Developing sustained dual-drug therapy for tendon sports injuries

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NANYANG
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**Developing Sustained Dual-drug Therapy
for Tendon Sports Injuries**

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School of Materials Science and Engineering

2016

Developing Sustained Dual-drug Therapy for Tendon Sports Injuries

School of Materials Science and Engineering

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Abstract

Tendon plays an important role in regulating body locomotion and providing additional stability to the body. However, tendon is susceptible to injuries and the healing process could be devastating along with the several issues, namely adhesion formations, slow healing and failure at fixation sites, which have deferred the success of proper tendon healing via tendon tissue engineering. This dissertation thus aims to create a sustained dual-drug therapy to address these issues. For adhesion formation, naproxen sodium (NPS) has been shown to be able to avoid this symptom through inhibiting inflammation process. Therefore, so as to eliminate the side effects of NPS such as drowsiness, headache and nausea, NPS was loaded into an electrospun aligned poly (l-lactic acid)/poly (ϵ -caprolactone) scaffold and its release from the scaffold was optimized to achieve controlled release through adjusting the polymer compositions and water percentage in hexafluoroisopropanol (HFIP). A 2-week sustained release was achieved through modulating the NPS distribution in the fibers of a scaffold which possesses aligned morphology and optimal mechanical properties. Notably, the scaffold exhibited no cytotoxicity and tenocytes could respond to the topographical cues given by the scaffolds, forming a network of cells which mimic the native tendon tissue arrangement. Although NPS is needed to prevent adhesion formations, it has been shown to inhibit the proliferation of tenocytes. Hence the second objective of this dissertation is to release growth factor to promote cell proliferation, and at the same time tackle on the slow healing issues during tendon healing. Insulin-like growth factor 1 (IGF-1) was thus loaded into electrospun poly (lactic-co-glycolic acid)/poly (ϵ -caprolactone) aligned scaffolds for its ability to promote cellular proliferation and maturation. HFIP was identified as the electrospinning solution for its ability to better maintain IGF-1 stability. The released IGF-1 was shown to have maintained its bioactivity and could trigger downstream cellular response such as promoting tenocytes proliferation and up-regulating the tendon specific gene markers expression (i.e.

scleraxis and tenomodulin). Most importantly, the released IGF-1 has compensated the inhibitory effect of NPS, through enhancing the proliferation and differentiation of tenocytes on the combined scaffolds (which were fabricated through sequential electrospinning of NPS scaffolds and IGF-1 scaffolds). In addition, IGF-1 scaffold has been utilized as the platform to tissue engineer C2C12 murine myoblast, tenocytes and TE85 human osteosarcoma together, with the intention of enhancing interaction between different cells in order to solve the issue of failure at the fixation sites, where regeneration is difficult at this translation region. Through medium compatibility optimizations, three types of cells could proliferate and differentiate in a common medium, in which their configuration on the scaffold mimicked the actual musculoskeletal tissue arrangement in the body. These results indicate that the scaffolds have promising potential in enhancing tendon regeneration *in vivo*, with their IGF-1 and NPS releasing abilities and desirable tendon TE characteristics.

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Table of Contents

Abstract	i
Acknowledgements	iii
Table of Contents	v
Table Captions	xi
Figure Captions.....	xiii
Abbreviations	xxi
 Chapter 1 Introduction.....	 1
1.1 Background and problem statement.....	2
1.2 Objectives and scope.....	4
1.3 Dissertation overview.....	7
1.4 Findings and outcomes/originality	8
References.....	8
 Chapter 2 Literature Review	 13
2.1 Tendon	14
2.1.1 Structure and function	14
2.1.2 Sports injuries, tendinopathies and healing process	15
2.2 Current Treatments.....	16
2.2.1 Medicinal intervention	16
2.2.2 Surgical intervention	16

2.3	Tendon tissue engineering (TE)	17
2.3.1	Scaffolds	17
2.3.2	Cells	18
2.2.1	Chemical stimulations	19
2.2.1	Mechanotransduction	20
2.4	Osteotendinous and myotendinous junctions	20
	References	21
Chapter 3	Experimental Methodology	25
3.1	Materials selection	26
3.2	Materials fabrication	26
3.2.1	Preparation of electrospinning solutions	26
3.2.1.1	Solutions for NPS scaffold	26
3.2.1.1	Solutions for IGF-1 scaffold	26
3.2.2	Fabrication of electrospun scaffolds	27
3.3	<i>In vitro</i> drug release studies	28
3.3.1	Release of naproxen sodium (NPS)	28
3.3.2	Release of insulin-like growth factor 1 (IGF-1)	28
3.3.3	Determination of IGF-1 stability in different solvents	29
3.4	Materials Characterization	29
3.4.1	Morphological assessment of electrospun scaffolds	29
3.4.2	Contact angle and wetting tension	30
3.4.3	Surface properties of electrospun NPS scaffolds	30
3.4.4	Mechanical properties of electrospun scaffolds	30
3.4.5	Hydrolytic degradation of electrospun scaffolds	31

3.5	Cell Studies	32
3.5.1	Cell culture.....	32
3.5.1.1	L929 on NPS scaffold	32
3.5.1.2	C2C12 on IGF-1 scaffold.....	32
3.5.1.3	Tenocytes on IGF-1 scaffold	33
3.5.1.4	TE85 on IGF-1 scaffold	33
3.5.1.5	Tri-culture of C2C12/Tenocytes/TE85 on IGF-1 scaffolds	34
3.5.2	Cell quantification.....	34
3.5.2.1	PicoGreen assay	34
3.5.2.2	AlamarBlue assay	35
3.5.3	Cell morphology analysis	35
3.5.4	Immunocytochemistry	35
3.5.5	Western blotting for determination of IGF-1 bioactivity	36
3.5.6	Real time quantitative polymerase chain reaction	37
3.5.7	Assessment of mineralization by Alizarin Red S staining	38
3.6	Statistical analysis	38
	References.....	39

Chapter 4 Sustained-release of Naproxen Sodium from Electrospun-aligned PLLA–PCL Scaffolds

4.1	Introduction	42
4.2	Materials and Methods	44
4.3	Results	44
4.3.1	Aligned fibrous scaffolds to mimic tendon micro-environment.....	45

4.3.2	NPS released sustainably up to two weeks	48
4.3.3	Scaffold hydrophilicities affected NPS release profiles	49
4.3.4	Co-solvent affected NPS distribution inside the fibers	51
4.3.5	Scaffolds yielded optimal mechanical properties	52
4.3.6	NPS scaffolds exhibited no cytotoxicity on L929	54
4.3.7	Tenocytes exhibited elongated morphology but proliferation was slightly inhibited under NPS influence	55
4.4	Discussion	57
4.5	Summary	62
	References	62

Chapter 5 Controlled release of IGF-1 from Electrospun Scaffolds Enhanced Tenocytes Proliferation and Differentiation.....67

5.1	Introduction	68
5.2	Materials and Methods	70
5.3	Results	70
5.3.1	HFIP as the solvent for electrospinning	70
5.3.2	Controlled IGF-1 release from the electrospun scaffolds	73
5.3.3	Mass loss and change in molