrospun fibers further improve the degree of regeneration. These fibrous scaffolds may serve as a reservoir to release the embedded drugs, such as growth factors and DNA, in a sustained manner for long-term applications [14]. Altogether, such biofunctional electrospun fibrous scaffold may hold great potential as implantable devices for neural tissue engineering.

1.2. HYPOTHESIS

In this thesis, we hypothesize that the electrospun fibers endowed with biochemical cues may serve as a potential scaffolds for nerve regeneration. Recent studies identified small interfering RNA (siRNA) as a promising tool for various applications in regenerative medicine including nerve repair [15]. By encapsulating siRNA into the electrospun fibers, we aim to develop a scaffold-mediated gene therapeutic platform which controls cellular behaviors via RNA

interference (RNAi) in a precise manner, in order to facilitate regeneration process in the nervous system. This scaffold-based silencing system takes the advantages in providing localized availability of biomolecules to adherent cells for long-term effect. Furthermore, fibrous topography offers an additional control over cell fate by influencing neurite extension and axon growth.

1.3. OBJECTIVE

The overall objective in this thesis is to construct a biofunctional nanofibrous scaffold and to evaluate the potential application of siRNA delivery in axon regeneration for long-term therapy. To accomplish this goal, the following objectives are set:

1. To understand the electrospinning process and to investigate fabrication methods for bio-functional electrospun fibrous scaffolds.
 - a. Study the relationship of electrospinning parameters and the morphology of the resulting fibers.
 - b. Fabricate biofunctional scaffolds via drug-loading approaches and modifying fiber property by uni- or co-axial electrospinning.
2. To develop a nanofibrous scaffold-based siRNA delivery system for long-term therapeutic application *in vitro*.
 - a. Investigate the feasibility of siRNA delivery by electrospun polymeric nanofibers.
 - b. Design and modify polymer properties to achieve sustained release of siRNA for long-term applications.
3. To investigate the topographic effect of electrospun nanofibrous scaffolds on the cellular behaviors of primary rat astrocytes *in vitro*.

- a. Compare astrocyte behaviors on nanofiber and flat topographies, including proliferation, apoptosis, and cell adhesion.
 - b. Evaluate the nanofiber topographic effect on siRNA uptake and the potential application of siRNA therapy on astrocytes for CNS regeneration.
4. To examine the biocompatibility of electrospun nanofibers of different topographies as implantation devices and the resulting foreign body reactions in response to fiber topographies both *in vitro* and *in vivo*.

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CHAPTER 2

THE APPLICATION OF ELECTROSPUN NANOFIBROUS SCAFFOLDS IN NERVE TISSUE ENGINEERING

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SUMMARY

The repair process in the nervous system is complicated and brings great challenges to researchers. Tissue engineered scaffolds provide an alternative approach for neural regeneration. Sub-micron and nano-scale fibrous scaffolds, which mimic the topography of natural extracellular matrix (ECM) can be potential scaffold candidates for neural tissue engineering. In this chapter, the application of electrospun fibers in the field of nerve regeneration is reviewed. Electrospinning is a versatile method producing fibers with diameters ranging from several micrometers to hundreds of nanometers. These biocompatible fibrous scaffolds are able to manipulate biological processes related to neural regeneration in culture, including axonal outgrowth, Schwann cell migration and stem cell differentiation. The nerve conduits constructed from electrospun fiber mat can be introduced at lesion sites to promote peripheral nerve regeneration by implantation. These functional nanofibrous scaffolds may serve as powerful tools for neural tissue engineering.

2.1. INTRODUCTION

The nervous system in human species has very limited regeneration capability, and as a result, patients who endure nerve injuries or traumas often suffer from the loss of sensory or motor function and neuropathic pains. Plenty of therapeutic approaches have been attempted in order to facilitate nerve regeneration. In the peripheral nervous system (PNS), direct end-to-end surgical reconnection is a common treatment for nerve transection injuries when the injury gap is small. Nerve autografts are considered as the ‘golden standard’ for bridging larger nerve defect gaps. However, the shortage of donor grafts, the potential loss of function at donor sites and the requirement of multiple surgeries are among the reasons that constrain the use of autografts. Allografts and xenografts [1-3] are also taken into consideration to substitute autologous nerve grafts. These treatments are yet hampered by immunological rejections and chances of disease transfer. Nerve regeneration in the central nervous system (CNS) is even more challenging, with the inhibitory environment formed at injury sites frequently restricting nerve regeneration [4]. Current methods of treatment are often not effective enough to restore nervous function in the CNS.

Due to the above reasons, tissue engineered scaffolds may serve as an alternative implantation devices to facilitate neural repair. The physical and chemical properties of artificial grafts can be tailored based on purpose-specific applications. Biodegradable and biocompatible materials are often used to reduce the occurrence of immune responses. By controlling scaffold morphology, architecture and components, suitable topographical and biochemical cues may be provided to promote tissue regeneration. These

artificial scaffolds can also be modified to provide a permissive substrate for axons to regenerate beyond the injured area in CNS tissue engineering [5].

A common approach in tissue engineering is to mimic the architecture of the natural extracellular matrix (ECM). The ECM plays an important role in regulating cellular behaviors by influencing cells with biochemical signals and topographical cues [6-7]. It has two main components: polysaccharides and fibrous proteins. As such, nanofibrous constructs have been used extensively as potential tissue engineering platforms. It is generally hypothesized that a close imitation of the ECM will provide a more conducive environment for cellular functions ranging from adhesion, migration, proliferation to differentiation [8]. Meanwhile, nanofibers exhibit a unique property of extremely high surface area-to-volume ratio which facilitates the release of biochemicals for drug delivery purposes, including proteins, drugs, and nucleic acids [9-11]. Additionally, the large surface increases the contact area between cells and the scaffolds, thereby enhancing drug uptake by cells. Therefore, nanofibrous scaffolds may also be used as a potential drug carrier for chemical therapy.

Various approaches have been developed to produce nanofibers. In particular, electrospinning is one of the widely-studied fiber-fabrication methods as applied to neural tissue engineering. The technique of electrospinning is a simple method of producing continuous fibers with diameters ranging from several microns to hundreds of nanometers [12-14]. The flexibility of the technique is evident from the ease of forming fibers out of a wide range of materials including natural and synthetic polymers, composites and ceramics [15-16]. Like conventional fiber spinning processes, parameters such as polymer solution concentration and viscosity, and polymer solution

dispense rate may be controlled during fabrication to alter the dimensions of the fibers. In addition, because of the nature of electrospinning, electrical field strength and field pattern may be varied to manipulate the architecture and morphology of the resulting scaffolds. For a comprehensive review on the electrospinning process and approaches to control scaffold architecture, one may refer to the works by Chew *et al.* [12] and Murugan *et al.* [17].

Due to the versatility of the electrospinning technique, electrospun scaffolds have found applications in various areas of tissue engineering ranging from cardiovascular [18] to musculoskeletal tissue engineering [19] and stem cell engineering [20]. The large surface area-to-volume ratio property of these scaffolds ensures that any incorporated bioactive agents are released efficiently and that the contact between cells and these biochemicals are maximized.

The feasibility of using electrospun fibrous scaffolds as substrates for neural tissue engineering have been investigated by several research groups [21-23]. Comparisons between substrates made out of polymer films versus electrospun fibers have been tested both *in vitro* and *in vivo* to demonstrate the efficacy of these fibers in enhancing nerve regeneration. *In vivo*, axonal extension within electrospun fibrous nerve conduits and partial functional reconnection have been demonstrated [24-25]. Altogether, these findings indicated that electrospun fibers are potential candidates for nerve repair applications [26-27]. A summary of electrospun fibrous scaffolds used in nerve regeneration studies is listed in Table 2.1.

Table 2.1. Electrospun nanofibrous scaffolds for nerve regeneration

Polymer	Solvent / Medium	Diameter	Cells / Nerve injury model	Reference
<i>In vitro</i>				
Poly(L-lactic acid) (PLLA)	10 w/v% , chloroform	500 nm (R / A)	Rat DRG	[28]
	1 wt% , DCM: DMF (7: 3 v/v)	(272 ± 77) nm (R)	Neural stem cell line C17.2	[21]
	1 – 5 wt%, DCM: DMF (7:3 v/v)	250 nm (1 wt%, R); 1.5 µm (3 wt%, R); 300 nm (2wt%, A); 1.5 µm (5 wt%, A)	Neural stem cell line C17.2	[29]
	10 w/v%, HFP	100-500 nm (R)	PC12 cells	[30]
	10 w/v%, THF: DMF (1:1 v/v)	(0.51 ± 0.38) µm (R)	Mouse embryonic cortical neurons	[22]
	15 w/v%, DCM: THF (7:3)	(4.3 ± 0.5) µm (R)	Rat Schwann cell (RT4-D6P2T)	[31]
Poly(ε-caprolactone) (PCL)	16 wt%, DCM: methanol (4:1 v/v)	(1.03 ± 0.03) µm (A); (2.23 ± 0.08) µm (R)	Human Schwann cells	[32]
	20 w/v%, DCM: DMF (4:1 v/v)	250 nm (A)	Mouse embryonic stem cell	[33]
	9 wt%, chloroform: methanol (75:25 w/w)	(559 ± 300) nm (A)	Chick DRG explants; neurons from dissociated chick DRG; rat Schwann cells; rat OECs	[34]
	15 wt%, chloroform: methanol (75:25 v/v)	(630 ± 40) nm (R)	Rat Schwann cell	[35]
	6 wt%, HFP	(431 ± 118) nm (R)	Neural stem cell line C17.2	[36]
	12 w/v%, DCM: DMF (1:1 v/v)	(0.95-1.26) µm (R)	Rat Schwann cell line (RT4-D6P2T)	[31]
	6 wt%, HFP	PCL/gelatin 50:50: (113 ± 33) nm, PCL/gelatin 70:30: (189 ± 56) nm (R)	Neural stem cell line C17.2	[36]
PCL/Collagen	(75:25 wt%), HFP	(541 ± 164) nm (A)	Chick DRG explants; neurons from dissociated chick DRG; rat Schwann cells; rat OECs	[34]
PCL/Chitosan	(75: 25 wt%), HFP and TFA/DCM	(190 ± 26) nm (R)	Rat Schwann cell	[35]
Poly(D,L-lactide-co-glycolic acid) (PLGA)	50 wt%, DMF: THF (1:1 v/v)	3-5 µm (R / A)	PC12 cells	[37]
	10 w/v%, THF: (1:1 v/v)	(0.76 ± 0.30) µm (R)	Mouse embryonic cortical neurons	[22]

Table 2.1. (continued)

Polymer	Solvent / Medium	Diameter	Cells / Nerve injury model	Reference
Poly-L-lactate (PLA)	3 wt%, chloroform	(524 ± 305) nm (A)	Rat embryonic DRG	[38]
Poly(e-caprolactone-co-ethyl ethylene phosphate) (PCLEEP)	12 wt%, DCM	(5.01 ± 0.24) µm (A)	PC12 cells	[39]
Poly(3-hydroxybutyrate) (PHB)	14 w/v%, chloroform	(3.7 ± 1.7) µm (A)	Rat Schwann cell line (RT4-D6P2T)	[23]
Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)(PHBV)	14 w/v%, chloroform	(2.3 ± 2.1) µm (R)	Rat Schwann cell line (RT4-D6P2T)	[23]
Poly (acrlonitrile-co-methylacrylate) (PAN-MA)	18 w/v%, DMF	(400-600) nm (A)	Rat DRG; rat Schwann cells	[40]
Copolymer of methyl methacrylate (MMA) and acrylic acid (AA) (PMMAAA)	8 wt%, acetone	(450 ± 88) nm (R)	Rat embryonic cortical neural stem cells	[41]
Polydioxanone (PDS)	10 w/v%, HFP	2-3 µm (R / A)	Rat primary astrocytes; rat embryonic DRG	[42]
Polyamide	Commercially available	180 nm (R)	Rat cerebellar granule neurons, rat cerebral cortical neurons, rat hippocampal and ventral spinal cord neuronal cultures, rat DRG	[43]
Chitosan	8 wt%, TFA: DCM (75:25 V/V)	(450 ± 48) nm (R)	Rat Schwann cell	[35]
<i>In vivo</i>				
Poly(e-caprolactone-co-ethyl ethylene phosphate) (PCLEEP)	12 wt%, DCM	(5.08 ± 0.05) µm (A)	Rat sciatic nerve, 15 mm gap	[44]
PCL/PLGA	5.5wt% PCL and 4wt% PLGA, chloroform: methanol (3:1 v/v)	140 - 500 nm (R)	Rat sciatic nerve, 10mm gap	[25]
Poly(ε-caprolactone) (PCL)	15 w/w%, chloroform	2.5 - 8 µm (R)	Rat sciatic nerve, 10mm gap	[25]
Poly (acrlonitrile-co-methylacrylate) (PAN-MA)	18 w/v%, DMF	400 - 600 nm (A)	Rat tibial nerve, 17mm gap	[40]
Polyamide	Commercially available	180 nm (R)	Laminectomy at T8 in rats	[24]
Chitosan	6.4 w/v%, TFA: DCM (4:1 v/v)	(700 ± 502) nm (R)	Rat sciatic nerve, 10mm gap	[45]

Legend: A: aligned, non-woven fibrous mesh; DCM: dichloromethane; DMF: dimethylformamide; DRG: dorsal root ganglia; HFP: 1,1,1,3,3,3-hexafluoroisopropanol; OEC: olfactory ensheathing cells; R: random, non-woven fibrous mesh; THF: tetrahydrofuran; TFA: trifluoroacetic acid.

2.2. EFFECTS OF ELECTROSPUN MATERIALS ON NERVE REGENERATION

Material choice plays a vital role in ensuring the success of neural tissue engineering strategies. Appropriate material degradation rate and mechanical properties help minimize inflammatory response and prevents nerve compression situations, while providing the required support and guidance to regenerating axons. With these requirements in mind, the search for the suitable scaffolding material remains and many have been attempted via electrospinning. These materials may be broadly categorized into three groups: 1) synthetic materials; 2) natural materials; and 3) biosynthetic and blend materials.

2.2.1. Electrospun biocompatible synthetic materials

Biocompatible synthetic materials are attractive for constructing neural tissue engineering scaffolds because of the ease in tailoring the degradation rate and mechanical properties of these materials to suit the applications. Polyesters such as poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly(caprolactone) (PCL), poly(3-hydroxybutyrate) (PHB) and their copolymers have been extensively investigated as electrospun scaffolds applied to neural tissue engineering as listed in Table 2.1. Polyesters can be degraded by hydrolysis of the ester bond leading to degradation products that can be resorbed through metabolic pathways, causing minimal toxicity to the hosts [46]. Other synthetic polymers such as polydioxanone (PDS), poly(acrylonitrile-co-methylacrylate) (PAN-MA) have also been reported as materials for electrospun fibers to induce nerve growth. In general, these

synthetic materials support neural cell growth, but their ability in terms of supporting cell proliferation and viability differs due to their distinct properties.

Sangsanoh *et al.* [31] studied the cytocompatibility of several biodegradable polymeric electrospun fibers and solution-cast films by culturing rat Schwann cells on these platforms. After 24 h, the rank of cell viability on various substrates was: PCL film > TCPS > PCL fibers > PLLA fibers > PHBV film > chitosan (CS) fibers $\approx$ CS film $\approx$ PLLA film > PHB film > PHBV fibers > PHB fibers. The rank, however, was different at day 3: TCPS > PHBV film > PLLA film > PCL film > PLLA fibers > PHB film $\approx$ PCL fibers > CS fibers > CS film > PHB fibers > PHBV fibers. While it was clear that scaffold architecture and chemistry affect Schwann cell viability, the exact relationship between these factors and Schwann cell behavior could not be elucidated from the study due to the lack of obvious experimental trends. An additional size effect was also present due to the different fiber diameters of different electrospun materials, which varied from 130 nm to 3.7 μ m. More in-depth comparison studies are thus required in order to understand the mechanisms of how material property changes may affect neural cell behaviors.

2.2.2. Electrospun natural materials

Natural materials have good biocompatibility, consist of biomolecular recognition sites, and may share similar properties with the soft tissues which they are replacing [47]. Collagen, gelatin, laminin and chitosan [48-50] are among the examples of electrospun natural materials that have been used for nerve reconstruction. Wang *et al.* [27, 45] developed chitosan micro/nanofiber mesh tubes for treatment of a 10 mm rat sciatic nerve gap. It was observed that

sensory function begun to recover as early as 8 weeks after implantation of the nano/microfiber mesh tubes. Regenerating axons also elongated through the lumen of the chitosan tubes. In spite of the regeneration obtained in these fibrous scaffolds, the weak mechanical properties of natural electrospun constructs may limit their application as nerve guide conduits. In order to maintain structural integrity during both operation and post-operation periods, sufficient mechanical strength is required of a nerve conduit. As evident from Wang's study, tube collapse may occur when the degree of acetylation of chitosan is lower than 93%. Meanwhile, the use of natural materials is also jeopardized by its high cost and possible immunogenicity.

2.2.3. Electrospun biosynthetic and blend materials

Biosynthetic and blend materials serve to combine the merits of both synthetic and natural materials, with improved mechanical properties over natural polymers and enhanced biocompatibility over synthetic ones. These unique properties can be achieved by covalent binding, surface adsorption or blending synthetic and natural polymers.

Ahmed *et al.* [43] surface-modified polyamide nanofibers by covalent attachment of fibronectin type III repeat D of human tenascin C, D5 and extended version D5'. These peptides were identified to promote neurite outgrowth for cerebellar granule neurons [51]. After covalent modification with these neuroactive peptides, neuronal cell attachment, neurite generation and neurite extension were significantly enhanced as compared to plain electrospun polyamide fibrous scaffolds. Collagen was also conjugated onto a copolymer of methyl methacrylate (MMA) and acrylic acid (AA) (PMMAAA) electrospun

nanofibers by Li *et al.* [41]. The collagen content ranged from 0% to 5.7% in the electrospun fibers. It was observed that at Day 2 and Day 4, the neurite length of cortical neural stem cells increased in accordance with collagen content. At the same time, cell viability was enhanced with respect to the increase in collagen content at Day 4. This indicated that the collagen-conjugated electrospun fibers could improve the attachment and viability of the cultured neural stem cells. The advantages of biosynthetic materials were also demonstrated by Patel *et al.* [28] through the immobilization of laminin onto PLLA nanofibers for the culture of rat dorsal root ganglia (DRG) tissues. After culturing for 6 days *in vitro*, cells on laminin-conjugated PLLA fibers had significantly longer neurite length as compared to cells grown on plain PLLA fibers.

Due to the uniqueness of electrospinning, synthetic and natural materials can be mixed as a blend to achieve improved chemical and physical properties. By choosing suitable solvents, synthetic and natural materials may be dissolved into the same medium and uniform mixtures can be obtained for electrospinning. Prabhakaran *et al.* [35] blended PCL with chitosan to produce electrospun nanofibers. These blended scaffolds (PCL/CS) had a narrow distribution of fiber diameter and improved hydrophilicity as compared to PCL fibers. With the appropriate hydrophilicity, cell adhesion and spreading became more rapid and more effective [52]. Moreover, the tensile strength and ultimate strain of the PCL/CS scaffolds increased in comparison with chitosan fibers. Their study demonstrated that the proliferation rate of rat Schwann cells on PCL/CS nanofibers was significantly higher than that on PCL nanofibers. Blend electrospun fibers were also fabricated by Ghasemi-Mobarakeh *et al.* [36]. By

comparing PCL/gelatin electrospun scaffolds at 100:0, 70:30 and 50:50 weight ratios, the presence of gelatin improved the hydrophilicity of PCL scaffolds. C17.2 neural stem cells showed longer neurite length and higher proliferation rate on 70:30 PCL/gelatin fibers in comparison with PCL constructs. Although the inclusion of gelatin positively influenced neural stem cell behavior, there appears to be a limit of ~50 wt% gelatin that one may include into PCL fibers, as indicated by this study. 50:50 PCL/gelatin fibrous constructs, while demonstrating higher hydrophilicity, also possessed loose and weak fibrous structures and enhanced degradation rate that may not be favorable for nerve regeneration. Schnell *et al.* [34] compared PCL and PCL/collagen nanofibers as nerve regeneration scaffolds. They found that both kinds of scaffolds were favorable substrates for neurite outgrowth and glial migration from DRG explants. However, Schwann cell migration, neurite orientation and process formation of Schwann cells, fibroblasts and olfactory ensheathing cells improved on PCL/collagen fibers.

Finally, the effects of the mode of biopolymer inclusion to synthetic electrospun fibers on neural cell behavior were evaluated by Koh *et al.* [30]. The authors studied three different methods of incorporating laminin into PLLA fibers - covalent binding, physical adsorption and blended electrospinning. In terms of supporting PC12 cell proliferation and enhancing neurite outgrowth, blended electrospinning was the most facile and efficient method as compared to the other two modification approaches.

In all studies, it appears that the presence of biopolymers, although drastically changing the degradation rate and mechanical performance of synthetic polymeric scaffolds, significantly helps in promoting neural cell

proliferation and neuron extension. Such materials may find useful applications in promoting nerve regeneration *in vivo*.

2.3. IN VITRO STUDIES

2.3.1. Effects of electrospun scaffold architecture on nerve regeneration

2.3.1.1. *Neurons & axonal outgrowth*

Neurite outgrowth and cell migration are indispensable processes for nerve repair. Guided axonal extension towards designated targets to reform synaptic connections can help in the restoration of nerve functions. The migrated glial cells to the injured sites can secrete neurotrophic factors and support regenerated neurons to help nerve regeneration. Accordingly, the ability to enhance neurite outgrowth, direct axon extension, and facilitate cell migration is an essential factor to be included during the design of tissue engineering scaffolds for nerve repair. While micro- and nano-patterned substrates have been extensively studied to understand the effects of contact guidance on neural cell behavior and function [53-54], electrospun scaffolds with aligned fibers are also gaining importance for their promise in providing topographical cues for nerve regeneration and as direct implantable devices.

Compared to randomly oriented fibers, aligned fibers are able to promote neurite outgrowth. DRG cultured on PLLA randomly oriented nanofibers showed no neurite outgrowth after 6 days. In contrast, significant extension of neurites from DRG explants was observed on aligned fibers [28]. In addition, many studies have proved that neurons cultured on aligned electrospun fibers have longer neurite lengths than those on random fibers [34, 38, 42].

The aligned fibrous scaffolds serve as contact guidance for directing neurite orientation and cell alignment. It is found that neurites emanated radially from DRG tissues when cultured on randomly oriented electrospun fibers, generating a round appearance. In contrast, on aligned fibrous substrate neurites grew in a radial manner initially when they emanated out from DRGs, but would follow the orientation of the fibers as they continued to grow. In addition, neurites that grew in the direction of the underlying fibers had a faster growth rate than those perpendicular to the aligned fiber axis or those grown on randomly-oriented fibers. The same growth behavior was observed in astrocytes. On randomly oriented fibers, however, astrocytes were positioned more haphazardly. A branched net shape structure was observed due to the lack of specificity in the direction of projections by the processes of the astrocytes. In contrast, astrocytic processes were well aligned, following the orientation of the fiber axis when cultured on aligned fibrous substrates [42].

Corey *et al.* [38] also found that ganglia changed its shape as a result of culture on aligned electrospun fibers. They explained that the ganglia deformation in the direction of the fibers was encouraged by the tension, which was generated by neurites following the fiber orientation. This elongated morphological change was also observed for human Schwann cells cultured on micron-sized electrospun fibers [32], indicating that the diameter of the fiber, at least up to 2 μm , is not a crucial factor in influencing cell alignment. The alignment of glial cells would further enhance the rate and extent of neurite outgrowth [55]. Additionally, these morphological alterations may elicit functional changes that will influence cellular phenotypes, ranging from

apoptosis to proliferation, cell migration, differentiation, contractility and gene expression [56-58].

2.3.1.2. Schwann cells

Schwann cells support peripheral nerve recovery by secreting neurotrophins and producing ECM molecules which elicit axonal regrowth [59]. The migration and formation of aligned Schwann cells to form bands of Büngner have to precede the infiltration of axons following nerve injuries. However, Schwann cell migration is often the rate-limiting step. Therefore, one of the approaches to enhance peripheral nerve recovery is to imitate the formation of bands of Büngner *in vitro*.

The possibility of guiding Schwann cell migration by aligned fibers has been studied *in vitro* by Schnell *et al.* [34]. By culturing DRG explants on blended PCL/collagen (4:1 w/w) aligned fibrous scaffolds, rat Schwann cells were observed to migrate away from the DRG along the direction of the aligned fibers. After 7 days of culture, more than 1 mm distance of migration on PCL/collagen aligned fibers was seen. Although aligned fibrous scaffolds could induce a more obvious alignment of the cells, random fibrous substrates also provide contact guidance to some extent, as the cells may stretch the cytoskeleton across multiple fibers and follow the fiber orientation. Chew *et al.* [32] found both randomly-oriented and aligned electrospun PCL fibers to decrease the expression of neurotrophin and neurotrophic receptors of human Schwann cells after 7 days of *in vitro* culture, indicating a more mature phenotype adopted by cells cultured on fibrous substrates. Aligned fibrous scaffolds, however, enhanced Schwann cell maturation more than randomly-

orientated fibers, as more substantial up-regulation of the gene expression of myelin specific marker, P0, was observed in cells grown on aligned fibers.

2.3.1.3. Stem cells

Stem cells have recently been considered and studied as one of the cell-based therapies in neural tissue engineering. Stem cells are able to differentiate into various cell types. These stem cells can be transplanted to the injured sites in the nervous system, replacing missing cells and activating endogenous cells to perform self-repair [60].

In effort to understand stem cell-substrate interactions, various cell types have been analyzed on electrospun scaffolds. The differentiation rates of neural stem cell C17.2 cultured on PLLA electrospun fibers were found to be similar on random and aligned fibers, but were dependent on fiber diameters [29]. By counting the number of cells that exhibited elongated shape under the laser scanning confocal microscope, the differentiation rate of C17.2 after 2 days *in vitro* was quantified. On nanofibers with average diameter of 300 nm, cells demonstrated a differentiation rate of 80%. In contrast, the differentiation rate of C17.2 on micron-sized fibers with average diameter of 1.5 μm was only 40% [29].

The differentiation of embryonic stem (ES) cells on the electrospun nanofibers was also explored [33]. ES cells were cultured in the presence of retinoic acid, which could induce ES cell differentiation along the neural lineage. Both random and aligned fibrous substrates were supportive of stem cell neural differentiation into neurons, oligodendrocytes and astrocytes. Interestingly, the aligned nanofibers discouraged the differentiation of ES cells

into astrocytes. Therefore, aligned fibers might be a potential therapeutic approach for CNS injuries, as astrocytes have been found to be the main reason for glial scar formation. Similar finding was also reported with self-assembled nanofibers minimizing astrocytic differentiation [61]. Altogether, the use of nanofibrous scaffolds as stem cell culture platforms may be promising in CNS repair, supplying neurons and at the same time, inhibiting glial scar formation.

In general, multiple works have shown that cells do respond to the unique fibrous architecture of electrospun scaffolds. In most studies, cellular response evaluation remains restricted to understanding cell proliferation and morphological changes with respect to topographical signals. Only few recent studies have attempted to elucidate in detail the functional changes of neural cells in terms of gene expression and cellular differentiation [32, 61]. More analysis is, therefore, required to ensure that the observations of cell shape changes do correspond to useful cell functions that will indeed aid nerve regeneration *in vivo*. At the same time, deeper understanding of the mechanisms, such as cell signaling processes, behind the corresponding cellular responses to topographic stimuli will also help in facilitating precise control of cellular functions and stem cell differentiation using electrospun fibers.

2.3.2. Effects of controlled release of biochemicals from electrospun scaffolds on nerve regeneration

Neurotrophic factors have profound effects on neural development and functions. These factors are usually expressed in large amounts in the presence of nerve injuries and play crucial roles in supporting neural cell survival and axon regrowth [62-63]. Multiple delivery methods have been extensively

investigated to introduce these factors to the injury sites in both CNS and PNS, such as in the form of microspheres [64-65] and polymer matrices [66-67]. Electrospun nanofibers may serve as a promising delivery vehicle due to its simultaneous ability to provide scaffolding function and contact guidance that may be necessary for nerve regeneration.

Using a copolymer of caprolactone and ethyl ethylene phosphate, PCLEEP, Chew *et al.* [39] demonstrated that by directly blending human β nerve growth factor (NGF) into PCLEEP polymer solution using electrospinning, protein-encapsulated electrospun fibers could be obtained. The encapsulated protein was found to be dispersed uniformly throughout the fibers in aggregate form due to phase separation. A sustained release of bioactive NGF from aligned electrospun fibers was obtained for at least three months *in vitro*. Following that, the efficacy of such biofunctional electrospun scaffolds was evaluated in an *in vivo* study by the same group [44]. By using human glial cell-derived neurotrophic factor (GDNF)-encapsulated aligned electrospun fibrous conduits, sciatic nerve regeneration in a rat model was significantly enhanced. The total number of myelinated axons, area of regenerated nerve cross-section and G ratio value were significantly larger than the control groups comprising of plain electrospun scaffolds and PCLEEP films. Detailed information on the study is discussed in the next section. Altogether, the ability of incorporating growth factor signaling and contact guidance within an electrospun scaffold demonstrates the uniqueness of this fabrication technique and its potential in enhancing nerve regeneration.

In a separate study, surface modification of electrospun scaffolds with growth factors was adopted instead. Patel *et al.* [28] explored the effects of

immobilizing basic fibroblast growth factor (bFGF) onto nanofibers on DRG neurite extension *in vitro*. As compared to plain aligned electrospun fibers and bFGF-immobilized randomly oriented fibers, the highest neurite outgrowth was achieved on a combination of aligned fibers with either immobilized bFGF or soluble bFGF introduced directly into the cell culture medium. In addition, the conjugated biofunctional fibers presented several advantages over delivering bFGF in soluble manner. First, only a small amount of bFGF was required to achieve similar effects as soluble bFGF in medium. Secondly, the electrospun fibrous scaffolds can act as a delivery vehicle for specific targets, without inducing systemic effects.

The studies highlighted above clearly demonstrated the efficacy of electrospun nanofibrous scaffolds as biofunctional substrates that combine topographical and biochemical signaling that may be vital for enhancing neural tissue regeneration.

2.4. IN VIVO STUDIES

Random electrospun nanofibers have been applied for the regeneration of both CNS and PNS injuries. Meiners *et al.* [24] investigated the feasibility of using polyamide nanofibers for over-hemisection spinal cord injury in rats. A laminectomy was made at thoracic level 8 spinal vertebrae and the nanofibrous fabric was crumpled into the lesion site. After 3 weeks, immunohistochemical staining showed axonal growth at injury sites and the axons extended onto the nanofibers. Unfortunately, this implantation method led to the self-folding of the scaffolds, and the regenerated axons followed the surface contours of the random folds, which were not targeting the distal stump. Certain orientations of

the scaffolds should, therefore, be considered for implantation, such as employing multilayered tubular structures [68].

Promising results of using random electrospun fibers for PNS injuries have also been reported. Panseri *et al.* [25] fabricated PLGA/PCL electrospun fibrous tubes to bridge a 10 mm nerve gap in the rat sciatic nerve. After 4 months, regenerated tissues filled the inner lumen of the fibrous tubes and bridged the nerve gaps. Neurite outgrowth was found along the longitudinal conduit axis. At the same time, myelination and collagen IV deposition were detected in concurrence with regenerated axons. Von Frey test was carried out to evaluate sensory function recovery after implantation every two weeks. Rats were placed on metal mesh floor and a series of Von Frey hair was applied to the plantar surface of the hind paw. Brisk withdrawals or paw lickings were accounted as positive responses. A decreasing trend of sensitivity threshold was observed in nerve conduit-treated animals as the test progressed, whereas no positive response to stimuli was seen in the untreated injured animal group. Electromyography tests revealed that 70.6% of the fibrous tube-treated rats showed an initial reinnervation in plantar muscles, while no CMAP (compound muscle action potential) was present in non-treated animals. However, both mean values of CMAP and MCV (motor conduction velocity) were lower in treated animals as compared to healthy animals, indicating a relatively early phase of nerve regeneration.

Aligned fibers are able to enhance neurite outgrowth and direct neurite extension *in vitro*. In order to evaluate the effectiveness of such scaffolds in enhancing nerve regeneration and functional recovery via contact guidance, several *in vivo* studies have been carried out.

Kim *et al.* [69] stacked both randomly-oriented and aligned electrospun fiber sheets into *polysulfone* to form guidance conduits for nerve repair in a 17 mm rat sciatic nerve transection model. After 16 weeks in aligned fibrous constructs, the number of axons per cross-section was significantly higher than in conduits comprising of randomly-oriented fibers. In aligned fibrous conduits, the regenerated axons were myelinated and extended into the distal end. Behavioral assessment by grid walking test and electrophysiology test were carried out after 16 weeks to measure functional recovery. When provided the same level of stimulation, similar compound action potentials (CAP) and muscle contractions were observed in regenerated nerves treated with autografts and aligned constructs. However, in animals where the nerves are bridged by random constructs, no CAP was detected. Grid walking tests demonstrated that in animals that received autografts and aligned constructs, significantly fewer foot slips were recorded than in random construct- or saline filled polymeric construct-treated animals. These findings showed that aligned fibrous constructs had comparable performance in reducing the functional deficits after nerve injury as autografts. It is believed that contact guidance played an indispensable role in the regeneration process observed in the study. The stacked structure of the aligned fiber conduit was three-dimensional and therefore maximized the topographic directional cues.

Using an alternative approach of rolling up a sheet of aligned electrospun fibers in different directions, hollow nerve guide conduits were obtained by Chew *et al.* [44]. These nerve conduits comprised of hollow centers with aligned fibers oriented either longitudinally or circumferentially along the inner surface of the conduits. After 3 months of implantation to bridge

a 15 mm gap in the rat sciatic nerve, both fibrous conduits had higher numbers of myelinated axons and larger nerve cross-sectional area as compared to plain polymer tubes without electrospun fibers. Interestingly, electrospun fibers of both longitudinal and circumferential orientation resulted in similar extent of nerve regeneration and functional recovery. In this case, it seems that contact guidance was not the reason for promoting axon regeneration but rather the increased surface area for cell adhesion.

Although many *in vitro* studies have verified that aligned fibers are able to drastically enhance neurite outgrowth rate and direct axon extension, only few *in vivo* studies have actually been carried out to verify the usefulness of such contact guidance on nerve regeneration and functional recovery. Therefore, the concrete conclusion of aligned fibers in enhancing nerve repair *in vivo* by either contact guiding cues or enhanced cell attachment area cannot be derived until more research has been implemented.

2.5. CONCLUSIONS

Nerve regeneration is a complex process and a challenging field for researchers. From our limited knowledge of the nervous system to the achievement of partial neural functional recovery, impressive progress has been made during the past decades. However, much remains unexplored and the journey to accomplishing the final goal of fully regenerating functional nervous systems continues.

While topographical features from sub-micron- and nano-fibers, aligned or randomly oriented, clearly affect neural cell morphology and function, detailed in-depth studies are still required. In particular, our current

understanding of the effects of fibrous architecture on cellular function is still limited to the evaluation of cell morphological and viability changes. Detailed mechanistic evaluation of cellular functions such as Schwann cell myelination, glial scar formation, neuron synapse formation, neural cell signal transduction and neural stem cell differentiation are required to facilitate the precise control of cellular functions by nanofibrous topographical signals. The size effect of topographical features on cellular function is also unknown. The ideal size of nanofibers that should be coupled with the cell type of interest remains to be elucidated and such may only be found when other parameters such as material chemistry are decoupled from the experimentations. Finally, in terms of *in vivo* studies, minimization of the variability in test methods used by different groups will also enable more fruitful comparisons between experimental outcomes.

Nanofibrous scaffolds can serve as powerful tools in the tissue regeneration processes. The fibrous structure closely resembles the natural environment that cells grow in and provides appropriate physical cues for manipulating cellular functions. Electrospinning is a versatile and economical way of mass-producing fibrous constructs. It also enables incorporation of biochemicals to provide a synergistic effect in promoting nerve regeneration. Altogether, such biofunctional nanofibrous scaffolds hold great potential as direct implantable devices and as basic neural cell biological study platforms that may be necessary for neural tissue engineering.

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CHAPTER 3

ELECTROSPINNING OF BIOCOMPATIBLE POLYMERS AND INVESTIGATION OF TWO DRUG LOADING METHODS FOR BIOFUNCTIONAL SCAFFOLDS

SUMMARY

A variety of different polymers can be easily fabricated into fibrous morphology via the electrospinning technique, providing fibers at micro- and nano-scales for various applications. Polycaprolactone (PCL) is a biocompatible and biodegradable polymer which is widely used in tissue engineered scaffolds. In this study, PCL was chosen as a model polymer to understand electrospinning process. The relationship of electrospinning parameters and the property of the resulting fibers were investigated and discussed in this chapter. A comprehensive list of parameters was analyzed, including operational conditions, intrinsic property of polymer solution and the setup of collection devices. In addition, the methods and processes of fabricating bio-functional scaffolds based on PCL were explored and discussed. We investigated different approaches for incorporating drugs, such as proteins and siRNA, into electrospun fibers, including uni- and co-axial electrospinning. The properties of the PCL electrospun fibers could also be modified by forming core-shell structures with other biopolymers.

3.1. INTRODUCTION

Electrospinning is a versatile and simple method producing fibers with diameters ranging from several nanometers to micro-size. A broad repertoire of synthetic polymer and natural materials can be utilized and processed into fibrous structure by the electrospinning method. The similarity of the electrospun nanofibrous structure to that of the natural extracellular matrix (ECM) makes electrospun fibers a promising scaffold for applications in tissue engineering and regenerative medicine. Polycaprolactone (PCL) is one of the most commonly used polymers in the polyester family for tissue engineering purposes. The remarkable properties of PCL, including non-toxic biocompatibility and slow degradation rate [1], are exceedingly suitable for long-term applications such as implantation and drug delivery [2]. Additionally, the properties of PCL can be tailored for specific purposes by modifications such as copolymerization. For instance, a copolymer poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP), which is used in a following chapter, was synthesized to attain improved hydrophilicity and degradability for desirable drug-delivery purpose [3].

In addition to providing fibrous and porous topographic surface mimicking native ECM, electrospinning also allows drug delivery to present favorable chemical signals for regenerative purposes. Biomolecules ranging from antibiotics, growth factors, DNA and anticancer drugs have been reported to be successfully loaded into electrospun fibrous scaffolds and released to achieve considerable therapeutic effect [4]. Several distinct loading methods are attempted via conventional or modified electrospinning setup, including physical adsorption [5], chemical immobilization [6-7], encapsulation [3] and

co-axial delivery [8]. In particular, core-shell fibrous structure can be produced by a novel design using co-axial electrospinning. The sheath and core solutions are designed to be dispensed homocentrically under the same electrostatic field, forming core-shell fiber structures using different materials [9-10]. This core-shell fibrous structure is of special interest for drug delivery purposes. The shell solution may consist of a polymer solution in organic solvent system, while the core solution is usually in a milder condition of the aqueous phase to preserve the bioactivity of the embedded biomolecules. Furthermore, this fabrication system reduces phase separation between the polymer and drug solutions, which usually occurs in the uni-axial electrospinning, facilitating even drug distribution and eliminating burst release problem. However, the combinations of the core and shell solutions must be wisely chosen and optimized for a stable spinning process, concentric core-shell structures and desirable drug-release kinetics [11]. Compared to complicated optimizations involved in co-axial electrospinning, a simple alternative for fabrication of drug-loaded fibers is using drug-polymer mixture solution as a blend by uni-axial electrospinning [12]. This approach provides an easy and straightforward way for fabrication of bio-functional scaffolds. Despite of the controversy over the loss of bioactivity, a variety of biomolecules were successfully incorporated into electrospun fibrous scaffolds with satisfying functionality [3, 13]. Moreover, sustained drug release can be achieved with appropriate polymer selection or polymer modification.

Small interfering RNA (siRNA) is an attractive tool in decreasing the expression of target genes. The use of siRNA becomes an effective platform to promote and direct the growth of functional tissues [14-15]. However,

successful application of siRNA faces several challenges, including low cellular uptake, bio-instability and RNase degradation [16]. Compared to viral vectors, non-viral delivery of siRNA in the form of particulate polymer- or lipid-siRNA complexes are advantageous in less immunogenic concerns, improved gene packaging ability and amenable formulation for specific applications such as prolonged circulation, storage stability and targeted delivery [17]. Polyethylenimine (PEI) is recognized as a potent non-viral vector for gene delivery both *in vitro* and *in vivo* [18]. Non-covalent bonds, which are formed between the cationic amine group on PEI and the negatively charged group on nucleic acid, enable the formation of polymer-nucleic acid particles at nano-scales. These nanoparticles are then taken up by cells through endocytosis and efficiently regulate gene expression via ‘proton-sponge effect’ [19]. Higher siRNA silencing efficiency can be achieved in the form of PEI/siRNA nanoparticles *in vitro* [20]. Furthermore, siRNA is protected from nucleolytic degradation even in the presence of RNase *in vitro* or serum nucleases *in vivo* upon PEI complexation [21]. By encapsulating PEI/siRNA nanoparticles into electrospun fibers, it is possible to fabricate bio-functional scaffolds with gene therapeutic effect for long-term applications. The fibrous scaffolds serve as a PEI/siRNA reservoir and release the nanoparticles in a sustained manner, avoiding the needs for repeated administration. Meanwhile, scaffold-mediated transfection facilitates local availability of PEI/siRNA complex to adherent cells on scaffolds, which may also promote the cellular uptake and silencing efficiency [22].

In this chapter, the electrospinning parameters affecting the property of the resultant electrospun PCL fiber were investigated. The fabrications of bio-

functional scaffolds, including drug-loading scaffolds and core-shell fibers, were also studied and discussed.

3.2. MATERIALS AND METHODS

3.2.1. Understanding the electrospun parameters and the effects on electrospun fiber property

Concentration effect was studied by dissolving PCL (Mw: 65,000, Aldrich, USA) in a solvent mixture of dichloromethane (DCM) (VWR, EC): acetone (VWR, EC) (volume ratio: 4:1) at 8 wt%, 10 wt% and 12 wt%. Water-soluble polymer polyethylene glycol (PEG) (Mw: 3350, Sigma, USA) was added as a porogen at a concentration of 20 mg/mL with respect to PCL solutions in the aim of facilitating drug release in the following experiments. Steady spinning processes were obtained with a voltage of 5-6 kV (GAMMA high voltage research, US) and a needle-collector distance of 8-10 cm, with a constant polymer flow rate at 2 mL/h controlled by syringe pumps (New Era pump systems Inc., US).

Solvent mixtures of different combinations of chloroform (Sigma-Aldrich, USA) and methanol (Panceac, EU) were used to investigate solvent effect. Twelve wt% of PCL with 20 mg/mL PEG was dissolved in chloroform: methanol mixtures at 6:4, 8:2 and 9:1 (volume ratio). This polymer concentration was pre-optimized to fabricate electrospun fibers with uniform diameter. Electrospinning was performed with high voltage of 6.5-8.5 kV with a constant dispense rate at 1.6 mL/h. The distance between needle and the collector was 7-8 cm.

The flow rate effect was evaluated using 12 wt% of PCL in chloroform: methanol solvent system mixed at 9:1 ratio. The flow rate was set from 0.6 mL/h to 3.0 mL/h, while other operational parameters remained unchanged to ensure identical fabrication condition.

Fiber orientation can be manipulated by varying the rotation speed using a rotating disk as the collecting device. To evaluate the relationship between fiber alignment and the rotational speed, electrospun fibers were collected on rotating disk at speed of 0 rpm, 1000 rpm and 2000 rpm using 12 wt% PCL in 4:1 DCM: methanol. The voltage applied on the polymer solution was 5-6 kV with a distance of 8-10 cm. The polymer dispense rate was kept constant at 2 mL/h.

PCL fiber morphology was evaluated under scanning electron microscope (SEM) (JOEL, JSM-6390LA, Japan). Fiber diameter was determined by averaging 100 fiber diameters measured using ImageJ (NIH) based on SEM images and was represented as mean $\pm$ standard error (SE) of the mean. Fast Fourier transform (FFT) was used to assess PCL fiber alignment obtained at various rotation speeds. Briefly, SEM images were firstly transformed into FFT output images using ImageJ to show the distribution of pixel frequency. Then graphic depictions were produced by laying a circular projection of FFT images. Radial summation of pixel intensity was thereafter quantified using 'Oval Profile' plugin from 0-360° within the circular projection. FFT data were normalized to the baseline value as zero and the values are in calculated in arbitrary units. Due to the symmetry of FFT, identical distribution of pixel intensity was shown in the range of 0-180° and

180-360 °. Therefore the output of FFT graphic result was plotted in the range of 0-180 °.

3.2.2. Fabrication of bio-functional electrospun fibers with core-shell structure

3.2.2.1. PCL/BSA core-shell electrospun fibers

A simple and straightforward setup for co-axial electrospinning was designed to produce core-shell electrospun fibers, as shown in Figure 3.1A. Shell and core solutions were individually loaded into outer and inner syringes composed of a 5 mL- and 1 mL-syringe. The flow rates of two solutions were controlled separately by two syringes pumps. Needles of 20G and 30G were placed concentrically for shell and core solutions, which connected to outer and inner syringes respectively. Under the electrostatic field, core-shell electrospun fibers were generated and deposited on the grounded collector.

Fig. 3.1. Configuration of a simple coaxial electrospinning setup designed for co-axial electrospinning to produce core-shell fibers. A: Components of the co-axial syringes. B: Schematic drawing of co-axial electrospinning setup.

PCL was dissolved in DCM: methanol solvent mixture (3:2, v/v) at PCL concentration of 12 wt%. Five milliliter of PCL polymer solution was loaded into outer syringe as the shell solution. Bovine serum albumin (BSA) (Sigma) was used in this work as a model protein to investigate drug delivery in the core solution. Other electrospinning components were the same as that of uni-axial electrospinning setup as illustrated in Fig. 3.1B. The combinations of inner and outer flow rates were studied to attain stable spinning process. Optimized flow rates were found at 1-3 mL/h and 0.2 mL/h for outer and inner solutions, respectively. Other operational parameters, including voltage, distance between the needle and the collector, were adjusted in accordance to flow rate settings. Ten wt% of BSA dissolved in PBS was loaded into inner syringe as the core solution for the investigation of drug delivery purpose. To visualize the BSA encapsulation along PCL fibers, 6 mg of FITC-BSA (Sigma, US) was mixed into 1 mL of 10 wt% BSA solution in PBS. Fluorescent fibers were deposited on glass slide and fibers were thoroughly rinsed with PBS to remove the un-encapsulated FITC-BSA. The distribution of FITC-BSA along the fibers was observed under fluorescent microscope (Zeiss, Axiocert 200M, Germany). Core-shell fiber morphology was evaluated under SEM by collecting fibers on stationary Al foil.

3.2.2.2. PCL/gelatin core-shell electrospun fibers

An improved design of co-axial electrospinning setup is illustrated in Fig 3.2. This configuration separated inner and outer syringes. This eliminated the problem of frictions generated between two syringes, which usually cause imprecise control of flow rates occurred in the former design (Fig. 3.1A). The

shell solution was dispensed into a customer-designed cylindrical chamber via a Teflon tube. The inner solution came out through a 22G needle which was placed homocentrically to the cylindrical chamber containing the shell solution. The chamber nozzle was charged with high voltage and the core-shell electrospun fibers were then produced under the same electrostatic field.

Fig. 3.2. Improved design of coaxial electrospinning setup for core-shell structured electrospun fibers.

Gelatin (Bovine type B, Sigma, US) is a natural biomaterial and showed improved biocompatibility for tissue engineering applications in comparison with synthetic biopolymers. It is found to support cell adhesion and growth onto tissue engineered scaffolds [23]. PCL/gelatin core-shell fibers may present novel properties for specific applications. Gelatin as the shell fiber may enhance biocompatibility for cell adhesion and proliferation while PCL as the core fiber may improve the mechanical strength of the scaffolds. To fabricate PCL/gelatin core-shell fibers, 10 wt% of gelatin dissolved in 2,2,2-trifluoroethanol (TFE) (Fluka, China) was used to sheath 16 wt% of PCL

(Mn=80,000, Sigma, US) in TFE. The flow rates of outer and inner solutions were pre-optimized and set at 2 mL/h and 1 mL/h respectively. Electrospinning process was performed under a voltage of +13kV/-4.5kV between the polymer solutions and the stationary collector, with a nozzle-collector distance of 12 cm. The surface morphology of the core-shell fibers were observed under SEM. To examine the cross-section of core-shell fibers, fiber mats were first rolled into cylindrical tubes and immersed in liquid nitrogen for 1 min. Then the fiber tubes were fractured with a sharp blade to expose the cross-sections of the core-shell fibers. The fractured specimen was air dried and then examined under field emission scanning electron microscope (FESEM) (JEOL JSM-6700F, Japan).

3.2.3. Fabrication of bio-functional scaffolds by delivering PEI/siRNA nanoparticles in electrospun fibers

3.2.3.1. *Fabrication of PEI/siRNA-encapsulated electrospun fibers*

Branched PEI (Aldrich, Germany) with molecular weight of 25 kDa was used as a model non-viral gene delivery polymer in the present work. Negative control siRNA (Bioneer, Korea) was used for the optimization of loading level investigation and the control release study. The negative control siRNA was designed with a scrambled sequence as follows: sense: CCU ACG CCA CCA AUU UCG U (dTdT); antisense: ACG AAA UUG GUG GCG UAG G (dTdT). Briefly, 0.5 nmol of negative control siRNA was diluted in 5% glucose (Sigma) solution buffered with HEPES buffer (Hyclone) to a final concentration of 60 µg/mL and incubated at room temperature (RT) for 10 min. After that, 20 µL of 10 mM PEI solution was mixed with siRNA solution to reach a N/P ratio of 10

for effective complexation of PEI/siRNA nanoparticles [18] (1 μ g of DNA contains 3 nmol of phosphate and 1 μ L of 10 mM PEI solution is 10 nmol of nitrogen). The PEI/siRNA solution was vortexed and incubated for 10 min at RT, allowing PEI and siRNA to fully interact for particle formation. Double-stranded DNA oligos (dsDNA) (Research Biolabs, Singapore) with the same sequence as the negative control siRNA were used as a substitute of siRNA for characterizations of PEI/siRNA nanoparticles, including determinations of particle size and ζ -potential. FAM-labeled DNA oligonucleotides (FAM-ODN) (Research Biolabs, Singapore) with the same sequences as the negative control siRNA were synthesized as a substitute of siRNA for the visualization of siRNA distribution inside PCL fibers. PEI/dsDNA or PEI/FAM-ODN particles were prepared following the same procedure as described above.

PCL was dissolved in TFE to obtain a 14 wt% polymer solution. Porogen PEG was added into PCL solution of 0 mg/mL (PCL), 10 mg/mL (PCL-PEG10) and 20 mg/mL (PCL-PEG20) with respect to PCL polymer solution, aiming to investigate proper PEG concentration for desired PEI/siRNA release. To prepare PEI/siRNA-PCL solutions, 150 μ L of fresh PEI/siRNA was added into 0.5 mL of PCL polymer solution with certain amount of PEG. The mixture was vortexed and sonicated to obtain uniform emulsions. After that, the mixture solution was loaded into a syringe and dispensed out at a flow rate of 1 mL/h. The voltage applied on the polymer solution and the collector was +15-20 kV and -2-5kV, respectively. PEI/siRNA-encapsulated PCL scaffolds were stored at -20 $^{\circ}$ C until use.

3.2.3.2. Characterization of PEI/siRNA-encapsulated PCL electrospun fibers

Particle size and ζ -potential of the nanoparticles are important parameters affecting the efficiency of gene transfection [24-26]. Therefore, we studied particle size and ζ -potential of the PEI/siRNA nanoparticles using ZetaPALS (Brookhaven instruments, USA).

Fiber morphology was examined under SEM and fiber diameter was measured using ImageJ as described above. FAM-ODN was used for fluorescent visualization of the nanoparticle distribution inside the electrospun fibers. PEI/FAM-ODN-encapsulated fibers were collected on glass slides and then images were acquired under the fluorescent microscope.

3.2.3.3. *Release kinetics of PEI/siRNA nanoparticles from PCL nanofibers*

PEI/siRNA-encapsulated scaffolds were immersed in 3 mL of PBS (pH 7.2, GIBCO) in a 24-well cell culture plate. Sterile Teflon rings were placed on top of the scaffold to ensure the entire scaffold was well covered in PBS. The culture plate was placed in a shaker at 37 °C (Sartorius Certomat[®], Germany) during the controlled release study period of 180 hours. At certain time points, 1 mL of the supernatant was taken out and another fresh 1 mL of PBS was replenished. The supernatants were stored at -20 °C until use. To quantify siRNA concentration from PEI/siRNA nanoparticles, 1 μ g of dextran sulfate (DS) (Mw: 500kDa, Sigma, USA) was added into 300 μ L of PEI/siRNA supernatant to release siRNA from the complexes, based on preliminary studies for a complete displacement of siRNA. siRNA concentrations from the supernatants were determined by RiboGreen reagent (Quanti-IT[™] RiboGreen[®], Invitrogen, US) and the fluorescent intensity was measured using a microplate reader (Tecan[®], Infinite 200, Austria).

3.2.3.4. *The integrity of siRNA in the electrospun fibers*

In order to test the feasibility of delivering PEI/siRNA nanoparticles by electrospun fibers, siRNA was extracted from PEI/siRNA-encapsulated scaffolds immediately after fabrication and the integrity of the extracted siRNA was examined in a 2.5% agarose gel with ethidium bromide. In brief, PEI/siRNA-loaded scaffolds were dissolved in 1 mL of chloroform (Biomolecular grade, Sigma) and siRNA was then extracted 5 times by a total of 1 mL of DEPC-treated water (1st Base, Singapore). Following that, 1 μ g of DS was added into 15 μ L of the extraction solution to release siRNA from the nanoparticles for electrophoresis. Band positions of the encapsulated siRNA on gels were compared with fresh naked siRNA, which was used as the positive control, to examine the siRNA integrity.

In addition, we also evaluated siRNA integrity after the release study at 180 h. At the end of release study, PEI/siRNA-encapsulated scaffolds were washed in PBS and then dried under vacuum. The extraction procedures were carried out as described above. The siRNA extraction solution was concentrated for electrophoresis study using a lyophilizer (Alpha 1-2 LD Freeze dryer, Christ, Germany). The lyophilized powders were reconstituted in 150 μ L of DEPC-treated TE buffer (pH 8.0, 1st Base, Singapore). One microgram of DS was added to 15 μ L of the concentrated siRNA extraction solution to release siRNA from the complex. Samples were then loaded to a 2.5% agarose gel for the evaluation of siRNA degradation after release study of 180 h.

3.3. RESULTS

3.3.1. Understanding the electrospun parameters and the effects on electrospun fiber

Fiber morphological change with respect to polymer concentration is illustrated in Fig. 3.3. Bead-thread morphology was observed at low PCL concentration of 8 wt%. The beaded fibers disappeared and smooth fibers became dominant with increased polymer concentration. When PCL concentration reached 10 wt% and 12 wt%, beaded structures were completely removed and continuous PCL fibers were obtained under the similar operational parameters. The resulting diameters of 10 wt% and 12 wt% of PCL fibers were $1.40 \pm 0.02 \mu\text{m}$ and $1.73 \pm 0.04 \mu\text{m}$, respectively. It is observed that above the threshold concentration to obtain smooth and continuous fibers without bead formation, fiber diameter will increase with elevation in polymer concentration.

Fig. 3.3. SEM images show the concentration effect on PCL fiber morphology. The concentrations under evaluation were 8 wt% (A), 10 wt % (B) and 12 wt% (C) of PCL in 4:1 DCM: acetone. Scale bar = 20 μm .

The solvent effect is shown in Fig. 3.4. PCL electrospun fibers exhibited wide diameter distribution in 6:4 chloroform: methanol solvent mixtures while uniform fibers were obtained in 8:2 and 9:1 chloroform: methanol mixtures. The increasing portion of chloroform in the solvent mixture not only narrowed fiber diameter distributions (Fig. 3.4D), but also increased the diameters of the

resulting fiber. The average fiber diameters for PCL in 6:4, 8:2 and 9:1 solvent mixtures were $1.18 \pm 0.05 \mu\text{m}$, $2.42 \pm 0.03 \mu\text{m}$ and $2.53 \pm 0.03 \mu\text{m}$, respectively.

Fig. 3.4. SEM images show solvent effect on the electrospun the fiber morphology. PCL electrospun fibers were obtained from 12 wt% PCL solutions in chloroform: methanol mixtures at volume ratios of 6:4 (A), 8:2 (B) and 9:1 (C). Scale bar in A,B,C = 20 μm . D: Fiber diameter distributions of 12 wt% PCL in three different solvent mixtures.

The flow rate effect using of 12% PCL polymer solution in 8:2 chloroform: methanol is shown in Fig 3.5. The average diameters of fibers prepared at 0.6, 1.6 and 3.0 mL/h were $1.83 \pm 0.02 \mu\text{m}$, $2.42 \pm 0.03 \mu\text{m}$ and $2.33 \pm 0.06 \mu\text{m}$, respectively. Small fibers with narrow diameter distribution were yielded at low flow rate. Increment of the flow rate to 1.6 mL/h led to large fiber formation. However further increase in the flow rate to 3.0 mL/h resulted in wider diameter distribution ranging from 0.5 to 4 μm , as shown in Fig 3.5D.

Fig. 3.5. SEM images show 12 wt% PCL in 8:2 chloroform: methanol mixture at flow rate of 0.6 mL/h (A), 1.6 mL/h (B) and 3.0 mL/h (C). Scale bar in A,B,C = 20 μm. D: Fiber diameter distributions of 12 wt% PCL in 8:2 chloroform: methanol at different polymer flow rates.

The comparison of PCL fiber orientation in response to disk rotation speed is demonstrated in Fig. 3.6. Fiber alignment was described as the height and the shape of the peak generated in the FFT plot. Higher and narrower peak indicates more aligned fibers along the single axis of orientation. At 0 rpm, the fibers exhibited random orientation on the collector as shown in SEM image (Fig. 3.6A), and the normalized intensity value below 0.06 arbitrary units indicated even distribution from 0-180°. When the rotation speed was applied, the fiber alignment was observed from the SEM images (Fig. 3.6B,C), however in various degrees with respect to the speed employed. At 1000 and 2000 rpm, the plots of intensity distributions formed sharp peaks, indicating more fibers

were oriented along the same axis. The maximal values of normalized intensity from FFT plots were 0.23 and 0.31 arbitrary units for 1000 and 2000 rpm, respectively. The curves of fibers collected at high rotation speed exhibited narrower distribution compared to lower speed.

Fig. 3.6. The morphology of 12 wt% PCL in 4:1 DCM: methanol collected at rotational speed of 0 rpm (A), 1000 rpm (B) and 2000 rpm (C). Scale bar in A,B,C = 200 μm . D. FFT analysis of PCL electrospun fibers collected at different rotation speeds.

3.3.2. Fabrication of core-shell fibers

3.3.2.1. PCL/BSA core-shell electrospun fibers

The core-shell PCL/BSA fibers were shown in Fig. 3.7. PCL/BSA fibers exhibited large fiber diameter variations ranging from 200 nm to more than 2 μm (Fig. 3.7D), due to the instability of the co-axial electrospinning process. The difference in physical and chemical properties of core and shell solutions resulted in the unstable spinning jet. BSA was in aqueous phase and was not able to form fibrous structure alone. Moreover, FITC-BSA residues on the glass

slides were washed off by PBS. Therefore, the co-localization of the fluorescent image of BSA fibers and the optical image of PCL fibers indicated core-shell structure of PCL/BSA fibers (Fig. 3.7A,B,C).

Fig. 3.7. Characterization of PCL/FITC-BSA core-shell electrospun fibers. A: Fluorescent microscopy image of PCL/FITC-BSA fibers; B: Corresponding optical images of PCL/FITC-BSA fibers; C: Merged PCL/FITC-BSA image; D: Morphology of PCL/FITC-BSA core-shell fibers under SEM. Scale bar = 20 μm in (A,B,C) and 4 μm in D.

3.3.2.2. *PCL/gelatin core-shell electrospun fibers*

Uniform polymer fibers were obtained for both PCL and gelatin dissolved in TFE solvent, as shown in Fig. 3.8A,B. The fiber diameters of plain gelatin and PCL fibers were 1248 ± 17 nm and 383 ± 5 nm respectively. Co-axial electrospinning were carried out under the same operating parameters as plain fiber fabrication. As shown in Fig. 3.8C, uniform core-shell fibers were obtained with an average fiber diameter of 1041 ± 23 nm, which was comparable to that of gelatin fibers. To assess the core-shell structure of PCL/gelatin fibers, cross-section images were acquired under FESEM. A concentric representation of the core-shell structure of PCL-gelatin fibers was seen, as shown in Fig. 3.8D.

Fig. 3.8. The morphology of electrospun fibers of A: 10% gelatin in TFE; B: 16%PCL in TFE-PBS mixture; C: PCL-gelatin co-axial fibers. D and E: Cross-section images of core-shell PCL-gelatin fibers after freeze fracture at low (D) and high (E) magnifications. Core-shell structure in image E was indicated by black arrows. Scale bar = 5 μm in (A,B,C) and 2 μm in (D,E).

3.3.3. Delivery of PEI/siRNA nanoparticles by electrospun fibers

3.3.3.1. Characterization of PEI/siRNA-encapsulated nanofibers

The size of PEI/siRNA nanoparticles formed in DEPC-treated water was measured and an average particle size of 306.9 ± 19.1 nm was obtained. The ζ -potential of the resulting solution containing PEI/siRNA nanoparticles was 17.11 mV. Uniform and continuous electrospun fibers were obtained, as shown in Fig. 3.9. The average fiber diameters were of 692 ± 11 nm, 634 ± 13 nm and 846 ± 29 nm for PCL, PCL-PEG10 and PCL-PEG20, respectively. No obvious trend was observed in the fiber diameter with respect to PEG concentrations. However, as the PEG concentration increased in the polymer solution, the electrospinning jets became less stable, which may contribute to a wider fiber diameter distribution as observed.

Fig. 3.9. SEM images show the morphology of PEI/siRNA-encapsulated electrospun fibers at low (upper panel) and high (lower panel) magnifications. A,B: PCL; C,D: PCL-PEG10; E,F: PCL-PEG20. Scale bar = 10 μm in (A,C,E) and 2 μm in (B,D,F).

Fig. 3.10 demonstrated the distribution of the PEI/FAM-ODN particles within PCL fibers. PEI/FAM-ODN particles were present as punctate dots, which were sparsely distributed along PCL fibers. This may be due to low loading level of FAM-ODN compared to PCL amount. We chose this low amount of PEI/FAM-ODN compared to PCL solution because higher concentration of ODN/siRNA caused particle agglomeration during the preparation processes of PEI/siRNA-PCL polymer solutions. The aggregated nanoparticles had larger particle size which may exceed the diameter of the fibers, causing the spinning process extremely unstable to produce fibers with uniform diameters.

Fig. 3.10. Distribution of PEI/FAM-ODN particles in PCL electrospun fibers. A: Optical image of PCL fibers. B: Corresponding fluorescent image indicating FAM-ODN distribution. Scale bar = 20 μm .

3.3.3.2. *PEI/siRNA release kinetics from PCL and PCL-PEG electrospun fibers*

The release profile of PEI/siRNA nanoparticles from PCL and PCL-PEG nanofibers within 180 h was plotted in Fig. 3.11. The PEI/siRNA nanoparticles were released from the polymer fibers within 180 h in a sustained manner. The cumulative amounts of siRNA released from electrospun fibers at the end of release study (180 h) were 63.4 ng, 57.9 ng and 53.0 ng from PCL, PCL-PEG10 and PCL-PEG20 scaffolds respectively. However, we noticed that the total release within 180 h was relatively low as compared to the original loading level of 0.5 μmol . The released siRNA was less than 1% of the original loading amount. It is likely that the majority of the PEI/siRNA still remained entrapped within the scaffolds. Although PEG was added as a porogen to enhance siRNA release, no significant increase in siRNA release rate was observed with higher concentration of PEG in PCL fibers. A similar trend of siRNA release was shown for three types of PCL/PCL-PEG nanofibers. This result was opposite to our preliminary studies using DNA oligos, where higher

amount of PEI/DNA was released with elevated PEG concentrations (data not shown). This discrepancy may stem from the low siRNA concentration in the supernatant and the limited detection ability of RiboGreen reagent, in comparison with PicoGreen reagent which was used for DNA detection. Additionally, the presence of PEG seemed to interfere with RiboGreen detection while no interference was seen when working with PicoGreen reagent (data not shown). Nonetheless, the results suggest the feasibility of sustained release of PEI/siRNA nanoparticles from the electrospun nanofibrous scaffolds.

Fig. 3.11. Release profile of PEI/siRNA nanoparticles from PCL, PCL-PEG10 and PCL-PEG20 electrospun fibers within 180 h.

3.3.3.3. The siRNA integrity study

To evaluate the feasibility of siRNA delivery by electrospun nanofibers, the siRNA integrity was examined by extracting siRNA encapsulated in the nanofibers immediately after fabrication. As shown in Fig. 3.12A, siRNA extracted from newly prepared electrospun nanofibers showed the same band

position as the fresh naked siRNA, indicating the siRNA integrity was preserved inside the nanofibers. This result suggested that siRNA in the form of PEI/siRNA complex was able to stand the harsh conditions during the electrospinning process and the electrospun nanofibers may serve as a potential PEI/siRNA delivery system.

siRNA integrity was also examined at the end of the release study of 180 h (Fig. 3.12B). siRNA bands were shown in the RNA gel from PEI/siRNA extractions in all scaffolds. Compared to the positive control, there seemed to be a slight retardation of PEI/siRNA extracted from scaffolds after the release study probably due to incomplete dissociation of PEI/siRNA complex. Stronger band intensities were observed in siRNA extracted from PCL-PEG10 and PCL-PEG20. This was due to higher extraction efficiency of siRNA from PEG-containing PCL fibers. Therefore, the cumulative siRNA released was calibrated accordingly. Nevertheless, this result suggests that siRNA remained intact within the electrospun fibers after release study of 180 h. The siRNAs inside electrospun fibers was likely to be physically protected by the surrounding polymers.

Fig. 3.12. siRNA integrity study on 2.5% RNA agarose gel. A: PEI/siRNA extracted a from as-spun siRNA-encapsulated scaffolds. Lane 1: dsRNA ladder; lane 2: positive control (naked siRNA); lane 3: PEI/siRNA extraction. B: PEI/siRNA extracted from scaffolds after controlled release of 180 h. Lane 1: dsRNA ladder; lane 2: positive control (naked siRNA); lane 3: PEI/siRNA extracted from 14 wt% PCL; lane 4: PEI/siRNA extracted from 14 wt%PCL-PEG10; lane 5: PEI/siRNA extracted from 14%PCL-PEG20.

3.4. DISCUSSION

3.4.1. Electrospinning process and the properties of the resulting fibers

Electrospinning is a versatile technique to produce fibers under the control of electrostatic forces. This flexible technique has been widely used for various applications, such as filtration, wound dressing and material reinforcement [27-28]. In particular, the use of electrospun fibers as tissue engineered scaffolds attracts increasing attentions for regenerative applications [4, 29]. The fabrication of electrospun fibers is a complex process which is influenced by a comprehensive list of variables, such as polymer concentration, flow rate, solvent, etc. These parameters may also interact with each other and further complicated our understanding of the electrospinning process. Nevertheless, some general trends can be observed and summarized based on detailed investigations [30]. In our work, PCL was used as a model polymer to

investigate the relationship between the operating parameters and the electrospun fiber properties.

Polymer concentration affects the fiber formation and the morphology of the resultant fibers. During the electrospinning process, it is believed that the surface tension, electrical forces, and viscosity of the polymer solution are playing crucial roles in influencing fiber morphology. Changes in polymer concentration may alter solution viscosity which affects the electrospinning product. It is reported that the ideal viscosity for electrospinning is within 800 to 4000 cP [31]. Below 800 cP, polymer jets tend to break into droplets. As observed for 8 wt% of PCL solution in 4:1 DCM: acetone, surface tension played the leading role and droplets were therefore formed to reduce the surface area. In contrast, when the viscosity is higher than 4000 cP, the applied electrical force is unable to overcome surface tension of the viscous solution. At a threshold concentration within the acceptable range, beads can be eliminated and continuous fibers are produced with smooth surface. In our case, the increase of PCL concentration to 10 wt% led to an elevated viscosity where continuous fibers with smooth surfaces were obtained. Further increase in PCL concentration to 12 wt% resulted in large fiber diameters due to higher viscosity [30].

Another critical determinant of fiber morphology is the solvents system. Fiber morphology differs even when the same polymer was used but in distinct solvent systems. Solvent volatility shares the responsibility for the difference observed in fiber morphology by influencing the phase separation process [32]. In our studies, the volume ratio of chloroform and methanol affects the fiber

morphology and the presence of methanol in the polymer solution resulted in smaller fibers but lead to wider diameter distribution.

The flow rate also exerts impact on fiber size and scaffold structure [33-34]. Thin and uniform fibers were obtained at low flow rate while fibers with larger diameter and wider diameter range were fabricated under higher flow rate. However, low solution dispensing rate usually leads to inefficiency in the fiber production process compared to higher polymer flow rate. Further increase in flow rate may also cause instability during the electrospinning process and the resultant fibers may show wider fiber diameter distribution.

In terms of regenerative medicine, scaffold topography is an indispensable variable in regulating cellular behaviors. For instance, micro-patterned substrate facilitates cell elongation and alignment along the grooves directed by the contact guidance, in comparisons with cells on two-dimensional flat surface [35-36]. Various setups have been designed to control fiber assembly and topography. In particular, the application of rotating target is one commonly used collecting device for fabricating aligned electrospun fibers [37]. Fiber alignment is dependent on the rotation speed. In our studies, PCL fibers were observed to exhibit certain degree of alignment at 1000 rpm, and highly aligned fibers were obtained at rotation speed of 2000 rpm. At low speed of 500 rpm or below, fibers were deposited in a random orientation.

3.4.2. Fabrication of core-shell electrospun fibers

The fabrication of core-shell fibers shows diversified potentials in various applications, such as producing micro/nanotubes [38-39], drug-loaded fibers [40-41], and hybrid nanofibrous materials [42-43]. Fibers with core-shell

structure have many outstanding advantages [44]. For instance, by constructing two materials into one composite, the mechanical properties can be improved for reinforcement purposes. In drug delivery system, unstable materials such as proteins and nucleic acid can be loaded as the core, which are physically insulated from harsh environmental conditions by the protective shell, to minimize bioactivity loss. Moreover, it may also serve as a dual-functional scaffold which combines the advantageous merits of the core and shell components for tissue engineering. In this study, we examined possible applications for drug-delivery (BSA-loaded fibers) and construction of biocompatible tissue engineered scaffolds (PCL/gelatin fibers).

In co-axial electrospinning, the core fibers are believed to be produced by the stress generated from the shell solution which causes shearing of the core solution by viscous dragging [45]. In this way, non-spinnable materials or solutions are able to be fabricated into fibrous structure when encapsulated inside the shell polymer by co-axial electrospinning. As the shell and core solutions experience the same operational parameters, the interaction and dissimilarity between two solutions may play an essential part in fiber fabrication, including solution viscosity, solution concentration, solution miscibility and incompatibility, solution conductivity and solvent volatility [44]. In our work of two types of core-shell fibers, the combination of PCL/gelatin solutions was a more compatible system for stable spinning compared to PCL/BSA system. The low viscosity of BSA in the aqueous solution resulted in frequent breakage of core solution, leading to the formation of droplets and discontinuous fibers [46].

Although two different designs were proposed for co-axial electrospinning setups in the present work, they are conceptually identical for core-shell fiber fabrication. The concentric configuration of inner and outer syringes required minimal amount of shell polymer which can save material cost, while separate syringe system reduced piston frictions which may lead to a more precise control over flow rate. The latter one is more commonly used for the fabrication of core-shell fibers using electrospinning technique. However, improvements should be focused on solving the large dead space to save material cost, especially in the case of using costly polymers such as collagen.

3.4.3. Delivery of PEI/siRNA nanoparticles by electrospun nanofibrous scaffolds

siRNA is an effective and important component in RNA interference pathways which utilizes the cellular machinery to target complementary mRNA, leading to potent silencing of target genes [16, 47]. siRNA has been extensively used in the studies of gene functions [48-49], pathway mechanisms [50-51] and diseases therapeutics [52-53]. Encouraging achievements are also achieved in tissue engineering [54-56]. However, the use of siRNA is facing several challenges such as bio-instability and transient efficiency [57]. In our study, PEI was chosen as a non-viral vector to transport siRNA into cells. The nanoparticles of PEI/siRNA have been reported to present high transfection efficacy as compared to other conventional non-viral vectors [18]. Moreover, the PEI in form of PEI/siRNA nanoparticles acts as a physical barrier to protect siRNA from RNase digestion during transfection [26]. By encapsulating PEI/siRNA nanoparticles into electrospun nanofibers, a biofunctional

nanofibrous scaffold, which may continuously release PEI/siRNA particles for long term gene therapeutic application, was produced.

The particle size of PEI/siRNA plays an important role in cellular uptake and transfection efficiency [17, 26], and it also affects scaffold fabrication process and the release kinetics in the present work. The particle size is controlled by several parameters, including the type of PEI, concentration of PEI and DNA/siRNA, ionic strength of the solvent, mixing kinetics, etc. [19, 58]. However due to limited knowledge in uptake pathways, the effect of particle size on transfection efficiency remained unclear. In the nanoscale regime, it is generally believed that larger particle size could achieve higher transfection efficiency. Kunath *et al.* reported that larger particles of 590 nm with low molecular weight achieved higher uptake efficiency than smaller size of 156 nm with high molecular weight in several cell lines tested [59]. Larger DNA carrying capacity, elevated sedimentation and charge-shielding may contribute to the enhanced transfection efficiency. It is also reported that large particles may traffick through a slow, non-degradative, caveolae-mediated pathway, which lead to higher transfection efficiency of the large nanoparticles [60]. The particle size of branched PEI with molecular weight of 25 kDa complexed with siRNA was 306.9 ± 19.1 nm, with a ζ -potential of 17.11 mV in our study. However, these particles formed were not stable and were prone to aggregation upon complexation, and this instability may affect the PEI/siRNA release from PCL nanofibers. Zeta-potential reflects the net charge at the particle surface of the particle and the value may differ with a series of variables such as type of PEI and the complexation protocol. Although a few works were carried out to understand the complexation process and the

underlying mechanisms, the controversial and obscure findings suggest that further and in-depth studies still need to be performed.

Liao *et al.* [61] suggested that the incorporation of PEG into PCL fibers resulted in porous structure on the surface of PCL electrospun fibers. Pores of 200 nm formed on the large diameter PCL fibers (2-3 μm) improved drug release. However, no obvious pore formation was observed on PCL nanofibers in our studies. In contrast, we found that PEG leached out of the scaffolds and lead to a rougher fiber surface. It is likely that PEI/siRNA nanoparticles were released along the dissolution of PEG into the medium. Comparing the particle size of PEI/siRNA and the fiber diameter, it was likely that PEI/siRNA nanoparticles were trapped in the fibers due to particle-fiber interaction and the low diffusive rate in the PCL polymer. Coupled with the low degradation rate of PCL, complete release of PEI/siRNA was not attainable within the duration of this study. Modification of the polymer can be one potential solution. Polymers with higher degradation rate may facilitate the siRNA release. The siRNA concentrations in the supernatant remained low due to the slow release rate. Especially at later time points, siRNA concentrations were less than 1 ng/mL, which were below the detection limit of the RiboGreen reagent. Moreover, the addition of dextran sulfate to release siRNA from PEI polyplex for RNA quantification seemed to interfere with RiboGreen detection. These might attribute to the discrepancies observed in the siRNA release profile (Fig 3.11). Nevertheless, the differences were within the error bars and the results suggested that sustained release could be obtained from the scaffold-based delivery system.

Integrity of siRNA was preserved in PCL nanofibers for at least 180 h under physiological conditions. The electrospinning process did not affect siRNA integrity in the form of PEI/siRNA nanoparticles. Similar findings have been reported by delivering DNA by electrospun fiber [13, 62-63]. siRNA stood the harsh environmental conditions during electrospinning process and the integrity was also retained during the controlled release study, probably owing to the protection of PEI and PCL polymer fibers. These results indicate that electrospun fibrous scaffold is a feasible and potential platform for siRNA delivery. In addition, the local availability of siRNA provided by electrospun nanofibrous scaffolds may enhance cellular uptake and minimize side effect to adjacent cells for *in vivo* applications.

3.5. CONCLUSIONS

The morphology of the electrospun polymer fibers is strongly influenced by the parameters used during the spinning process. Therefore, in order to fabricate fibers with desired properties, it is of great importance to search for the optimal electrospinning conditions whenever a new polymer is used. The alignment of polymer fibers can be obtained by choosing an appropriately designed electrospinning setup and these highly aligned fibers showed significant effect in modulating cellular behaviors. In addition to providing topographic cues to influence cellular behaviors, the electrospun fibrous scaffolds are also capable of delivering drugs for the purpose of regenerative medicine. A range of biomolecules can be easily loaded into electrospun fiber and slowly released for a long-term therapeutic effect. Taken altogether,

electrospinning technique offers an alternative and promising approach for constructing bio-functional tissue engineered scaffolds.

3.6. REFERENCES

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CHAPTER 4

RNA INTERFERENCE BY NANOFIBER-BASED siRNA DELIVERY SYSTEM

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SUMMARY

siRNA delivery has found useful applications particularly as therapeutic agents against genetic diseases. Currently, the delivery of siRNA typically takes the form of nanoparticles. In order to expand the applications of these potent but labile molecules for long-term use required by tissue engineering and regenerative medicine, alternative delivery vehicles are required. A scaffold-mediated approach to siRNA delivery was developed in this chapter. By encapsulating siRNA within polycaprolactone (PCL) nanofibers (300-400 nm in diameter), controlled release of intact siRNA could be achieved for at least 28 days under physiological conditions with desirable siRNA bioactivity. This work demonstrates the potential of nanofibrous scaffold-mediated siRNA delivery for long-term gene-silencing applications. The combination of topographical features provided by nanofibrous scaffolds may provide synergistic contact guidance and biochemical signals to mediate and support cellular development in regenerative medicine.

4.1. INTRODUCTION

Gene silencing via siRNA delivery has developed tremendously over recent years. Its rapid advancement has led to new avenues in biomedical applications ranging from understanding of gene functions [1] and cell signaling pathways [2-3] to the treatment of cancer [4-5], infectious diseases [6-7] and genetic disorders [8-9]. Perhaps less obvious is the immense potential of siRNA gene silencing techniques in the field of tissue engineering, regenerative medicine and stem cell engineering. Various possible applications of gene silencing technology in tissue engineering have been identified. HOXB13 expression regulation for enhancing wound healing and Nogo receptor expression down-regulation in the central nervous system for improving nerve regeneration are a few examples [10]. The list continues to expand with improved understanding of the field.

In applications like tissue engineering and stem cell engineering, long term supply of biomolecules is often required. Unfortunately, one of the main bottlenecks in siRNA technology is the ability to provide sustained availability of siRNA for long term applications [11]. Non-viral vectors have been developed to help siRNA cross cell membranes. These include lipids [12], cationic polymers [13], proteins [14], and conjugation of small molecules to siRNA [15-16]. Although relatively high transfection efficiency and desired cellular behaviors have been reported using these vectors, their associated transfection effect is often transient [17]. The silencing effect can last for weeks in non-dividing cells and only 3-7 days in dividing cells [18]. A sustained siRNA delivery system, particularly in the form of scaffold-based delivery,

therefore, may represent an attractive alternative to enable the expansion of siRNA technology to regenerative medicine.

Scaffold-mediated gene delivery or reverse transfection has been studied for DNA delivery. Thus far, the common platforms utilized are typically in the form of hydrogels [19] or porous scaffolding materials formed by gas forming or particle leaching techniques [20]. These approaches ensure the localized and prolonged availability of genetic materials to cells. Nanofibrous scaffolds, in contrast, represent a novel class of potent materials for such applications. By providing a good representation of the nanofibrous architecture of the natural extracellular matrix, biomimicking topographical signals are presented to seeded cells. Such added morphological features provide an extra dimension for better control over cellular functions. We have previously demonstrated the efficacy of electrospun fibrous scaffolds in enhancing Schwann cell maturation *in vitro* solely by topographical signals [21]. Coupled with growth factors, enhanced nerve regeneration over a critical defect gap in a rat model was realized [22]. In this study, an siRNA delivery system by electrospun nanofibers is developed. Although subjected to harsh processing conditions during electrospinning, we demonstrate that the bioactivity of siRNA was retained over a prolonged period of time. By encapsulating labile siRNA molecules into polymer fibers, the nanofibrous scaffold may serve as an siRNA reservoir for sustained release to attain long-term gene therapeutic effect while providing architectural signals to direct and support seeded cells. We demonstrate that such local availability of siRNA by scaffold-based delivery also enhanced gene silencing as compared to passive uptake of siRNA even in the absence of transfection reagent.

4.2. MATERIALS AND METHODS

4.2.1. Electrospinning

Polycaprolactone (PCL) (Mw: 65000, Aldrich, USA) was dissolved in 2, 2, 2-trifluoroethanol (TFE) (≥ 99.0 , Fluka, China) to obtain a 14 wt% polymer solution. Polyethylene glycol (PEG) (Mw: 3350, Sigma, USA) was added at concentrations of 0 mg/mL, 20 mg/mL (8.85wt% to PCL) and 60 mg/mL (26.5wt% to PCL) with respect to 14 wt% PCL solution as a porogen to control siRNA release rate. The fibers were denoted as PCL, PCL-PEG20 and PCL-PEG60 respectively. Negative control siRNA with a scrambled sequence (sense: 5'-CCUACGCCACCAAUUUCGU(dTdT)-3'; antisense: 5'-ACGAAAUUGGUGGCGUAGG(dTdT)-3') (Bioneer, South Korea) was used for electrospinning optimization and siRNA release study. GAPDH siRNA (Silencer[®] GAPDH siRNA, Ambion, USA) was chosen as the model siRNA for the evaluation of siRNA transfection efficiency *in vitro*. FAM-labeled DNA oligonucleotides (FAM-ODN) (Research Biolabs, Singapore) and Cy5-labeled oligonucleotides (Cy5-ODN) (1st Base, Singapore) with the same sequences as the negative siRNA were synthesized as a substitute of siRNA for the visualization of siRNA distribution inside PCL fibers and *in vitro* cellular uptake study, respectively. Briefly, 500 μ L of PCL or PCL-PEG polymer solution was mixed with 100 μ L of 10 μ g siRNA/ODN in DEPC-treated TE buffer (pH 8.0, 1st Base, Singapore) for electrospinning. The uniform siRNA-polymer mixture was loaded into a syringe and the flow rate was fixed at 1.5 mL/hr by a syringe pump (New Era pump systems Inc., USA). High DC voltage (GAMMA high voltage research, USA) was applied to the polymer

mixture at a positive voltage of 12-15 kV. A stationary 5×5 cm² aluminum (Al) foil connected to a negative electrode of 4-6 kV was used as the collector. The distance between the needle and the collector was 13-14 cm. All electrospinning parameters were set after initial optimization studies to obtain uniform fibers. The electrospinning process was carried out at room temperature of 19 to 22.5 °C and the humidity was 55 to 63%.

4.2.2. Characterization of siRNA-encapsulated electrospun nanofibers

The morphology of siRNA-encapsulated PCL and PCL-PEG electrospun nanofibers was evaluated by field emission scanning electron microscopy (FESEM) (JOEL, JSM-6700F, Japan). To assess the performance of PEG as the porogen, fiber surfaces after siRNA release study of 49 days were also evaluated using FESEM. After the siRNA release study, the scaffolds were washed, dried under vacuum overnight at room temperature and then observed under FESEM. The average fiber diameter was determined using Image J (NIH, USA) based on FESEM images by measuring 100 fibers, and presented as mean ± standard error (SE) of the mean. The distribution of siRNA was visualized by observing the FAM-ODN-encapsulated PCL fibers under fluorescent microscope (Olympus, IX71, Japan).

In order to measure the loading efficiency, negative control siRNA-encapsulated PCL and PCL-PEG scaffolds were fabricated (n=3) by electrospinning. Thereafter, the scaffolds were cut into two halves with similar weight and each half was dissolved in 1 mL of chloroform (minimal 99%, Sigma, USA) for higher extraction efficacy. The encapsulated siRNA was then extracted by adding 200 µL of DEPC-treated TE buffer into the resulting

polymer solution and the aqueous phase was collected. This process was repeated three times. The concentration of siRNA in the extracted aqueous solution was then determined by RiboGreen[®] assay (Quanti-IT[™] RiboGreen[®], Invitrogen, USA) and the fluorescence intensity was read in a microplate reader (Tecan[®], Infinite 200, Austria). The extracted amount of siRNA from each scaffold was calculated from the siRNA concentrations. The efficiencies of extracting siRNA from the polymers were accounted for by using PCL or PCL-PEG solutions that were mixed with siRNA solutions at the same amount as that used for electrospinning. The polymer/siRNA mixtures were placed under vacuum at room temperature overnight to remove all solvents. Dry polymer/siRNA constructs (n=3) were cut into two halves with similar weight and each half was then dissolved in 1 mL chloroform and siRNA was extracted and quantified as described above.

4.2.3. siRNA release kinetics

Negative control siRNA-encapsulated scaffolds (n=3) were used for siRNA release study. The average weights of the scaffolds were 7.77 mg, 8.30 mg and 9.33 mg for PCL, PCL-PEG20 and PCL-PEG60 respectively. The difference in scaffold weight was due to the addition of PEG into the PCL solutions. Each scaffold was placed in one well of a 6-well cell culture plate and 5 mL of DEPC-treated PBS (pH 7.4, 1st Base, Singapore) was added per well. Autoclaved Teflon rings were placed on top of the scaffolds to ensure that all scaffolds were completely immersed in PBS. The culture plate was sealed with parafilm to reduce evaporation and placed in a 37 °C shaker with shaking speed of 60 to 90 rpm (Sartorius Certomat[®] R, Germany). At fixed time points, 2 mL

of supernatant was taken out from each well and was replenished with 2 mL of fresh PBS. The concentration of siRNA in the supernatant was determined by RiboGreen[®] reagent and the fluorescence intensity was read in a microplate reader. After the siRNA release study of 49 days, the remaining scaffolds were kept in -20 °C prior to the evaluation of the integrity of siRNA inside the fibers by gel electrophoresis.

4.2.4. Integrity analysis of siRNA in polymer nanofibers

The integrity of siRNA in the polymer fibers was tested using a 2.5% agarose gel with ethidium bromide, and the positions of the resulting bands were compared with fresh naked siRNA as the positive control. The integrity of siRNA that was released into PBS supernatant and siRNA that remained entrapped within the PCL or PCL-PEG fibers after 49 days of release study were evaluated separately. To study the integrity of siRNA within the supernatant, 7-9 mg of siRNA-encapsulated scaffolds were cut into small pieces of 2-5 mm in size and immersed in 2 mL of DEPC-treated PBS in a microcentrifuge tube. 100 µL of the supernatant were taken out at each time point. Following that, 18 µL of each supernatant from three types of scaffolds were loaded for gel electrophoresis. To test the integrity of siRNA that remained in the fibers after controlled release of 49 days, scaffolds were washed, dried under vacuum at room temperature overnight and then dissolved in 1 mL of chloroform followed by extraction with 200 µL of DEPC-treated TE buffer. Thereafter, 18 µL of extraction solutions were loaded into the gel.

4.2.5. Cell culture and siRNA transfection

Human embryonic kidney 293 cells (HEK 293) (P12-21) and mouse fibroblast NIH 3T3 cells (P33-34) were used in this study. HEK 293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone, USA), 10% fetal bovine serum (FBS) (Hyclone, USA), 1% penicillin-streptomycin (Gibco), supplemented with 1% MEM non-essential amino acids (Gibco) and 1% sodium pyruvate (Gibco). NIH 3T3 cells were maintained DMEM, 10% bovine calf serum (BCS) (Hyclone, USA) and 1% penicillin-streptomycin. Medium with serum and antibiotics was called complete medium in this work. Cells were maintained in humidified incubator at 37 °C with 5% CO₂. For transfection, cells were kept within 10 passages to ensure similar cellular activity for siRNA transfection. TransIT-TKO (MirusBio, USA) was used as the transfection reagent in this study. The siRNA transfection conduction with TransIT-TKO/GAPDH siRNA complex was optimized (n=3). Briefly, HEK 293 cells were plated into 24-well plate at a density of 1.2×10^5 cells/well in 500 μ L of complete medium one day before transfection. Transfections were carried out using a series of combinations of TKO amounts and siRNA concentrations following the TKO's protocol. Cells were incubated for 48 h and silencing efficiency was determined by real-time RT-PCR. The combinations of TKO ranging from 1-4 μ L per well in 24-well plate format and siRNA final concentration from 5-50 nM were evaluated. Optimal TKO amount of 1.5 μ L was determined for HEK 293 cells in 24-well plate format at a total medium volume of 300 μ L. Lower amount of TKO resulted in ineffective transfections while higher amount caused remarkable cell death. And a siRNA concentration of 20 nM was used in the present work to achieve sufficient silencing results.

4.2.6. Bioactivity analysis of siRNA released from nanofibrous scaffolds

GAPDH siRNA supernatants collected from the scaffolds at various time points were used to transfect HEK 293 cells, to test the bioactivity of the released siRNA from polymer nanofibers. GAPDH siRNA-encapsulated PCL and PCL-PEG polymer nanofibers (n=3) with average weight of 7-9 mg were sterilized under UV for 1 hr and were placed in 24-well cell culture plate. All scaffolds were immersed in 2 mL of DMEM, covered with autoclaved Teflon rings to ensure that the scaffolds were completely immersed in the medium. The plates were kept in humidified incubator at 37 °C with 5% CO₂. At time points of day 5, day 15 and day 30, 550 µL of siRNA supernatant was taken out for transfection and cell viability assay. Each well was then replenished with fresh DMEM. The GAPDH siRNA concentrations in the supernatants from nanofibrous scaffolds were determined by RiboGreen assay.

One day before transfection, HEK 293 cells were plated into 24-well plate at a density of 1.2×10^5 cells/well in 500 µL of complete medium. In the case of the positive control, 1.5 µL of TKO reagent was complexed with GAPDH siRNA at a final concentration of 20 nM for transfection following TKO's protocol. For scaffold supernatant transfection, 1.5 µL of TKO reagent was mixed with 150 µL of GAPDH siRNA supernatant for each well. During the transfection, the total volume of medium was 300 µL per well and 300 µL of fresh complete medium was added to all wells 24 hr later. Cells in the negative control received no treatment of siRNA or transfection reagent. Cells were kept in the incubator and GAPDH mRNA level and cell viability were analyzed 48 hr after transfection.

4.2.7. Uptake of GAPDH siRNA by cells seeded on siRNA-encapsulated scaffolds

GAPDH siRNA-encapsulated scaffolds with average weight of 7-9 mg were cut to fit the wells of 24-well plates and sterilized under UV for 1 hr. HEK 293 cells were directly seeded on the scaffolds with or without TKO reagent (n=3) at 1.2×10^5 cells/well in 500 μ L of complete medium. Teflon rings were used to hold scaffolds to the bottom of the wells and to ensure that cells were attaching onto the scaffolds. For the experimental group without transfection reagent, cells were cultured on the scaffolds without further treatment and RNA analysis was carried out 96 hr after seeding. For the TKO transfection group, 2.5 μ L of TKO was added into each well 24 hr following cell seeding. Cells were then incubated until 96 hr after seeding before RNA analysis.

4.2.8. RNA analysis and real-time RT-PCR

Two to three wells of cells in 24-well plate were pooled for each sample to obtain sufficient RNA for analysis. Cells were lysed and RNA was isolated using TRIzol[®] reagent (Invitrogen). RQ1 RNase-free DNase (Promega, USA) was applied to the isolated RNA to improve RNA quality. Reverse transcription was carried out using Sensisript[®] RT kit (Qiagen, Germany) by incubating RNA and the reagent mixture at 37 °C for 1 hr. GAPDH mRNA level was determined by real-time PCR using iQ SYBR green supermix (Bio-rad, CA) in a iCycler iQ5 real-time PCR detection system (Biorad, USA), with beta-actin as the internal control. Primer sequences for GAPDH gene were: forward 5'-ATCAGCAATGCCTCCTGCAC-3', reverse 5'-TGGCATGGACTGTGGTCATG-3' and the product size was 103 bp. Primer

sequences for beta-actin were: forward 5'-GGCACCCAGCACAATGAAGATCAA-3', reverse 5'-ACTCGTCATACTCCTGCTTGCTGA-3', and the product size was 134 bp. Our preliminary studies showed that these two primers had similar amplification efficiency under the parameters used and therefore $\Delta\Delta C_T$ method was chosen to compare mRNA levels. Real-time PCR condition used was as follows: 3 min at 95 °C, 40 cycles at 95 °C for 15 s, followed by 59 °C for 30 s. The entire GAPDH gene silencing experiment was repeated 3 times for each sample.

4.2.9. Cell viability assay

The viability of transfected cells was determined by WST-1 assay (Roche, Germany). To understand the influence of PCL nanofibrous scaffolds on cell viability, HEK 293 cells were seeded at 4.8×10^4 cells/well onto plain sterile PCL, PCL-PEG20 and PCL-PEG60 scaffolds and TCPS as the control in 48-well plate and maintained in incubator for 3 days before WST-1 assay (n=3). To evaluate cell viability during transfection, cells were plated in 48-well plate for scaffold-based transfection and 96-well plate for supernatant transfection. One day before transfection HEK 293 cells were seeded at densities of 4.8×10^4 cells/well and 2.4×10^4 cells/well respectively. Following that, same transfection procedure was implemented with proportionally reduced amounts of siRNA and transfection reagent (n=3). WST-1 reagent was then added to each well at 1:10 dilution 48 hr after transfection. The relative absorbance ($A_{450}-A_{690}$) was measured using a microplate reader (Bio-rad, Benchmark Plus™, Japan) after incubation of 3 to 4 hours at 37 °C.

4.2.10. Uptake of Cy5-ODN by cells seeded on ODN-encapsulated scaffolds

HEK 293 cells were directly seeded on Cy5-ODN-encapsulated polymer fibers to test cellular uptake. NIH 3T3 cells were also included to investigate the effects of cell type difference on cellular uptake. Due to possible photobleaching of Cy5, scaffolds were not sterilized under UV. Instead, 1% antibiotic-antimycotic (Gibco) solution was added into the culture medium to prevent contamination. HEK 293 and NIH 3T3 cells were seeded onto scaffolds at densities of 2×10^4 cells/cm² and 1×10^4 cells/cm² respectively in 500 μ L of complete medium. The cells were divided into three groups. Group 1 as the control group with cells cultured on glass cover slips with 20 nM naked Cy5-ODN as the negative control and 20 nM Cy5-ODN complexed with 2.5 μ L TKO as the positive control. In Group 2, cells were cultured on Cy5-ODN fibrous scaffolds without TKO. In Group 3, cells were cultured on Cy5-ODN fibrous scaffolds with 2.5 μ L of TKO per well. Two hours after plating for cell attachment, Cy5-ODN, Cy5-ODN/TKO complex or TKO solution was added into each well. Teflon rings were used to hold the scaffolds to the bottom of the wells and to ensure that cells were on the scaffolds. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde (95%, Sigma-Aldrich, USA) for 30 min at room temperature, permeabilized in 0.05% Triton X-100 (Sigma, USA), 50 mM glycine ($\geq 99\%$, Sigma, South Korea) for 20 min and stained with DAPI (Invitrogen) at 1: 3000 dilution for 30 min. The uptake of Cy5-ODN was evaluated by a confocal microscope (Zeiss, LSM 510 Meta Laser Scanning Confocal Microscope, Germany).

4.2.11. Statistics

One-way ANOVA was carried out to calculate *p* value. The Tukey post hoc test was chosen when variances were homogenous otherwise the Games-Howell test was used. Error bars represent the standard error (SE) of the mean.

4.3. RESULTS

4.3.1. Characterization of siRNA-encapsulated electrospun nanofibers

Three types of PCL nanofibers encapsulating siRNA with or without PEG, were fabricated and designated as PCL (pure PCL fiber), PCL-PEG20 (20 mg/mL PEG to PCL) and PCL-PEG60 (60 mg/mL PEG to PCL) respectively. Uniform nano-sized PCL and PCL-PEG fibers with encapsulated siRNA were obtained by electrospinning (Fig. 4.1A-C). The average fiber diameters of PCL, PCL-PEG20 and PCL-PEG60 were (309.4 ± 7.0) nm, (335.0 ± 7.0) nm and (423.5 ± 9.8) nm, respectively. Fiber diameter increased with increasing loading levels of PEG ($p < 0.05$). In the case of PCL-PEG60 scaffolds, nanofibers appeared less cylindrical and fibers occasionally fused at junction sites. The FAM-labeled oligonucleotides (FAM-ODN) fluorescence image (Fig. 4.1D-F) indicated even distribution of siRNA or ODN along the polymer fibers. The distribution of FAM-ODN presented punctate dots within the fibers, due to low loading level of FAM-ODN as compared to the polymer (0.005 wt% of entire fibrous scaffold). To quantify the loading efficiency of encapsulating siRNA into electrospun nanofibers, extraction and loading efficiency analysis was carried out. The loading efficiencies of siRNA-encapsulated fibrous scaffolds were $51.9 \pm 1.3\%$, $65.8 \pm 1.5\%$ and $67.4 \pm 1.7\%$ for PCL, PCL-PEG20 and PCL-PEG60 fibers respectively.

Fig. 4.1. Characterization of siRNA-encapsulated PCL and PCL-PEG electrospun nanofibers. FESEM images show the morphology of siRNA-encapsulated fibers for PCL (A), PCL-PEG20 (B) and PCL-PEG60 (C). D-F: Distribution of FAM-ODN in PCL electrospun fibers. D: Optical image. E: Fluorescent image. F: Merged image. Scale bars in (A-C) = 5 μm and (D-F) = 20 μm .

4.3.2. Release of siRNA from polymer nanofibers

siRNA release was monitored for 49 days and a sustained release of siRNA from the polymer nanofibers was detected for at least 28 days (Fig. 4.2A). Prolonged incubation of siRNA scaffolds did not show detectable siRNA release by RiboGreen assay, which has a detection limit of 1 ng/mL. The cumulative amounts of siRNA that were released with respect to the loaded siRNA amount were 3.01%, 12.55% and 26.30% for PCL, PCL-PEG20 and PCL-PEG60, respectively. The siRNA release rate increased with elevated PEG concentration, indicating that the presence of PEG enhanced siRNA release. PCL fibers exhibited smooth surfaces (Fig. 4.2B), whilst rough surfaces of

PCL-PEG fibers were observed after siRNA release of 49 days (Fig. 4.2C, D). This is likely due to the leaching of PEG into the aqueous solution during the release study.

Fig. 4.2. Release of siRNA from electrospun nanofibers. A: Release kinetics of siRNA within 49 days in PBS at 37 °C. FESEM images show surface morphology of PCL fibers (B), PCL-PEG20 fibers (C) and PCL-PEG60 fibers (D) after siRNA release study of 49 days. Scale bars in (B-D) = 1 μ m.

4.3.3. Integrity analysis of siRNA in polymer nanofibers

Agarose gel electrophoresis was used to assess the integrity of siRNA in the electrospun fibers. siRNA remained intact despite the harsh processing conditions of electrospinning and also throughout the period of sustained release (Fig. 4.3.). A single band of siRNA was detected in the supernatants from the three types of polymer nanofibers at all time points (day 7, 14, 21, 28 and 49) (Fig. 4.3A-C). The molecular weight of siRNA recovered from the supernatants corresponded to that of naked siRNA, indicating that structurally intact siRNA was continuously released from the fibers for at least 49 days

under physiological conditions. The siRNA band intensity increased with increasing concentrations of PEG, which corresponded to the trends observed from the siRNA release profiles (Fig. 4.2A). However, siRNA band intensity was not observed to increase over time. It is possible that low cumulative siRNA release coupled with simultaneous siRNA degradation in the supernatant resulted in no obvious change in the siRNA concentration. The low detection sensitivity of ethidium bromide used for electrophoresis may have also attributed to the limited band intensity resolution.

At the end of siRNA release study of 49 days, all scaffolds were dissolved in chloroform and siRNA that remained entrapped within the polymer fibers was extracted to test for signs of degradation by gel electrophoresis. Due to low siRNA concentrations in the extraction solutions and the limited extraction efficiency, only light siRNA bands were detected (Fig. 4.3D). In the case of PCL-PEG60 samples, the lack of detection of siRNA bands might be due to the higher release rate of siRNA from these scaffolds, along with the low detection sensitivity of ethidium bromide. Nonetheless, the results clearly indicated that the remaining entrapped siRNA retained its integrity after the release study.

Fig. 4.3. Integrity of siRNA in polymer nanofibers tested by 2.5% agarose gel electrophoresis. Gel electrophoresis results of siRNA supernatants from PCL fibers (A), PCL-PEG20 fibers (B) and PCL-PEG60 fibers (C) of various time points. D: Gel electrophoresis results of siRNA samples that were extracted from nanofibrous scaffolds after siRNA release study of 49 days.

4.3.4. Bioactivity analysis of siRNA released from nanofibrous scaffolds

GAPDH gene silencing was chosen as the model gene for evaluating the efficacy of nanofibrous scaffold-mediated siRNA transfection. We first optimized GAPDH siRNA/TKO transfection condition with siRNA concentration ranging from 5 nM to 50 nM. Naked siRNA in the absence of TKO was included as a control. As shown in Fig. 4.4, GAPDH siRNA ranging from 10 nM to 50 nM achieved around 80% gene silencing efficiency on HEK 293 cells. However, when siRNA decreased to lower concentration of 5 nM, no observable GAPDH down-regulation was achieved, indicating a threshold siRNA concentration lay between 5 nM and 10 nM for effective silencing. However, naked GAPDH siRNA in the absence of transfection reagent was unable to affect GAPDH expressions.

Fig. 4.4. GAPDH siRNA transfection with various concentrations with TKO reagent and naked siRNA transfection. N20nM: naked 20 nM siRNA transfection. NEG: negative control. (Mean $\pm$ SE, n = 3)

Next, we performed experiments to determine if the released siRNA retained its bioactivity by transfecting released GAPDH siRNA collected from the supernatants of the three types of scaffolds at various time points (day 5, day 15 and day 30) into HEK 293 cells. The concentrations of GAPDH siRNA for transfection in 24 well culture plate were 14-20 nM, 41-63 nM and 108-173 nM for supernatants from PCL, PCL-PEG20 and PCL-PEG60 fibers, respectively. Efficient silencing effect was observed for all scaffolds at each time point (Fig. 4.5) with silencing efficiency of 61-81%, which was comparable to that of the positive control comprising siRNA-TKO complex introduced by conventional bolus delivery. This showed that siRNA released in the supernatant remained at least partially bioactive for a minimum of 30 days under physiological conditions. Although siRNA concentrations varied in the supernatants derived from the three types of scaffolds as indicated in the siRNA release profile (Fig. 4.2A), the difference in transfection efficiency among the three scaffolds was not significant. Similarly, the silencing effect did not show

significant changes over time for all scaffolds. This suggested that the electrospun nanofibrous scaffolds provided efficient protection for the encapsulated GAPDH siRNA over at least 30 days under physiological conditions.

Fig. 4.5. GAPDH mRNA level of supernatant transfection in HEK 293 cells. GAPDH siRNA supernatants were collected from PCL, PCL-PEG20 (PEG20) and PCL-PEG60 (PEG60) fibrous scaffolds at day 5, 15 and 30 and complexed with TKO reagent for transfection. POS: positive control of cells on culture plate subjected to conventional siRNA-TKO transfection. NEG: negative control of cells with no treatment. (Mean $\pm$ SE, $n = 3$)

4.3.5. Uptake of GAPDH siRNA by cells seeded on siRNA-encapsulated scaffolds

HEK 293 cells seeded directly on GAPDH siRNA-PCL and siRNA-PCL-PEG fibrous scaffolds showed significant gene silencing as compared to the negative control with cells seeded on culture plate in the absence of siRNA and TKO reagent. This significant down-regulation of GAPDH gene expression was consistently observed in cells with ($p < 0.01$) and without ($p < 0.01$) the use of transfection reagent (Fig. 4.6.). In drastic contrast, gene silencing via bolus delivery of soluble naked siRNA was only observed in the presence of TKO

(Fig. 4.4.). In the absence of TKO, GAPDH silencing efficiencies were 22.3%, 18.6% and 25.8% that of HEK 293 cells cultured directly on plain PCL, PCL-PEG20 and PCL-PEG60 fibers, respectively. With TKO reagent, the GAPDH gene silencing effect was greatly enhanced to 76.4%, 74.5% and 65.3% that of cells seeded directly on PCL, PCL-PEG20 and PCL-PEG60 scaffolds, respectively. GAPDH gene silencing efficiencies on PCL and PCL-PEG20 fibers with TKO reagent was comparable to the positive control (20 nM of soluble siRNA transfection with TKO) where 83.9% silencing effect was attained. A slightly lower silencing effect was seen in cells cultured on PCL-PEG60 fibers, which was significantly different from the positive control ($p < 0.05$), but insignificant when compared to PCL and PCL-PEG20 fibers. These results clearly indicated the efficacy of siRNA-encapsulated nanofibrous scaffolds in achieving scaffold-based gene silencing.

Fig. 4.6. GAPDH mRNA expression levels by HEK 293 cells seeded directly on siRNA-encapsulated scaffolds. Cells were cultured on three types of fibrous scaffolds containing GAPDH siRNA in the absence or presence of TKO reagent. Transfection was carried out with or without TKO transfection reagent. * indicates $p < 0.05$ compared to the negative control and # indicates $p < 0.05$ in paired groups. PCL, PEG20, PEG60: cells seeded on siRNA-encapsulated fibers comprising PCL, PCL-PEG20 and PCL-PEG60 without TKO reagent respectively. PCLT, PEG20T, PEG60T: cells seeded on siRNA-encapsulated fibers comprising PCL, PCL-PEG20 and PCL-PEG60 with TKO reagent. POS: positive control of cells on culture plate subjected to conventional siRNA-TKO transfection. NEG: negative control of cells with no treatment. (Mean $\pm$ SE, $n = 3$)

4.3.6. Cell viability

The cell viability on different types of PCL nanofibrous scaffolds was shown in Fig. 4.7. HEK 293 was most viable on plain PCL nanofibers, with a normalized cell viability of 0.94 which was comparable to cells on TCPS. Cells cultured on PEG containing nanofibers exhibited lower cell viability of 0.56 and 0.44 for PCL-PEG20 and PCL-PEG60 nanofibers, respectively. The presence of PEG in the PCL nanofibers significantly reduced the viability of HEK 293 cultured on nanofibrous scaffolds, in comparison with that on TCSP ($p < 0.05$). It is possible that the increased concentration of PEG inside PCL

greatly elevated the wettability/hydrophilicity of the fibrous scaffolds and led to poorer cell attachment and lower viability on PEG-containing fibers.

Fig. 4.7. Cell viability of HEK 293 cells cultured on various substrates. PCL-S: PCL fibrous scaffold. P20-S: PCL-PEG20 fibrous scaffold. P60-S: PCL-PEG60 fibrous scaffold. TCPS: tissue culture polystyrene. * indicates $p < 0.05$ compared to TCPS samples. (Mean $\pm$ SE, $n = 3$)

The TransIT-TKO transfection reagent appeared to induce slight toxicity to the cells compared to the negative control with cells seeded on culture plate in the absence of siRNA and TKO reagent (Fig. 4.8.). The viabilities of cells that were transfected using siRNA supernatant were 67-76% as compared to the negative control. These results, however, were not significantly different from the positive control that comprised of siRNA transfection with TKO, which was 77-82% of the negative control. No apparent difference was observed among the three types of scaffolds at all time points.

Fig. 4.8. Cell viability of transfected HEK 293 cells in GAPDH siRNA supernatant transfection which was collected at day 5, 15 and 30. POS: positive control of cells on culture plate subjected to conventional siRNA-TKO transfection. NEG: negative control of cells with no treatment. (Mean $\pm$ SE, n = 3)

When cells were seeded on siRNA-encapsulated scaffolds for GAPDH siRNA transfection, the cell viability was affected in two ways: the cytotoxicity of the transfection reagent and the surface chemistry effect of the three different fibrous scaffolds on cell attachment. Cells seeded directly on PCL-PEG scaffolds appeared to show lower cell viability as compared to cells that were transfected with siRNA collected from supernatants (Fig. 4.9.). Unlike supernatant transfection, cell viability on the siRNA-encapsulated scaffolds was PEG concentration-dependent. This corresponded with the trend observed when cells were cultured on plain PCL, PCL-PEG20 and PCL-PEG60 fibers without siRNA encapsulation (Fig. 4.7.). GAPDH siRNA-encapsulated PCL fiber alone was supportive of cell growth and cell viability on this type of scaffold was not significantly different from that on tissue culture plate without any treatment.

Fig. 4.9. Cell viability of HEK 293 cells in scaffold-based GAPDH siRNA transfection. HEK 293 cells were cultured on three types of fibrous scaffolds containing GAPDH siRNA. Transfection was carried out with or without TKO transfection reagent. * indicates $p < 0.05$ and ** means $p < 0.01$ compared to the positive control. # means $p < 0.01$ compared to the negative control. PCL, PEG20, PEG60: cells seeded on siRNA-encapsulated fibers comprising PCL, PCL-PEG20 and PCL-PEG60 without TKO reagent respectively. PCLT, PEG20T, PEG60T: cells seeded on siRNA-encapsulated fibers comprising PCL, PCL-PEG20 and PCL-PEG60 with TKO reagent. POS: positive control of cells on culture plate subjected to conventional siRNA-TKO transfection. NEG: negative control of cells with no treatment. (Mean $\pm$ SE, $n = 3$)

4.3.7. Uptake of Cy5-ODN by cells seeded on ODN-encapsulated scaffolds

HEK 293 cells and NIH 3T3 cells were seeded on Cy5-labeled oligonucleotides (Cy5-ODN) encapsulated nanofibers and confocal microscopy was used to demonstrate uptake of released material by cells directly cultured on the nanofibers. Pseudo colors were applied to the confocal images to enhance color contrast for clearer visualization. Red was used to substitute for blue for nuclei staining by DAPI while green was used to substitute for red emission by Cy5-ODN. No observable uptake of Cy5-ODN introduced by passive uptake was seen when HEK 293 cells were seeded on glass cover slips without transfection reagent (Fig. 4.10A). This indicated that naked soluble

Cy5-ODN was not able to cross HEK 293 cell membrane. It is also possible that the Cy5-ODN was enzymatically degraded in the cell culture medium due to the presence of nucleases in added serum. In contrast, cellular uptake of Cy5-ODN aggregates was observed when Cy5-ODN was complexed with TKO reagent (Fig. 4.10E). Slight uptake of Cy5-ODN was seen when HEK 293 cells were seeded on Cy5-ODN-encapsulated PCL and PCL-PEG fibrous scaffolds in the absence of TKO reagent (Fig. 4.10B-D). The Cy5 signals appeared diffused and the intensity appeared to increase with increasing PEG loading level. On the other hand, ODN aggregates were clearly visible around cell nuclei when TKO was added (Fig. 4.10F-H), with no observable difference in signal intensity between the three types of fibers.

Slight uptake of Cy5-ODN was seen in NIH 3T3 cells that were cultured on glass cover slips in the absence of TKO reagent (Fig. 4.10I), while strong signals of Cy5-ODN aggregates were detected with the addition of TKO reagent (Fig. 4.10M). Dispersed Cy5 signals were also observed within 3T3 cells that were cultured on Cy5-ODN-PCL and PCL-PEG fibrous scaffolds in the absence of transfection reagent (Fig. 4.10J-L). Similar to the observation in HEK 293 cells, Cy5 signal intensity increased with increasing PEG loading levels. In contrast, Cy5-ODN signals existed in aggregate form when TKO was added into the cell culture medium (Fig. 4.10N-P). The Cy5 signal intensity in this case, did not appear to change significantly with PEG loading level. Compared to HEK 293 cells, NIH 3T3 cells appeared to uptake Cy5-ODN more readily in the absence of transfection reagent (Fig. 4.10A-D and 4.10I-L), suggesting cell-type variation in direct cellular uptake of ODN. In both cell types examined, when transfection reagent was absent, the degree of ODN

uptake was greater in cells cultured on scaffolds as compared to cells exposed to soluble ODN in the medium (on glass cover slips). This clearly demonstrated that cellular uptake of ODN was higher by scaffold-mediated reverse transfection than via bolus delivery.

Fig. 4.10. Cy5-ODN uptake by HEK 293 cells and NIH 3T3 cells. Cells were seeded on cover slips (control) and Cy5-ODN scaffolds without or with transfection reagent TKO. Pseudo color green indicates the distribution of Cy5-ODN and red signals present cell nuclei by DAPI staining. A-H: Cy5-ODN uptake by HEK 293 cells on cover slips (A, E), ODN-PCL fibers (B, F), ODN-PCL-PEG20 fibers (C, G) and ODN-PCL-PEG60 fibers (D, H) without (A-D) or with transfection reagent (E-H). I-P: Cy5-ODN uptake by NIH 3T3 cells on cover slips (I, M), ODN-PCL fibers (J, N), ODN-PCL-PEG20 fibers (K, O) and ODN-PCL-PEG60 fibers (L, P) without (I-L) or with transfection reagent (M-P). Scale bars = 20 μm.

4.4. DISCUSSION

Converse to the traditional approach of manipulating cellular functions by overexpression of biochemical signals for tissue regeneration [23-25], RNA interference (RNAi) adopts the opposite strategy by silencing target genes. This method is attractive particularly in areas where regeneration of tissues is often hindered by inhibitory factors, such as in the case of the central nervous system [26]. By incorporating biomimicking nanofibers with siRNA, such scaffold-based gene silencing systems provide an attractive platform for orchestrating cellular functions and differentiation through a combination of topographical and biochemical signals. These nanofibers can serve as potential biofunctional scaffolds for gene therapy and regenerative medicine. Electrospinning is an easy and versatile method of producing nanofibers [27]. Encapsulating biomolecules within electrospun fibers has been proven as a feasible and convenient way for sustained delivery of growth factors [28], DNA [25] and drugs [29]. Polymer fibers provide physical protection to the encapsulated biomolecules, preserve their bioactivity over prolonged periods of time and enable local release of biomolecules at target sites. This versatile scaffold fabrication technique is also useful for siRNA encapsulation as demonstrated in this study.

PCL is a biocompatible polymer with a low degradation rate suitable for long-term tissue engineering applications [30]. In previous Chapter, PEI/siRNA complex was delivered in PCL fibers. However, the release rate was hindered by the low diffusivity of the complexes and the nanoparticle-polymer interaction. By encapsulating naked siRNA within PCL fibers, an enhanced release rate of bioactive siRNA could be achieved under physiological

conditions for at least 28 days in a sustained manner. Although the release of siRNA from plain PCL fibers remains low even after 7 weeks, resulting in a cumulative siRNA release of only 3.01% of loaded siRNA amount, the release profile of siRNA could be altered through the inclusion of PEG as the porogen. siRNA is a macromolecule with an average molecular weight of 13.3 kDa. While the exact mechanism for the low release of siRNA from plain PCL fibers remains to be elucidated, it is possible that physical entrapment of siRNA within PCL matrix may have hindered the diffusion of siRNA from the fibers. Recent studies have also suggested that the encapsulated molecules inside PCL are mainly released based on desorption instead of diffusion [31-32]. Coupled with the low degradation rate of PCL, complete release of encapsulated siRNA was not attainable within the duration of this *in vitro* study. Drug release rate depends on the characteristics of the encapsulated molecule [33] and the properties of the polymer, such as molecular weight and degradation rate [34-35]. Different release rates of encapsulated biomolecules have been reported in various polymeric electrospun nanofibers, such as PCL and its derivatives [28, 36], poly(lactide-co-glycolide) (PLGA) [37] and poly(vinyl alcohol) (PVA) [38]. Thus, by modifying the choice of polymer and polymer properties, such as enhancing the degradation rate or swelling ratio of polymer matrix, appropriate release kinetics of siRNA may be achieved.

In common with other polymer-based delivery systems, nanofibers physically protect biomolecules encapsulated within them from loss of functionality [25, 39]. Although siRNA is prone to degradation, we showed that by encapsulating these labile molecules within PCL fibers, the structural integrity of siRNA could be maintained under physiological conditions for at

least 7 weeks. Furthermore, by using GAPDH siRNA released from polymer nanofibers at various time points to transfect HEK 293 cells, we showed that GAPDH siRNA was bioactive throughout 30 days of sustained release. This demonstrates the feasibility of utilizing electrospun nanofibers as an alternative delivery vehicle of siRNA for long-term therapeutic applications.

In GAPDH siRNA supernatant transfection experiments, equivalent silencing effects were observed in HEK 293 cells although siRNA concentrations differed in the supernatants derived from PCL, PCL-PEG20 and PCL-PEG60 samples. Although the exact mechanism behind this observation remains to be elucidated, it may be possible that this resulted from the saturation of the RNA-induced silencing complex (RISC) as a consequence of excess siRNA present in the supernatant [40-41]. The excess siRNA was unable to take effect due to the inability to combine with RISC in the cytoplasm. Saturation of the binding capacity of TKO reagent may be an additional factor. We found that GAPDH siRNA at concentration of 10 nM appears to be the threshold amount necessary for observable GAPDH gene silencing in HEK 293 cells using TransIT-TKO reagent for *in vitro* transfection via bolus delivery. Above this concentration, no further increase in silencing efficiency was observed. It is possible that when siRNA concentration exceeds 10 nM, the amount of TKO became the efficiency-limiting factor and the ability of transfection reagent to transport siRNA into cells became saturated. Naked soluble GAPDH siRNA alone showed no silencing effect in HEK 293 cells *in vitro*. In contrast, silencing effect was observed in cells cultured on GAPDH siRNA-encapsulated PCL and PCL-PEG scaffolds even in the absence of transfection reagent. These results concurred with Cy5-ODN uptake by HEK

293 cells, showing an enhanced cellular uptake of naked siRNA provided by scaffold-mediated reverse transfection. Such enhanced cellular uptake and transfection is likely due to the localized concentration and presentation of siRNA to cells, akin to the mechanism of reverse transfection of DNA [42].

In the scaffold-based GAPDH siRNA transfection, the GAPDH gene was more effectively silenced once transfection reagent was added into the medium. Besides the conventional complexing procedure in protein-free medium, TKO reagent was also able to complex with siRNA, which was released into the culture medium from the fibers even in the presence of serum and antibiotics. This resulted in high silencing efficiencies that were comparable to the conventional transfection method. It seemed that in the current delivery system, transfection reagent might still be required and a potential improvement to the current system may, therefore, involve the co-encapsulation of TKO reagent into the nanofibers with siRNA. By choosing the appropriate polymer and scaffold design, sustained release of TKO and siRNA from the fibers may be attainable for long-term gene regulation.

The relatively lower silencing effect observed in cells cultured on PCL-PEG60 scaffolds may be due to the lower viability of cells on this type of scaffold. The cell viability in the scaffold-based transfection contained the synergic influence of the cytotoxicity caused by transfection reagent and the surface chemistry effect of three types of fibrous scaffolds. Low molecular weight PEG ($M_w = 3350$) used in this study is known to have good biocompatibility and low cytotoxicity. Dissolved PEG in siRNA supernatant did not induce noticeable toxicity in the transfected cells (Fig. 4.8.). However cell viability was significantly lower than the positive control with TKO reagent

($p < 0.01$) when cells were cultured on PEG-containing PCL fibers while PCL fiber alone did not affect cell growth. It is possible that the inclusion of PEG increased the wettability/hydrophilicity of PCL-PEG fibers, which resulted in reduced 293 cell attachment [43]. Without appropriate attachment to the substrate, cell proliferation was hindered and the morphology of cell nuclei showed signs of nuclear fragmentation. Low cell viability may ultimately have affected the siRNA uptake and resulted in a diminished silencing effect as seen on PCL-PEG60 scaffolds.

GAPDH gene was chosen as the target for siRNA silencing in the present work. GAPDH gene is a house keeping gene and knockdown of this gene using siRNA is an obvious and straightforward indication of the feasibility of delivering siRNA by nanofibers. However GAPDH gene is not appropriate for long-term study. The reduction in GAPDH affects metabolic processes in cells and leads to poor proliferation and low cell viability. We observed that after conventional GAPDH siRNA/TKO transfection, cells were not able to recover from the knockdown of GAPDH gene. Cell proliferation remained low and eventually died several days after transfection. We are currently working on other genes to test the long-term effect of this siRNA delivery system by nanofibers.

In an attempt to elucidate the possible mechanisms involved in the reverse transfection of siRNA by electrospun fibers, we visualized HEK 293 cellular uptake of oligonucleotides using Cy5-ODN. NIH 3T3 cells were also included to investigate the effects of cell type on cellular uptake of oligonucleotides. As demonstrated by confocal microscopy, cellular uptake of naked soluble ODN introduced by passive uptake was negligible in HEK 293

cells (Fig. 4.10A), which corroborated findings of the GAPDH gene silencing study. Passive uptake of soluble ODN was obvious in NIH 3T3 cells, albeit in low amounts (Fig. 4.10I). This result highlights the cell-type dependence of oligonucleotides uptake by cells. The low cellular uptake of Cy5-ODN are likely due to the enzymatic degradation of oligonucleotides in the presence of serum and the inability of negatively charged ODN to pass through the cell membrane without the aid of transfection reagent. In contrast, we observed enhanced cellular uptake of Cy5-ODN in both HEK 293 and 3T3 cells when cells were seeded directly onto siRNA-encapsulated scaffolds, even in the absence of transfection reagent. This clearly demonstrated that cellular uptake of ODN was higher by reverse transfection than via passive uptake of naked ODN. Cy5 signal intensity increased with increasing PEG content (Fig. 4.10B-D and J-L). This enhanced cellular uptake of ODN is likely due to the increased local availability of ODN as cells were directly in contact with the ODN-encapsulated scaffolds. Although scaffold-mediated Cy5-ODN uptake showed a PEG concentration-dependent behavior in the absence of transfection reagent, GAPDH gene silencing efficiency did not vary significantly with PEG content (Fig. 4.6.). Meanwhile, when cells were directly cultured on Cy5-ODN scaffolds without TKO reagent, it is observed that almost all cells showed Cy5 signal, indicating high ODN uptake efficiency in both cell types. However, in the GAPDH gene silencing results, by directly seeding cells on siRNA-scaffolds without TKO, only around 20% silencing effect was seen. Combining these two observations, it seems that only partial siRNA which entered into cells was able to take effect while others may have been enzymatically degraded within the cytoplasm. Similar finding was also reported by Lingor *et*

al.[44]. They found that the uptake of naked siRNA in primary hippocampal neurons did not show silencing effect and the endosomal degradation of internalized naked siRNA was postulated as the possible reason. In the presence of transfection reagent, Cy5 signal was presented in aggregate form within the cell cytoplasm, as opposed to the more diffused signals observed in the absence of TKO. The aggregates are likely due to the formation of Cy5-TKO complex. There was no observable change in Cy5 signal with increased PEG concentration, suggesting once again the possibility of saturation of TKO's ability to complex siRNA, and that the amount of TKO may be the efficiency-limiting factor. In general, the lower ODN uptake by both cell lines in the absence of TKO, suggests that the transfection reagent may still be necessary in this delivery system for *in vitro* applications. However, naked siRNA has been shown to be efficiently taken up by various tissues such as skeletal muscle [45], liver [6] and the central nervous system [46] *in vivo* in the absence of transfection reagent. Therefore, whilst the exact process of local delivery of siRNA by electrospun fibers *in vivo* remains to be elucidated, the results are nonetheless promising.

4.5. CONCLUSIONS

This work introduces a scaffold-based approach for localized siRNA delivery. Local siRNA delivery exhibits remarkable potential in therapeutic applications due to possible lower dosage requirement and reduced toxicity to other tissues. Releasing siRNA from tissue engineering scaffolds can work as an important complement to local siRNA deliveries, providing suitable environment for tissue regeneration as well as modulating cellular behaviors by

RNA interference. Combined with topographical cues provided by nanofibrous scaffolds, such biofunctional substrates can offer both biochemical signals and contact guidance to seeded cells.

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CHAPTER 5

THE EFFECT OF NANOFIBER TOPOGRAPHY ON ASTROCYTE BEHAVIORS *IN VITRO* AND THE POTENTIAL APPLICATION OF siRNA DELIVERY FOR CNS REGENERATION

SUMMARY

The physiology of the nervous system presents great challenges to nerve repair after injury. In particular, astrocytes serve as major contributors to limited axonal regrowth through the secretion of growth inhibitory chemicals and the formation of glial scar. Unfortunately, detailed understanding of astrocyte behavior and interaction with micro-environmental signals remain limited. In this chapter, nanofiber topographical effects on astrocyte phenotypic changes, including the uptake of small-interfering RNA (siRNA) and the corresponding gene-silencing efficiency, were investigated to gain insights to construct a novel scaffold to deliver siRNA for CNS regeneration. Our observations suggested that electrospun nanofibers may serve as potential scaffolds for nerve injury repair by enhancing astrocyte apoptosis and reducing cell proliferation without altering cellular activation as compared to flat surfaces. Nanofibers may further facilitate the use of gene silencing to enhance CNS regeneration.

5.1. INTROCUCTION

The regenerative ability of human nervous system after injury is extremely limited, especially in the central nervous system (CNS). Traumatic damages may lead to permanent neurologic deficits which greatly reduce life quality of the patients. One of the major obstacles for CNS regeneration is the glial scar formed at the injury site, which consists predominantly of reactive astrocytes and extracellular matrix (ECM) molecules, especially chondroitin sulfate proteoglycans (CSPGs) [1-2]. Axon regeneration beyond glial scar is prohibited by these inhibitory molecules and the physical barrier of glial tissue [3]. In particular, the activity of astrocytes plays a crucial role in glial scar formation and several therapeutic approaches have attempted to inhibit astrogliosis so as to attenuate scar formation for CNS regeneration [4]. Unfortunately, detailed understanding on astrocyte behavior and the interaction with the micro-environmental signals remain limited. The study of the astrocyte-substrate interactions may, therefore, contributes to the basic knowledge for optimal scaffold design for CNS repair.

Cells encounter a wide variety of distinct signals in the body from the surrounding environment, which influence and regulate cellular behaviors and functions. The imposed topography is one influential element which can affect cell morphology, adhesion, migration, proliferation, apoptosis and even gene expressions [5-6]. Therefore, in a tissue engineering approach, it is desirable to modulate cell responses in a predictable manner by the topography of the engineered scaffolds. To understand the cell-substrate interactions, scaffolds with various topographies at micro- and nano-scale have been investigated, including grooves and ridges, pitches, and fibers [7-10]. Among different

formats of tissue engineered scaffolds, increasing studies have been carried out using electrospun fibrous scaffolds.

Electrospinning is a versatile fiber fabrication technique where a broad range of source materials can be utilized for purpose-specific applications. Various sizes of the electrospun fibers can be readily prepared by altering fabrication parameters and the composition of the polymer solutions [11]. Particularly, a close representation of the architecture of the nature ECM can be obtained using the electrospun nanofibrous scaffolds, providing suitable topographic signals for cell growth. Moreover, chemical cues, such as growth factors [12-13], drugs [14-15], DNA [16] and small-interfering RNA (siRNA) [17], can be incorporated into the electrospun fibrous scaffolds to achieve a synergistic effect for regenerative purposes.

In this study, primary cortical astrocytes were cultured on poly (caprolactone-co-ethyl ethylene phosphate) (PCLEEP) nanofibers to evaluate the effects of nanofiber topography on astrocyte proliferation, apoptosis and phenotypic changes. The choice of polymer stems from its controllable degradation rate and enhanced hydrophilicity that facilitated siRNA delivery [13]. As a starting step to evaluate the potential of using siRNA and nanofibers for CNS injury repair, the uptake and silencing efficiency of ChPF siRNA in astrocytes in response to nanofiber topography was also evaluated.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of PCLEEP scaffolds

The PCLEEP copolymer was synthesized with 1% ethyl ethylene phosphate (EEP) (Mw: 40000 g/mol) through bulk ring-opening polymerization of ϵ -caprolactone and ethyl ethylene phosphate (EEP) (Sinopharm Chemical Reagent Co., Ltd., China) according to a procedure described by Wang *et. al* [18]. Briefly, ϵ -caprolactone and EEP [19] were mixed in a fresh flamed and nitrogen-purged round-bottomed flask and then the polymerization was performed at 120 °C for 48 h with Sn(Oct)₂ as a catalyst in a glovebox with a water content less than 0.1 ppm. The resultant polymer was dissolved in dichloromethane and precipitated in cold ethyl ether: methanol (10:1, v/v), filtrated and dried under vacuum. The precipitated polymer was further purified by dissolving in tetrahydrofuran (THF) and precipitated in water at room temperature, filtrated and freeze-dried to produce the final product. PCLEEP was synthesized with a molecular weight of 40 kDa.

PCLEEP nanofibers were fabricated by electrospinning technique. In brief, PCLEEP polymer solution was prepared by dissolving in 2,2,2-trifluoroethanol (TFE) (Fluka, China) at a concentration of 22 wt% and then mixing with TE buffer (pH 8.0, 1st Base, Singapore) at 10:1 (volume ratio). The polymer solution was loaded into a syringe and the flow rate was set at 1.5 mL/h, which was controlled by a syringe pump (New Era pump systems Inc. US). Electrostatic field was applied by a high DC voltage power supply (GAMMA high voltage research, US) with +10 to 12 kV on the polymer solution and -1 to -3kV to the collector. PCLEEP nanofibers were then ejected from a 21G needle and deposited onto an aluminum foil strip, which was attached to a custom-made rotating disk at low rotation speed (100-300 rpm). The distance between the needle and the collector was 9-10 cm. PCLEEP films

were prepared as a two-dimensional (2D) control by solvent-casting. Briefly, 0.66 g of PCLEEP polymer was dissolved in 9 mL of chloroform (Sigma-Aldrich, USA). The resulting polymer solution of 4.7 wt% was poured onto a horizontally placed glass petri dish and air-dried overnight. Scaffolds were punched into circular shape with diameter of 16 mm and 20 mm to fit into 24- or 12-well tissue culture plates respectively for cellular studies. The fabrication process of PCLEEP scaffolds was carried out at humidity of 56-64% and temperature of 19-23.5 °C. Scaffolds were stored in -20 °C with desiccants until use to prevent hydrolysis and degradation.

5.2.2. Characterization of PCLEEP scaffolds

Surface morphology of PCLEEP scaffolds was evaluated under a scanning electron microscopy (SEM) (JEOL, JSM-6390LA, Japan). For nanofibers, fiber diameter was determined using ImageJ (NIH) based on SEM images. One hundred fibers were measured to obtain the average fiber diameter, which was expressed as mean $\pm$ standard error (SE) of the mean.

5.2.3. Cell culture

E18 old rat embryos were sacrificed in a CO₂ chamber according to current Institutional Animal Care and Use Committee (IACUC) guidelines. The brains were transferred to a 10 cm petri dish containing Earle's balanced salt solution (EBSS) (Invitrogen) using microdissecting scissors. While steadying the brain with the forceps, the cerebrum was separated from the cerebellum and the brain stem, and the cerebral hemispheres were separated from each other by gently teasing along the midline fissure with the sharp edge of a second forceps.

The cortex was deflected and peeled away, leaving behind the white matter. The meninges were gently peeled from the individual cortical lobes that were immediately placed into a fresh 10 cm petri dish containing EBSS. After that, the cortices were dissociated into a cell suspension by enzymatic digestion using papain at concentration of 25 units/mL (Worthington) in EBSS and HEPES buffer. Digested cells were then filtered through a 70 μ m cell strainer and spun down at 1800 rpm for 3 min. Cells were resuspended into MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2% glucose, and 1 % penicillin-streptomycin (GIBCO). Cells were plated in 25 cm² tissue culture flasks at a concentration of 5×10^6 cells in 5 mL of culture medium and incubated at 37°C for 72 hours. Neuronal cells were removed by constant agitation overnight. The medium was changed at this time every 48 hours until the monolayer is achieved in the flask. Primary rat cortical astrocytes were maintained in DMEM medium (Hyclone) supplemented with 10% FBS (Hyclone) and 1% penicillin-streptomycin. Cells of passage 2-11 were used in the study.

5.2.4. Proliferation and apoptosis assay

The proliferation and apoptosis rates of primary rat astrocytes cultured on PCLEEP nanofibers and films were evaluated at Day 1, Day 3 and Day 7 (n=3). Nanofiber and film scaffolds were sterilized by 30-min soaking in 70% ethanol followed by 1-h UV radiation. Sterile scaffolds were washed twice with PBS (pH 7.4, GIBCO) and equilibrated in DMEM medium for at least 30 min before use. Thereafter, astrocytes were seeded onto pre-equilibrated scaffolds at a seeding density of 0.4×10^5 cells/well in 24-well plate. Sterile Teflon rings

were placed on top of scaffolds to hold them to the bottom of the plate and ensure cells growing on the scaffold surface. Culture medium was changed at Day 3 for cells collected at Day 7.

The proliferation rate of astrocytes was determined by measuring DNA synthesis using 5-ethynyl-2'-deoxyuride (EdU) (Click-iT™ EdU Imaging Kits, Invitrogen). One day before sample collection, EdU solution was added into cell culture at a final concentration of 10 μ M for an overnight incubation. Exceptionally, EdU solution was directly mixed with cell suspension during seeding for Day 1 sample. Negative control was prepared by astrocyte seeding on glass cover slips omitting EdU addition. At specific time points, cells on scaffolds were fixed in 10% formalin (Sigma) for 30 min at room temperature (RT). After two washes in 3% BSA (Sigma) in PBS, cells were permeabilized in 0.5% Triton X-100 (Sigma), 50 mM glycine (Sigma) for 20 min at RT. Following that, cells were incubated in Click-iT reaction cocktail for 30 min at RT, protecting from light. Then the cells were counterstained in propidium iodide (PI) (Invitrogen) for 30 min in dark. Lastly, cells on scaffolds were mounted onto glass slides and stored in a dark container at 4 °C before image analysis. The staining of EdU and PI staining were carried out following the manufacturer's protocols with minor modifications.

The apoptosis rate of astrocytes was determined using deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay (Click-iT™ TUNEL Alexa Fluor® Imaging Assay, Invitrogen). The detection of cell apoptosis is based on the incorporation of modified dUTPs into the fragmented DNA in cells observed under fluorescent microscope. Cells culture and preparation were performed the same way as EdU assay described above and the TUNEL assay

was carried out following the manufacturer's protocol. Briefly, at specific time points, cells were fixed in 10% formalin at RT for 30 min. After two rinsings in deionized (DI) water, cells on scaffolds were permeabilized in 0.5% Triton X-100, 50 mM glycine solution for 20 min. To ensure appropriate staining, positive controls were prepared with astrocytes cultured on glass cover slips and treated with DNase I for 30 min at RT. Thereafter, all cell samples were rinsed with DI water and equilibrated in TdT reaction buffer for 10 min at RT. Cells were then incubated in TdT reaction cocktail for 60 min at 37 °C in a humidified incubator. Negative controls were prepared by seeding cells on glass cover slips omitting the addition of TdT reaction cocktail. Cells were then incubated in Click-iT reaction cocktail for 30 min at RT, avoiding exposure to light. Lastly, cells were counter-stained with PI and kept in dark at 4 °C before imaging.

5.2.5. Immunostaining

Glial fibrillary acidic protein (GFAP) immunostaining was performed to assess the morphological changes and cell attachment on PCLEEP scaffolds. Cell culture and sample preparation were described above. At time points of Day 1, Day 3 and Day 7, astrocytes cultured on scaffolds were fixed in 10% formalin. Cells on scaffolds were rinsed in Tris-buffered saline (TBS, pH 7.4-7.6) and then blocked three times in TBS++ which is composed of 0.23% Triton X-100 and 4.7% FBS in TBS, 10 min for each time. Following that, cells were incubated in anti-GFAP antibody (Millipore) solution (1:500 dilution in TBS++) at 4 °C overnight with constant agitation. After rinsing in TBST (TBS with 33% Triton X-100), samples were then incubated in the secondary

antibody (1: 500 dilution in TBS++) (Alexa Fluor[®] 488, Invitrogen) for 1 h at RT avoiding light. Lastly, PI was applied for nuclei staining.

5.2.6. Image analysis and quantification

Astrocytes on scaffolds stained for proliferation assay, apoptosis assay and GFAP immunostaining were evaluated under a confocal microscope (Zeiss, LSM 510 Meta Laser Scanning Confocal Microscope, Germany). For proliferation and apoptosis studies, fluorescent images were acquired at 10× magnification at six random fields per scaffold (n=3) for quantification. Cell numbers were manually counted with ‘Cell Counter’ function using ImageJ (NIH). An average number of approximately 300 cells were counted in each field. Cell morphology was evaluated by two parameters, cell area and cell shape index (CSI), based on GFAP immunostaining images. Six fields were captured randomly at 20× magnification for one scaffold (n=3). In total, 100 cells per sample were measured for area and perimeter analysis. Cell borders were outlined using ImageJ and cell area and perimeters were thereafter measured. Cell shape index was defined according to the formula $P^2/(4 \times \pi \times A)$, where P is the cell perimeter and A is cell area. The cell shape index value of 1 indicates a perfect circular cell shape while larger value suggests a more dendritic morphology.

5.2.7. SDS-PAGE and western blot analysis

Astrocytes were seeded on sterile nanofibers and films at a seeding density of 3×10^5 cells/well in 1 mL of culture medium in 12-well plate for protein analysis. At day 3, 1 mL of fresh culture medium was added to Day 7

samples to supply sufficient nutrients for cell growth. Cells were trypsinized at Day1, Day 3 and Day 7 by 0.25% trypsin/EDTA (GIBCO) and cell pellets were lysed using RIPA buffer (Sigma) supplemented with complete protease inhibitor cocktail (Roche). Protein concentrations were then determined using Micro BCA protein analysis kit (Pierce). Protein samples of 5-10 µg were mixed with Laemmli buffer (Biorad) with 5% 2-mercaptoethanol (Sigma) and boiled at 100 °C for 5 min before loading. Protein electrophoresis was performed in a 12% SDS-PAGE gel with a 4% stacking gel. The gel was subsequently blotted to PVDF membranes. Non-specific binding sites on membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffer saline with 0.1% Tween 20 (Sigma) for 1 hr at RT. Blots were then incubated in primary antibody which were diluted in 5% BSA for 2 h at RT or overnight at 4 °C: vimentin (1:15,000) (Millipore), GFAP (1:10,000) (Millipore) and β-actin (1:200) (Santa-Cruz). β-actin was used as the loading control. Thereafter, antibodies were labeled with HRP anti-mouse secondary antibody (1: 10,000) (Amersham Biosciences) by 1-h incubation at RT. Blots were developed using ECL Plus detection reagents (Amersham Biosciences) and visualized by quick exposures to x-ray films (Kodak).

5.2.8. Cy5-ODN uptake efficiency analysis

To visualize the cellular uptake, Cy5-labelled oligonucleotides (Cy5-ODN) (1st Base, Singapore) were used as a substitute of siRNA. Cy5-ODN was synthesized with scrambled sequences: sense: 5'-Cy5-CCTACG CCACCAATTTTCGTTT-3'; anti-sense: 5'-ACGAAATTGGTGGCGTAGGTT-3'). One day before transfection, primary rat astrocytes were seeded onto sterile

PCLEEP nanofibers and films at 0.5×10^5 cells/well in 24-well plate. Cy5-ODN was delivered to astrocytes with transfection reagent Trans-iT TKO (MirusBio, USA). In brief, 1.667 μL of TKO reagent was diluted in 50 μL of serum-free DMEM medium and was incubated for 10 min at RT. Then 15 pmol of Cy5-ODN was added into TKO solution and the mixture was gently mixed to allow complexation between Cy5-ODN and TKO. After 10-min incubation, 50 μL of Cy5-ODN/TKO complex was added dropwise to cell cultures in a total volume of 450 μL of culture medium, making a final concentration of Cy5-ODN to 30 nM. The negative controls on both nanofibers and films received the same amount of Cy5-ODN in DMEM medium in the absence of transfection reagent. Twenty-four hours after transfection, cell were fixed and labeled with GFAP antibody with a counterstaining in PI, following the same staining procedure as described in the previous section. The Cy5-ODN uptake was assessed under the confocal microscope. Ten random fields were captured at $63\times$ magnification per scaffold ($n=3$) under the same laser strength and operational parameters. The quantification of Cy5-ODN uptake was performed measuring Cy5-ODN intensity inside cells using ImageJ based on fluorescent images.

5.2.9. siRNA transfection efficiency analysis

Chondroitin polymerizing factor (ChPF) and GAPDH siRNAs (Ambion, US) were used in this work for the evaluation of siRNA transfection efficiency in astrocytes on nanofiber and 2D topographies. ChPF is a key enzyme involved in CSPG synthetic pathway which adds inhibitory glycosaminoglycan (GAG) side chains onto CSPG core proteins. Down-regulation of ChPF by siRNA was shown to effectively enhance neurite outgrowth *in vitro* [20]. One day before

transfection, astrocytes were plated onto sterile PCLEEP scaffolds at a density of 1×10^5 cells/well in 12-well plate (n=3). siRNA/TKO transfection complexes were prepared by mixing 3.33 μL of TKO with siRNA in 100 μL of serum-free DMEM medium. One hundred μL of siRNA/TKO complex was then added into cell cultures with a total culture medium volume of 900 μL . siRNA final concentrations of 0.2 nM, 2 nM and 30 nM were studied for comparison. Negative controls were included on both nanofibers and films with negative control siRNA (Ambion, US) complexed with TKO reagent. Medium was changed 24 h after transfection and the silencing efficiency was analyzed 48 h post transfection.

5.2.10. RNA isolation and real-time RT-PCR

Cells on scaffolds were lysed in TRIzol[®] reagent (Invitrogen) and RNA was isolated using RNeasy[®] Mini Kit (Qiagen) with a revised protocol. RNA was treated with DNase (RQ1 DNase, Promega) before reverse transcription. cDNA was synthesized using Sensiscript[®] RT Kit (Qiagen). The real-time PCR was carried out with iQ[™] SYBR[®] Green Supermix (Bio-rad) in StepOne[™] Plus Real-Time PCR system (Applied Biosystems). The primer sequences used were as follows: ChPF forward 5'-CAAGAAGGCCGTCAGAACTC-3', ChPF reverse 5'-GGTTTACAGCCACACCCAGT-3' with a product size of 116 bp; GAPDH forward 5'-TGATTCTACCCACGGCAAGTT-3, GAPDH reverse 5'-TGATGGGTTTCCCATTTGATGA-3 with a product size of 77 bp; β -actin forward 5'-GGGAAATCGTGCGTGACATT-3', β -actin reverse 5'-GCGGCAGTGGCCATCTC-3' with a product size of 76 bp. The similarity of the amplification efficiencies of three primers was confirmed by plotting

standard curves, and the $\Delta\Delta C_T$ method was used to analyze mRNA levels. Real-time PCR was programmed as follows: 3 min at 95 °C, amplification for 40 cycles at 95 °C for 15 s and 60 °C for 30 s. Melt curves were also plotted to ensure no primer-dimer formation for three genes.

5.2.11. Statistics

All statistics were carried out in SPSS. Independent-samples t-test was performed to calculate difference between films and nanofibers. The *p* values less than 0.05 were considered significantly different. All data are expressed as the mean $\pm$ standard error (SE) of the mean.

5.3. RESULTS

5.3.1. Characterization of PCLEEP scaffolds

PCLEEP nanofibers and films were prepared by electrospinning and solvent-casting methods respectively. The morphology of PCLEEP nanofibers and films is shown in Fig. 5.1. Uniform fibers were obtained with an average fiber diameter of 665 ± 11 nm. PCLEEP films exhibited flat and smooth surfaces with minor irregularities.

Fig. 5.1. The morphology of PCLEEP nanofibers and film prepared by electrospinning and solvent-casting methods. A: PCLEEP fibers (insert: higher magnification under SEM); B: PCLEEP films.

5.3.2. Cell proliferation and cell density

EdU is a nucleoside analog, which is readily incorporated into DNA of replicating cells and the rate of cell proliferation is, therefore, presented by the EdU incorporation rate. Astrocyte proliferation rates on PCLEEP films at Day 1 and Day 3 were 28.2% and 40.2%, which were significantly higher than that on PCLEEP nanofibers, where 9.9% and 18.9% of EdU incorporation rates were achieved. At Day 7, low proliferation rates of 8.5% and 9.3% were observed on nanofibers and films (Fig. 5.2A). Cell density on different topographies at Day 1, Day 3 and Day 7 is shown in Fig. 5.2B. Initial cell adhesion on nanofibers and films was comparable as reflected by cell density at Day 1. Cell number on films gradually increased during culture and reached a confluent density of 478 cells/mm² at Day 7 (Fig. 5.2B), which resulted in a low EdU incorporation rate on films at Day 7. A low percentage of EdU positive cells on nanofibers was also observed at Day 7. However, the significant lower cell density than that on the films indicated that topographic effect other than space constraint limited astrocyte growth on the nanofibers.

Fig. 5.2. Nanofiber topography inhibits astrocyte proliferation compared to films in culture. A: Proliferation rate of astrocytes measured by EdU incorporation. B: Total astrocyte numbers on nanofibers and films. * indicates $p < 0.01$. (Mean $\pm$ SE, $n = 3$)

5.3.3. Cell apoptosis

Cell apoptosis was determined by DNA fragmentation detected using TUNEL assay. As shown in Fig. 5.3, the apoptosis rates of astrocytes on nanofibers were 11.3%, 11.9% and 15.8% at Day 1, Day 3 and Day 7, respectively. In contrast, significantly lower apoptosis rates were observed on 2D substrates, where 5.0%, 7.1% and 4.9% of TUNEL positive cells were obtained at Day 1, Day 3 and Day 7, respectively.

Fig. 5.3. Apoptosis rate of primary astrocytes on PCLEEP films and nanofibers was evaluated by TUNEL assay at different time points. * indicates $p < 0.05$ and ** means $p < 0.01$. (Mean $\pm$ SE, $n = 3$)

5.3.4. Cell phenotypic change

The representative astrocyte morphology on PCLEEP nanofibers and films at 3 day *in vitro* is shown in Fig 5.4. Cell morphology was similar on Day 1 and Day 7 as compared to that on Day 3 as shown in the figure. Astrocytes on films adopted fully stretched morphology and were well spread on the 2D flat surface with GFAP filaments arranged in a delicate criss-cross pattern, showing a similar morphology as cells cultured on TCPS. In contrast, astrocytes exhibited elongated and ramified shape on the nanofibers with condensed GFAP filament arrangements, which is a more typical astrocyte appearance *in vivo* [21]. The topographic effect on cell area and cell shape index of astrocytes was shown in Fig. 5.5. Smaller cell area was observed when astrocytes were cultured on nanofibers, with a narrow area distribution below $2000 \mu\text{m}^2$. In contrast, astrocytes on the 2D surfaces exhibited larger cell area and wider area distribution which seemed to expand with time. On the other hand, cell shape

index showed that astrocytes adopted remarkably more dendritic morphology in response to nanofiber topography as compared to 2D surfaces. Furthermore, more astrocytes possessed larger CSI at later time points on nanofibers, while insignificant changes in CSI range was seen on films.

Fig. 5.4. GFAP immunostaining showed astrocyte morphology on PCLEEP nanofibers (A,B) and films (C,D) at 3 day *in vitro*. Nanofiber topography enhanced astrocyte elongation and stellation. Scale bar in (A,C) = 50 μm and (B,D) = 20 μm . Green: GFAP; red: nuclei.

Fig. 5.5. Evaluation of astrocyte morphology on PCLEEP nanofibers and films at Day 1, Day 3 and Day 7. A,B: Cell area distribution of astrocytes on nanofibers (A) and films (B) over time. C,D: Distribution of CSI respond to the nanofiber topography (C) and 2D flat surface (D).

The activation of astrocytes often comes with a ramified morphology change, similar to the results observed in Fig. 5.4A, B. However, changes in the GFAP morphology are not an essential indication of astrogliosis. Therefore, protein expressions of vimentin and GFAP in response to topographic difference were evaluated. As shown in Fig. 5.6, no observable change was found in astrocytes in response to different topographies. Vimentin and GFAP level remained the same on both Day 3 and Day 7.

Fig. 5.6. Vimentin and GFAP protein expression in astrocytes on PCLEEP nanofibers (FB) and films (FM) at Day 3 and Day 7 in culture. β -actin served as the loading control.

5.3.5. Efficiency of Cy5-ODN uptake and siRNA silencing

To visually track siRNA uptake by astrocytes, Cy5-labeled ODN with a scrambled sequence was used as a substituted (Fig. 5.7). In the absence of TKO reagent, no detectable Cy5 signals was found in astrocyte cytoplasm on both nanofiber and film substrates (Fig. 5.7A,C), indicating that naked Cy5-ODN was unable to cross cellular membrane. In contrast, in the presence of TKO reagent, Cy5-ODN was successfully introduced into astrocytes and distributed around cell nuclei in cytoplasm on both nanofibrous and 2D substrates. The Cy5-ODN uptake efficiency was quantified by the intensity of Cy5 signals presented in cytoplasm expressed in relative fluorescent units (RFU). A Cy5 intensity of 2.31×10^5 RFU/cell was achieved on 2D surfaces, which was significantly higher than that on nanofiber topography where 1.51×10^5 RFU/cell was obtained.

Fig. 5.7. Cy5-ODN uptake by astrocytes on PCLEEP nanofibers (A,B) and films (C,D). No observable Cy5-ODN signal was seen in naked Cy5-ODN transfections in the absence of transfection reagent (A,C). Cy5-ODN/TKO signals were detected around nuclei in cytoplasm on both nanofibers (B) and films (D). Green: GFAP; red: Cy5-ODN; blue: nuclei. Scale bar = 20 μ m. F: Quantification of Cy5 signal intensity revealed significantly higher Cy5-ODN uptake on films than on nanofibers. * indicates $p < 0.01$.

To evaluate siRNA silencing efficiency, two intracellular genes were targeted in this study, ChPF and GAPDH. Different from the observation in Cy5-ODN uptake, higher silencing efficacy was attained on nanofibers compared to that on films targeting ChPF gene (Fig. 5,8A). The ChPF mRNA levels were decreased by 74.4%, 76.0% and 75.0% at ChPF siRNA concentration of 0.2 nM, 2 nM and 30 nM, respectively. Meanwhile, ChPF gene in astrocytes on films was down-regulated by 54.1%, 51.7% and 69.0% at siRNA concentration of 0.2 nM, 2 nM and 30, respectively. Slight elevation in ChPF silencing was obtained with the highest siRNA concentration (30 nM), however difference was not significant. Nanofibers enhanced higher ChPF down-regulation at a wide siRNA concentration range as investigated, and significant differences were observed at lower siRNA concentration of 0.2 nM

and 2 nM ($p < 0.01$). On the other hand, the silencing result targeting GAPDH differed from ChPF siRNA transfection, as shown in Fig. 5.8B. The silencing efficiencies for GAPDH genes on nanofibers were 28.5%, 61.0% and 79.5% at siRNA concentrations of 0.2 nM, 2 nM and 30 nM respectively, while 41.7%, 58.8% and 73.3% were achieved on films. In a drastic contrast to ChPF silencing, an siRNA concentration-dependent silencing pattern was seen in the down-regulation of GAPDH gene on both nanofibers and films. At concentration of 30 nM, GAPDH genes were sufficiently knocked down (~70%) by siRNAs on both substrates. However, the silencing efficiency of GAPDH was greatly reduced as GAPDH siRNA concentration decreased, and a significant reduction ($p < 0.05$) was seen at 0.2 nM compared with siRNA concentration of 30 nM.

Fig. 5.8. siRNA silencing efficiency of target genes at different siRNA concentrations in astrocytes on PCLEEP nanofibers and films. A: ChPF siRNA transfection efficiency. ** indicates $p < 0.01$ between paired groups. B: GAPDH siRNA transfection efficiency. * means $p < 0.05$ compared to siRNA concentration of 30 nM on nanofibers and # indicates $p < 0.01$ comparing with siRNA concentration of 30 nM on films. Neg: negative control of cells transfected with negative control siRNA/TKO complex. (Mean $\pm$ SE, $n = 3$)

5.4. DISCUSSION

Topographic cues have long been recognized as an important stimulus influencing cellular behaviors, including cell adhesion, morphology, phenotypic changes and also gene expressions. Adequate biomimetic topography provided by scaffolds may modulate cellular behaviors and functions in a desired manner

for specific applications. Studies have already been performed employing electrospun nanofibers for nerve regeneration [22]. Electrospun nanofibrous scaffolds are able to enhance neurite extension and improved axon regeneration *in vitro* for the regeneration of peripheral nervous system (PNS) [23-24]. Furthermore, a certain degree of functional recovery was also observed by scaffold implantation to overcome critical gaps in sciatic nerve injury with both randomly-oriented and aligned electrospun fibers [25-27]. Apart from the positive influence presented to neurons, little is known about the nanofiber topographic effect on other cell types in the nervous system. Particularly, it is generally believed that astrocytes are the major contributor for the inhibitory glial scar, and therefore, it is desirable to diminish astrocyte activity to attenuate glial scar formation for CNS regeneration. It would be of special interest to understand the astrocyte-substrate interactions for the optimization of scaffold design. Accordingly, we investigated the nanofiber topographic effects on astrocyte behaviors, including cell proliferation, apoptosis, and phenotypic changes. The siRNA uptake efficiency was also studied to evaluate the potential application of RNAi modulation over cell fate in response to topographic cues. Our results suggest that the electrospun nanofibers may serve as a promising bio-functional scaffold for CNS repair.

Electrospinning is a versatile method to fabricate fibrous scaffold with diameters ranging from several nanometers to micron regime [28]. Compared to microfibers, nanofibers are more favorable for tissue engineering applications, as nature ECM and basement membranes are composed of various components such as fibers, pores, and pits at nano-scales [29]. In the present study, we produced electrospun nanofibers with an average diameter of 665 nm using a

synthetic copolymer PCLEEP. In comparison with the widely used biocompatible polymer polycaprolactone (PCL), PCLEEP includes more hydrophilic structure (ethyl ethylene phosphate) [13], which can fine-tune the biodegradability and hydrophilicity for drug delivery applications.

Diversified observations have been reported on the growth of various cell types cultured on distinct electrospun fibers. The understanding of the fiber topographic effect on cell proliferation and apoptosis is complicated by different variables, including cell type [30-33], the properties of the polymer of choice [34-35] and the resultant electrospun fibers [36-37]. In our work, the proliferation and apoptosis assays revealed that PCLEEP nanofibers discouraged cortical astrocyte growth and enhanced cell death, as compared to a 2D surface. The topographical effect elicited prompt cell responses, as drastic changes in the proliferation and apoptosis rates were detected as early as Day 1. Decreased cell viability on PCL electrospun fibers with diameter of 700 nm was also reported by Lord *et al.* for primary hippocampal astrocytes culture, in comparison with that on TCPS [38]. It has been suggested that topographical effect may influence a spatial organization of cytoskeleton-linked membrane receptors [39] and affect the conformation of protein bindings on the substrate surface [40], which in turn affect cellular functions. However, the exact mechanism remains to be elucidated. Astrocytes in healthy CNS tissue usually have little or no proliferation *in vivo* [41]. Therefore, these results implied the suppressive role of nanofiber topography on astrocyte growth *in vitro*. Our findings may provide partial reasons for the reduced scarring observed in rat cortices that were treated with nanofibers, while severe glial scar formation was induced by implanting flat substrates [42-44]. .

In our study, we found that astrocyte exhibited drastic morphology change on PCLEEP nanofibers in comparison with that on 2D flat surface within 7 days. Elongated and ramified morphology was observed on nanofibers, while stretched cell shape was seen on 2D flat substrate. On electrospun nanofibers, the protruding filapodia from astrocytes tended to attach on fiber surface or on the joint sites where several fiber converge, as shown in SEM images observed by others [10, 45]. Astrocytes are inclined to lay their filapodia and processes on the surface of the fibers for focal adhesion formation, dodging void space of the interfiber pores. In contrast, astrocytes completely attached to the flat substrates and exhibited a fully stretched morphology on PCLEEP film. This difference in cell adhesion and attachment may ultimately affected cell morphologies, however, the underlying mechanism is not well understood. It is noteworthy that the effect of nanofiber topography on cell morphology is also cell-type dependent, and the morphological changes may vary with polymer property [46] and fiber diameter [36-37]. Endothelial cells on terpolymer fibers [32] and human osteoblast-like MG63 cells on collagen nanofibers [47] exhibited fully spread morphology on the electrospun fibers, adopting similar cell shapes of that on the flat surfaces. Ramified morphological changes commonly relate to astrocyte activation [48]. Therefore, we investigated the expression of astrocyte specific proteins, vimentin and GFAP, which are usually up-regulated during astrogliosis [49-50]. Despite the morphological changes, we found that the nanofibrous scaffolds did not exert observable impact on the expressions of vimentin and GFAP as compared to the 2D control.

siRNA is a powerful tool in regulating cell functions by selectively silencing the target genes. Recently, increasing attentions have been drawn on applying siRNA technology to tissue engineering and regenerative medicine [51-52]. New perspectives for nerve regeneration utilizing siRNA have also emerged. Down-regulation of neurite outgrowth inhibitors Nogo and MAG in CNS to enhance nerve regeneration are a few examples [53-54]. A recent study identified a new way to alleviate CSPGs by using siRNA targeting ChPF, which is a key component in CSPG-GAG chain synthesis [20]. Silencing of ChPF gene significantly reduced CSPG production and enhanced neurite outgrowth from cerebellar granule neurons was achieved *in vitro*. In order to evaluate the efficacy of the siRNA-encapsulated nanofibrous scaffolds for CNS injury treatment, we first decoupled the signals by focusing on topographical effect on astrocyte behavior and gene-silencing extent. Despite the decrease in Cy5-ODN uptake in response to nanofibers, the resulting gene silencing efficiency was similar to the 2D control when cells were treated with an equivalent amount (30 nM) of ChPF siRNA. In fact, nanofiber topography significantly enhanced ChPF silencing efficiency at low siRNA concentrations (0.2 and 2 nM). These results suggest that while nanofiber topography decreased the internalization of siRNA by astrocytes as compared to a flat surface, it facilitated the downstream silencing process. After cellular uptake, siRNA undergoes several essential steps before binding to its complementary mRNA for cleavage. These include endosomal escape, the unpacking of siRNA-transfection reagent complexes, and the formation of RNA-induced silencing complex (RISC) [55,56]. Two different scenarios were observed in silencing GAPDH and ChPF genes (Fig. 5.8). On nanofibers, effective silencing of ChPF genes (74.4-76.0%) was

obtained with siRNA concentration ranging from 0.2 nM to 30 nM, while silencing efficiency decreased with lower siRNA concentration for GAPDH gene. In the real-time PCR experiment, we found that the C_T value of ChPF was around 5 cyclers larger than that of GAPDH with comparable amplification efficiencies, which indicated a lower amount of ChPF mRNA in astrocyte cytoplasm. As a result, the threshold siRNA concentration for an effective knock-down of ChPF gene would be lower than that for GAPDH, which may explain the difference in silencing ChPF and GAPDH shown in Fig. 5.8. The similar trend was also observed on films. It is likely that the enhanced silencing effect by topography can be undermined when large amounts of siRNA are present.

At present, detailed understanding of substrate topographical effect on gene-silencing is lacking. However, it is possible that nanofiber topography facilitated these downstream cytoplasmic processes more efficiently. Besides topographical effect, the availability of siRNA may also affect the final silencing outcome. At higher concentrations of siRNA (30 nM), the difference in gene silencing efficiency due to substrate topography was abolished. Further investigations using fluorescent tracking techniques such as fluorescence resonance energy transfer (FRET) and live cell imaging may provide useful insights to the possible mechanisms involved in our experimental observations.

Interestingly, electrospun nanofibers appear to inhibit neural stem cells from differentiating into astrocytes and promote neuronal differentiation [45]. In the differentiation media supplemented with 1 μ M of retinoic acid and 1% FBS, rat neural stem/progenitor cells (NSCs) seeded on poly(ethersulfone) (PES) nanofibers of 749 nm in diameter showed a negligible differentiation into

astrocytes and ~60% into neurons, whereas on TCPS, ~7% of the cells differentiated into astrocytes and ~40% into neurons. This result implies that the potential application of the electrospun nanofibers for nerve regeneration can also be coupled with stem cell therapy.

5.5. CONCLUSIONS

Astrocyte responses to the nanofiber topography was investigated and discussed in the present work, aiming to provide insights to the rational design of tissue engineered scaffolds for CNS regeneration. The nanofiber topography suppressed astrocyte growth and was able to enhance siRNA silencing efficiency. Altogether, we believe that the electrospun nanofibers may serve as a potential scaffold for gene silencing applications to promote CNS regeneration. Our next step is to develop siRNA-loaded electrospun nanofibrous scaffolds reducing CSPG after injury so as to create a permissive environment for nerve repair in the CNS.

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CHAPTER 6

THE TOPOGRAPHICAL EFFECT OF THE ELECTROSPUN

NANOFIBROUS SCAFFOLDS ON THE *IN VIVO* AND *IN VITRO*

FOREIGN BODY REACTION

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SUMMARY

Topographical cues play an important role in influencing cellular behavior and are considered to be significant parameters to be controlled in tissue engineering applications. The present work in this chapter investigated the biocompatibility with regard to scaffold architecture and topographical effect of nanofibrous scaffolds on the *in vivo* and *in vitro* foreign body reaction. Random and aligned polycaprolactone (PCL) nanofibers were fabricated by electrospinning technique. Primary monocytes isolated from human donors were cultured on different surfaces for *in vitro* evaluation. The *in vivo* study was carried out by implanting PCL nanofibers and film scaffolds subcutaneously in rats to test the biocompatibility and host response. It is observed that fibrous scaffolds elicited significantly thinner fibrous capsulation formed around the tissue-implants interface, compared to film implants. Additionally, cell infiltration was observed in aligned fibrous scaffolds both *in vitro* and *in vivo*. These results indicate that aligned electrospun nanofibers may serve as a promising scaffold for tissue engineering by minimizing host response, enhancing tissue-scaffold integration, and eliciting thinner fibrous capsules.

6.1. INTRODUCTION

The foreign body reaction is a normal component of the healing process which is observed after implantation of medical devices in the host. It is the end-stage of the inflammatory and wound healing response. Macrophage and foreign body giant cells play a crucial role in the foreign body reaction, and their responses toward implant materials have significant impact on the proper functioning of medical devices [1]. Inappropriate tissue response on the material surface often leads to device failure. The formation of the fibrous capsule during the foreign body reaction is one common barrier for normal function of certain medical devices, such as biosensors [2-3], drug delivery systems [4-5], eye implants [6], etc. Efforts have been made in order to reduce fibrous capsule thickness. Unfortunately, due to limited knowledge of the mechanism of the foreign body response to implanted materials, fulfilling complete functions of medical devices remains challenging. Material surface chemistry [7], physical properties [8], and morphological features [9] all play a part in modulating cellular reactions towards implant materials.

Biomaterial topography may have a significant impact on the foreign body reaction [10]. Porous structures have resulted in moderate tissue responses and enabled faster healing processes. The fibrous capsule formed on a porous surface was thinner compared to a dense solid implant surface [11-12]. Surface geometry has also been extensively studied to understand its influence on macrophage behavior and host responses. Micro-patterned grooves and ridges have been employed as substrates to understand macrophage behavior and foreign body reactions towards micro-sized topography [9, 13-14]. The study of nanotopography is actually more substantial as the natural extracellular

matrix (ECM) surrounding the cells is composed of nano-sized components [15]. A number of studies investigated macrophage responses or tissue reactions toward nano-sized surface geometry [16-19]. However, these studies are far from exhaustive in understanding the underlying mechanism. In the present work, we provide additional understanding of the nano-topographical influence of electrospun nanofiber scaffolds on the foreign body reaction both *in vitro* and *in vivo*. We used a biocompatible synthetic polymer, polycaprolactone (PCL), to fabricate aligned and random nanofiber scaffolds by electrospinning and PCL film were used as a control, to investigate the fiber topographic effect on the *in vivo* and *in vitro* foreign body reactions.

6.2. MATERIALS AND METHODS

6.2.1. Preparation of PCL scaffolds

Polycaprolactone (PCL) (Mw: 65000, Aldrich, USA) nanofibers were fabricated by electrospinning technique [20]. In order to obtain aligned and random nanofibers with similar fiber diameters, different solvent mixtures and electrospinning parameters were used (Table 6.1.). For random fibers, PCL was dissolved in 5:1(v/v) 2, 2, 2-trifluoroethanol (TFE) (≥ 99.0 , Fluka, China): deionized H₂O (DI H₂O) to obtain a concentration of 14wt%. 9.5wt% of PCL in 3:2(v/v) dichloromethane (DCM) (HPLC grade, VWR, EC): methanol (HPLC grade, Panceac, EU) was used for aligned fiber fabrication. The polymer solution was then loaded into a 3 mL syringe connected to a 22G needle. The flow rate of the polymer solution was controlled by a syringe pump (New Era pump systems Inc., USA). High DC voltage (GAMMA high voltage research, USA) was applied to the polymer solution and the ejected electrospun fibers

were collected using a grounded rotating drum wrapped with aluminum (Al) foil. Due to the weak mechanical property of aligned fibers along the perpendicular direction to the fiber axis, PCL film was attached on top of the Al foil and aligned fibers were directly deposited on the film. This supporting film allowed easy handling of aligned fibers for *in vitro* and *in vivo* studies. The electrospinning parameters for fiber fabrications are summarized in Table 6.1. The spinning process was carried out at room temperature 20-23 °C and the humidity was 51-56%. The PCL film was prepared using a hot press under a temperature of 65-68 °C and a compressive force of 12-16 N force (Carver press, model No.: 4128, USA).

Table 6.1. Electrospinning parameters for aligned and random PCL fibers

	Aligned	Random
Concentration (wt%)	9.5	14
Solvent mixture	3:2 DCM: methanol	5:1 TFE: DI H ₂ O
Flow rate (mL/hour)	1.5	1.5
Distance (cm) ^a	8-9	13-14
Voltage (kV)	13-15	16-18
Rotator speed (rpm)	2600-2700	200-250

^a: Distance from needle tip to collector surface.

6.2.2. Characterization of PCL scaffolds

The morphology of electrospun nanofibers was analyzed using field emission scanning electron microscopy (FESEM) (JOEL, JSM-6700F, Japan). The fiber diameter was determined using ImageJ based on SEM images by measuring 100-300 fibers, and represented as mean $\pm$ standard error (SE) of the mean. Scaffold porosity was calculated by measuring scaffold weight and thickness and then applying the following formulas:

$$Porosity = \left(1 - \frac{Apparent\ Density}{Bulk\ Density}\right) \times 100\%$$

$$Apparent\ Density = \frac{Scaffold\ Weight}{Scaffold\ Volume}$$

6.2.3. Cell culture

Monocytes were cultured using techniques similar to those used previously to assess monocyte/macrophage adhesion and fusion into foreign body giant cells [21-24]. The three types PCL scaffolds (random fibers, aligned fibers and film) were cut into circular samples with a diameter of 1 cm. The PCL scaffolds were then soaked in 70% ethanol for 30 minutes, washed three times with PBS for 5 minutes each, and then irradiated 20 cm away from a UV light source in a cell culture hood for 30 minutes. The PCL scaffolds and autoclaved glass cover slips (positive control), were then placed in four 24-well cell culture plates (n=4) for 4 time points of day 0 (2 hr after seeding), day 3, day 7 and day 10. The glass cover slips received a surface treatment of 0.5 mL of 25 µg/mL RGD peptide to promote cell adhesion [23]. After 30 minutes, the RGD peptide solution was aspirated and rinsed twice with Dulbecco's phosphate buffered saline at pH 7.4 (PBS++) to remove any non-adherent peptide. Silicone rubber rings were sonicated in 100% ethanol for 5 minutes, autoclaved, and inserted into each well to ensure the scaffolds remained at the bottom of the 24-well plates.

Primary monocytes were isolated from 5 human blood donors using centrifugation, Ficoll, and Percoll methods as previously described.[25-26] The initial monocyte concentration was determined by hemacytometer and then diluted to 10⁶ cells/mL using a solution of 10% autologous serum and

macrophage-serum free media (SFM) (Gibco). 0.5 mL of the monocyte solution was then plated on the material surfaces for an initial cell seeding of 5×10^5 cells/well and incubated at 37 °C and 5% CO₂ for 2 hours. After 2 hours, the plates were gently rinsed with 0.5 mL of PBS++ to remove non-adherent cells. The PBS++ solution was then aspirated and one of the four plates was terminated by fixation in a 2.5% buffered glutaraldehyde solution and refrigerated for 3 days to evaluate cell adhesion at day 0 (2 hr). The remaining three plates were re-fed with 1 mL of SFM and incubated. At 3 and 7 days, cells were re-fed with 1 mL of a 5% heat-treated autologous serum which harvested from donors' blood, 15 ng/mL IL-4, and SFM solution to induce foreign body giant cell formation [21-22]. At each time point, cells were fixed with 2.5% buffered glutaraldehyde and refrigerated before further evaluation.

6.2.4. Cell morphology and monocyte/macrophage density

To evaluate cell morphology, cells were fixed at each time point using buffered glutaraldehyde solution and rinsed with Millipore water three times for 5 minutes. The samples were then dehydrated using two 5-minute 1 mL/well applications of 30%, 50%, 70%, and 95% ethanol in water followed by two 15-minute applications of 100% ethanol. Then two 1 mL/well of 100% hexamethyldisilazane (HMDS) were applied to all samples for 15 minutes in order to lower surface tension and maintain cell shapes. After the final aspiration of HMDS, the remaining solution was allowed to evaporate off the samples in a fume hood. Dried samples were sputter coated and examined under analytical SEM (JOEL, JSM-6390LA, Japan) at 200× and 1000× magnification. The density of monocyte/macrophage on scaffolds was determined using the

200× SEM images while the 1000× images were taken to qualitatively assess cell morphology. Each image was divided into four quadrants (upper left, upper right, lower left and lower right) using Adobe Photoshop and the number of monocytes and macrophages were counted manually. Cell density in each area was calculated by dividing the number of cells counted by the surface area of the quadrant. These values were then averaged to calculate average adhesion density for the sample.

6.2.5. Implantation of scaffolds

PCL aligned fibers, random fibers and film were subcutaneously implanted, two per animal, into 12 week-old, female, Sprague Dawley rats (Charles Rivers Laboratories, North Wilmington, MA). These surgeries were performed in the Animal Resource Center (ARC) at Case Western Reserve University using protocols approved by the Institutional Animal Care and Use Committee (IACUC). Proper care for laboratory animals was provided under NIH guidelines both during and after the surgeries. Rats were fed adlibitum and post-operative care was provided by ARC veterinary technicians. The implants were then removed 1, 2, and 4 weeks after surgery and histological evaluation was performed.

For implantation, the PCL scaffolds were cut into 1.0×2.0 cm² sections. They were then soaked in 70% ethanol for 30 minutes, immersed in PBS three times for 5 minutes per rinse, and irradiated 20 cm away from a UV light source for 30 minutes in a sterile cell culture hood. The rats were anesthetized using Aerrane[®] isoflurane anesthetic liquid for inhalation (Baxter, Deerfield, IL) in a bell jar and received a continuous dosage throughout the surgery. The back of

the rats were shaved using an electric razor and remaining adherent hair was removed using water to sustain an aseptic surgery site. The site was then cleaned with Betadine. The subcutaneous implantation of biomaterials has been previously described in detail [27-30]. A 1.0 to 1.5 cm incision was made along the posterior midline of the back. Marcaine[®] topical anesthetic was then locally applied to the incision to help minimize potential post-operative pain. Two subcutaneous pockets were then cleared on either side of the midline below the panniculus carnosus in the connective tissue fascia using blunt dissection with rounded-tip scissors. One sample was placed into each pocket ensuring sufficient distance from the midline. The wound site was then closed using 9 mm surgical wound clips and cleaned using Betadine[®]. Each rat was returned to its cage and observed until it recovered from the anesthetic.

6.2.6. Histology and image analysis

After 1, 2, and 4 weeks the rats were euthanized using carbon dioxide and the samples were explanted with surrounding tissue intact. The removed tissue was pinned to a paraffin block and immersed in 10% buffered formalin phosphate solution (Fisher Scientific, Fair Lawn, NJ) for 48 hours. The tissue surrounding the implant was then sectioned into strips approximately 2 mm in thickness using a razor blade. Three strips were then placed into a histology cassette, processed, and stained with Hematoxylin and Eosin (H&E) or Masson's Trichrome. Aligned scaffolds were sectioned in two directions: perpendicular and parallel to the fiber orientation. H&E staining was used to assess the foreign body reaction on material surfaces and fibrous capsule formation. Masson's Trichrome staining was applied to confirm fibrous capsule

formation on scaffold surfaces. Histological stained slides were examined using light microscopy (Olympus, model No.: IX71). Thickness of fibrous capsule and cellular infiltration was measured using H&E stained images and confirmed with Trichrome stained images. A zero-to-four grading scale was used to assess the severity of inflammatory, wound healing, and foreign body reaction steps for comparison between materials.

6.2.7. Statistical analysis

For sample groups more than two, one-way ANOVA was carried out to calculate p value. The Tukey post hoc test was chosen when variances were homogenous otherwise the Games-Howell test was used. Independent-samples t-test was used for comparing porosity differences between aligned and random fibers. $p < 0.05$ was considered as significantly different. Error bar represents the standard error (SE) of the mean.

6.3. RESULTS

6.3.1. Properties of PCL electrospun nanofibers

FESEM images of aligned and random electrospun nanofibers are shown in Fig. 6.1. The diameters of aligned and random fibers were 506 ± 24 nm and 313 ± 5 nm, respectively. Both aligned and random fibrous scaffolds showed highly porous structures. The porosities were $82.6 \pm 1.0\%$ and $80.9 \pm 1.0\%$ for aligned and random fiber scaffolds, respectively. T-test showed that there was no significant difference between the porosities of the two PCL fibrous scaffolds.

Fig. 6.1. The morphologies of aligned (A) and random (B) PCL electrospun nanofibers. Scale bar = 1 μm .

6.3.2. Cell morphology and monocyte/macrophage density

Initial monocyte adhesion (day 0) on the four types of substrate surfaces is shown in Fig. 6.2. On aligned fibers, monocytes exhibited a round shape indicating the cells were not well attached to the fiber surface. Monocytes on random fiber, film and RGD-glass were well spread on the substrate surfaces 2 hours after seeding indicating good adhesion. Monocytes were able to migrate easily between fibers and cell infiltration was observed on aligned fibers. However, no cell penetration was seen on the randomly oriented nanofiber scaffolds.

Fig. 6.2. SEM images show the morphology of monocytes at day 0 (2 h) on PCL aligned fibers (A), PCL random fibers (B), PCL films (C) and RGD-coated glass cover slips (D). Scale bar = 10 μm .

The monocyte/macrophage density on each substrate surface is summarized in Fig. 6.3. The initial cell adhesion at day 0 (2 hr) on the aligned fiber scaffolds was significantly lower than other three groups, while the random fiber scaffold was most supportive for monocyte adhesion. The cell density on the RGD-glass was slightly higher than on the PCL film, however the difference was not significant. Cell density decreased on all substrate surfaces over time as monocytes differentiated into macrophages and then fused into foreign body giant cells in the presence of IL-4. In general, random fiber scaffolds showed a higher cell density over 10 days as compared to aligned fiber scaffolds, film and RGD-glass.

Fig. 6.3. *In vitro* adherent monocyte/macrophage cell density on PCL nanofibers, PCL film and RGD-glass as a function of time. (Mean $\pm$ SE) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison between paired groups.

6.3.3. Foreign body reaction and fibrous capsule formation

At week 4, fibrous capsules were formed on the surfaces of three PCL scaffolds *in vivo* (Fig. 6.4). Intense foreign body reaction was observed on random fiber surfaces (Fig. 6.4C, D) as large foreign body giant cells and activated macrophages were seen on the random fiber scaffolds. Whereas on aligned fiber scaffolds and film, the foreign body reaction was more quiescent. The thicknesses of fibrous capsules on aligned fiber scaffolds, random fiber scaffolds, and film were $7.55 \pm 0.54 \mu\text{m}$, $4.13 \pm 0.31 \mu\text{m}$ and $37.71 \pm 0.25 \mu\text{m}$, respectively. The fibrous capsule on PCL film was about 5 to 9 fold thicker than on fiber scaffold surfaces. On both fiber scaffolds, only a thin layer of collagen was deposited on the scaffold surface while a thick collagen layer formed on the film surface (Fig. 6.5).

Fig. 6.4. Histological images showing the fibrous capsules formed on PCL scaffold surfaces at week 4. A,B: Aligned fibers. C,D: Random fibers. E,F: Film. Left, H and E staining. Right, Masson's Trichrome staining. Three types of scaffolds are indicated on the images. The paired arrows indicate the aligned scaffold with cell infiltration. The open arrows show the strong macrophage/foreign body giant cell reaction on the random fiber scaffold. Scale bar = 50 μ m.

Fig. 6.5. The thickness of fibrous capsules formed on different PCL scaffolds at week 4.

(Mean $\pm$ SE) * indicates statistical significance, $p < 0.001$.

6.3.4. Cellular infiltration

Cellular infiltration has rarely been reported on electrospun nanofibers. Electrospun fiber scaffolds usually demonstrate a two-dimensional behavior as the pore sizes on the surface are too small for cells to penetrate. However, *in vivo* cellular infiltration was observed in this study on aligned fiber scaffolds (Fig. 6.6). Macrophages were able to migrate into aligned fiber scaffolds as early as one week after implantation (Fig. 6.6A, B). A complete penetration was achieved in the aligned fiber scaffolds as flat macrophage edge was observed, indicating the macrophages reached the surface of the supporting PCL film. Tissues surrounding the aligned fiber scaffolds were observed in two orientations: parallel and perpendicular to the aligned fiber axis. With the parallel orientation, macrophages showed the same orientation along the fiber axis (Fig. 6.6F, H). The perpendicular orientation showed no cell alignment as expected and the hollow spaces observed histologically between cells were

cross-sections of PCL fibers (Fig. 6.6E, G). Besides macrophage infiltration, fibroblasts, which are early components of the fibrous capsule, also infiltrated the aligned fibers. During the inflammatory and healing processes, fibroblasts will move to the surface of the implants, and then deposit collagen on the material surface to develop fibrous capsules. In Fig. 6.6H, collagen produced by infiltrating fibroblasts was identified inside the aligned fiber scaffold and on the surface of the PCL film. This suggests that the aligned fiber scaffolds are able to promote tissue-scaffold integration *in vivo*.

Fig. 6.6. Cell penetration on aligned fibers with perpendicular (left) and parallel (right) orientation. A,B: Week 1. C,D: Week 2. E,H: Week 4. G and H are Trichome staining of E and F, respectively. The paired arrows showed the apparent macrophage penetration into the aligned fibers. The thick black arrow head on Fig. 6.6H shows collagen deposition. Scale bar = 50 μ m. Aligned fibers were supported on PCL film.

6.3.5. *In vivo* biocompatibility assessment

PCL is a biocompatible polymer which has been extensively studied as a tissue engineering material. In this work, the biocompatibility of the aligned and random PCL fiber scaffolds and the PCL film was clearly indicated by the very

early resolution of the acute and chronic inflammation (Table 6.2). No or minimal acute and chronic inflammation was seen at 2 weeks. Rapid onset of granulation tissue and foreign body reaction responses were observed following acute and chronic inflammation. Expected normal healing responses, i.e., granulation tissue and fibrous capsule formation, and the normal foreign body reaction were present at week 2 and week 4. However, the different topographies of the PCL scaffolds influenced the process of healing. The PCL film had the least complexity and the healing response was the least severe and resolved faster compared to the fiber scaffold implants. The aligned fiber scaffold showed the next best biocompatibility in terms of healing response and foreign body reaction. The random fiber scaffold had the slowest healing process.

Table 6.2. *In vivo* biocompatibility: inflammatory and wound healing response

Samples	Acute Inflammation	Chronic Inflammation	Granulation Tissue	Foreign Body Reaction
Week 1				
Aligned	0	0	+1	+2
Random	0	+1	+1.5	+3.5
Film	0	0	+1.5	+2
Week 2				
Aligned	0	0	+2.5	+2
Random	0	0	+3	+3
Film	0	0	+2	+2
Week 4				
Aligned	0	0	+1	+1
Random	0	0	+1	+1.5
Film	0	0	0	+1

Score: 0: None; +1: Minimal; +2: Mild; +3: Moderate; +4: Extensive.

n=2 for Week 1 Aligned; n=3 for Week 1 Film and Week 2 Aligned; n=4 for all others

6.4. DISCUSSION

The foreign body reaction and fibrous capsule formation on the surface of implant material are important parameters in assessing the biocompatibility of the implants. In this work, we investigated these responses toward different scaffold architectures made from a widely used biocompatible material, polycaprolactone (PCL). Topographic cues are able to influence cellular behavior independent of chemical cues [31]. Therefore, the form of the medical device may ultimately affect its functionality. We fabricated three types of PCL scaffolds, i.e., aligned nanofiber, random nanofiber and plain film, and evaluated monocyte/macrophage behavior *in vitro* and foreign body reaction and fibrous capsule formation *in vivo*.

Monocyte/macrophage showed different adhesion abilities on the three types of PCL scaffolds. Cell density was lowest on the aligned fiber scaffolds while highest on the random fiber scaffolds. From the SEM images (Fig. 6.2), aligned fibers exhibited larger inter-fiber spacing compared to randomly oriented fiber scaffolds. This larger inter-fiber spacing gave a smaller fiber surface area for initial cell adhesion and resulted in a decreased cell density on the aligned fibers. On the other hand, cells showed the best attachment on the random nanofiber scaffold where the inter-fiber spacing was too small for cell infiltration and monocytes were able to spread on the surface. Compared to PCL film and RGD-glass surfaces, random nanofibers had a large surface-to-volume ratio and therefore exhibited a larger surface area for cells to attach.

The formation of the fibrous capsule is dependent on the properties of the implanted biomaterial and the thickness of the fibrous capsule can have a significant influence on the functionality of the implanted materials *in vivo*. In the present study, we found that the fiber scaffold surface induced thinner

fibrous capsule formation when compared to the smooth surface of the PCL film. The thickness of the fibrous capsule on the film surface was $37.7 \pm 0.3 \mu\text{m}$ while that on fiber scaffold surfaces was less than $8 \mu\text{m}$. It has been reported that porous ceramic implants showed thinner fibrous capsules and healed faster than impervious ceramic implants [12]. Scaffolds with lower porosity are more likely to induce dense fibrous capsule formation [32] and the thickness of fibrous capsules can be greatly reduced when the implants are more porous [33]. In the present study, nanofiber scaffolds with highly porous structures and porosities larger than 80% greatly enhanced tissue-scaffold interactions and allowed cellular infiltration.

Randomly-oriented electrospun fibers with nano-ranged diameters showed limited ability for cell infiltration [34-35]. However, in our study, *in vivo* cellular infiltration was observed on aligned fiber scaffolds with diameter of $506 \pm 24 \text{ nm}$ while the random PCL fiber scaffold failed to support cell penetration. Comparing the SEM images of aligned and random fibers (Fig. 6.1), we found that the random fibers have closer inter-fiber connections compared to aligned fibers, which may restrict fiber movement. In the aligned fiber scaffold, however, fibers with decreased inter-fiber connections showed more fiber flexibility and larger inter-fiber spaces could be obtained. Monocyte/macrophages were able to infiltrate between the aligned fibers (Fig. 6.2A). Moreover, collagen was also found to in the aligned fiber scaffold (Fig. 6.6H), indicating the infiltration of fibroblasts. Therefore, the aligned nanofiber scaffold demonstrated the capability of enhancing tissue-scaffold integration *in vivo*.

Intense macrophage and foreign body giant cell activity were found on random fiber scaffolds at all time points. 4 weeks after implantation, large foreign body giant cells still remained on random fiber surfaces (Fig. 6.4D), indicating that random fiber surface is less favorable in the host as compared to other two scaffold surfaces. From the histology aligned fibers and film generated a mild foreign body reaction on the surface within 4 weeks and films induced the lowest intensity of macrophages and foreign body giant cells. The biocompatibility of the three types of PCL scaffolds were evaluated from the histology slides. For all scaffolds, acute inflammation was not observed. Acute inflammation normally resolves less than one week on biocompatible biomaterials, such as PCL. Chronic inflammation was only seen on random fibers at week 1 and resolved thereafter. Granulation tissue appeared on all scaffolds from week 1 and then the intensity increased at week 2 as expected with the healing response, and finally dropped to nil. The random fiber scaffold triggered the strongest granulation tissue response while the least response was observed on the film. The foreign body reaction was similar to the granulation tissue response except that it did not disappear but become a stable tissue component around the implant. The foreign body reaction was most severe on the random fiber scaffold and least on the film. This indicated that the healing process was slowest on the random fiber scaffold and fastest on the film. The aligned fiber scaffold generated a moderate healing process in our study. Protein adsorption on implant material surface is the main reason for cell-associated inflammation and foreign body reaction [36-37] which relates to the surface area of the implant material. Increased surface area exposed to the blood may attract more blood proteins onto the surface, resulting in a higher degree of

cellular response. Nanofibers including random and aligned architecture exhibit high surface-to-volume ratios, and the surface area is tremendously enlarged as compared to plain film. By absorbing more foreign body reaction-associated proteins on to the fiber scaffold surfaces, it was likely to take a longer time for the healing process. However, the difference of foreign body reactions between aligned and random fibers is still obscure. One possible reason is that the cellular infiltration into the aligned fibers reduced the severity of foreign body reaction. With improved tissue-scaffold integration, the aligned fiber scaffold appeared more 'biocompatible' to the host.

6.5. CONCLUSIONS

This study demonstrated that nano-topographical cues have a significant influence on the *in vitro* and *in vivo* foreign body reaction to PCL nanofiber scaffolds. The electrospun PCL nanofibers with aligned and random orientation along with PCL films induced different inflammatory and wound healing responses. Aligned fiber scaffolds allowed cellular penetration *in vivo* and may serve as a promising scaffold for tissue engineering by minimizing host response and enhancing tissue-scaffold integration.

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CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

7.1. CONCLUDING REMARK

Tissue engineering provides powerful tools to improve and repair tissue functions in various areas, including nerve regeneration. Despite the progress and improvements achieved within the past decades, successful recovery from nerve damages or neural diseases remains challenging and more efforts are required for effective regeneration.

In this thesis, we aimed to construct a bio-functional scaffold by electrospinning technique as a potential platform for long-term siRNA therapy for nerve regeneration. First, we investigated the relationship between electrospinning parameters and the properties of the resultant fibers. The understanding of this relationship is of great value in fabricating purpose-specific scaffolds in a predictable and controllable manner. Our initial attempts in producing bio-functional scaffolds, including core-shell fibers and nanoparticles-embedded scaffolds, demonstrated the versatility of electrospinning for various applications.

To functionalize fibrous scaffolds for desired manipulation over cells, we then developed a robust nanofibrous scaffold-mediated siRNA delivery system for long-term RNAi treatment. Interestingly, enhanced cellular uptake of siRNA was observed on the nanofibers, probably due to the localized concentration and availability to cells presented by the underlying scaffolds.

Topographic effect also exerts influential impact on regulating cellular behaviors and functions. We therefore investigated the nanofiber topographic

effect on the cellular behaviors of astrocyte. Astrocyte is the major component of the glial scar which inhibits axon regeneration in the CNS, and accordingly the understanding of astrocyte response to the underlying substrates may have important implications for the purpose of CNS regeneration. Our results demonstrated that the nanofibrous scaffolds suppressed astrocyte proliferation and enhanced apoptosis. Furthermore, improved siRNA uptake in astrocytes was observed on the nanofiber topography. These results suggested that electrospun nanofiber may serve as a promising scaffold for CNS regeneration with therapeutic potentials via siRNA delivery.

Tissue engineered scaffolds must be safe and biocompatible for implantation, and any corresponding tissue-scaffold interactions should not hinder the normal functionality of the implanted device. Therefore, the foreign body reaction towards electrospun nanofibers was studied. In particular, aligned and randomly-oriented fiber topographical effects were analyzed. Both random and aligned PCL nanofibers demonstrated better biocompatibility as compared to a two-dimensional solid surface *in vitro* and *in vivo*. Moreover, fiber orientation was observed to elicit different foreign body reactions. Specifically, aligned nanofibers resulted in superior cell-scaffold interactions as compared to randomly-oriented fibers, in terms of cell infiltration and fibrous capsule formation. This observation, along with the nanofiber topographic effect on astrocyte, laid the foundation of utilizing nanofibrous scaffolds for CNS regeneration. Together with the feasibility of delivering siRNA via nanofibers to regulate cell behaviors and functions, we demonstrated the potential application of the bio-functionalized electrospun nanofibers for neural tissue engineering.

7.2. FUTURE DIRECTIONS

The conclusions in this thesis, albeit promising findings for neural tissue engineering, have been primarily presented with non-definitive terms for general application, as more detailed works need to be performed before we can apply these results to clinic trials.

Advances in the area of applying bio-functional electrospun nanofibers to nerve regeneration will be interdisciplinary. First, it would be advantageous to fabricate bio-functional scaffolds with controlled incorporation of chemical and topographical cues into electrospun fibers. For siRNA delivery, a mild fabrication process would be favorable for minimizing the loss of bioactivity. Co-axial electrospinning might be one alternative approach. A stable environment can be provided by the inner core for sensitive biomolecules, and the outer polymer may also serve as a physical barrier to protect the drugs from enzymatic digestion. A controllable releasing rate of the embedded drugs is also an important consideration for desirable delivery. Appropriate polymer selection and modification should be performed for various needs. In this study, we demonstrated that the topographical cues could provide contact guidance and direct cells behaviors in specific manners for tissue engineering purposes in terms of fiber diameter and orientation. However, more topographic parameters should be investigated. Cellular infiltration into the scaffolds should also be encouraged, such as by controlling pore size or constructing 3D scaffolds, in order to expand our knowledge on cell-scaffold interactions.

As mentioned in the Discussion sections of the preceding chapters, a deeper insight of cell-substrate interactions are required for in-depth

understanding in basic cell biology and optimization of scaffold design. Cells are capable of sensing different topographical cues presented by the surrounding microenvironment, and as a response, cells exhibit distinct cellular behaviors including cell growth, proliferation, attachment and differentiation. It would be of great interest to know how these transformations are triggered from environmental signals. It is believed that various signaling pathways in cells, such as tyrosine phosphorylation [1] and Rho activation [2], are responsible for these cellular changes, however deeper information waits to be unveiled.

Lastly, the long-term therapeutic effect of the siRNA-delivering nanofibrous scaffolds should be verified in the animal model aiming for nerve regeneration. Cells behave in different ways as they grow on a two-dimensional surface or surrounded by ECM components in three dimensions, and therefore the performance of the bio-functional scaffolds tested with cell culture is prone to vary when applied *in vivo* [3]. It is essential to examine the *in vivo* therapeutic effect and proper adjustments or modifications need to be made to the current drug delivery system before clinical trials. CSPG produced at CNS injury sites strongly prohibits axon regeneration. The removal/digestion of CSPG is able to significantly improve neurite extension and axon regeneration. Interfering CSPG synthesis by siRNA is one potential approach to decrease CSPG production. Target genes in astrocytes have been identified, including chondroitin polymerizing factor (ChPF) [4], chondroitin 4-sulfotransferases (C4ST-1) and chondroitin 6-sulfotransferases (C6ST-1) [5-6]. In addition, myelin related inhibitors, such as myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgp) in Schwann cells or oligodendrocytes [7], are also potential therapeutic targets. The down-

regulation of these genes could create a more permissive environment for axon regrowth and CNS repair. Nerve conduit can be fabricated from the siRNA-embedded nanofibers and implanted for the treatment of spinal cord injury to evaluate the potential application in the animal model.

7.3. REFERENCES

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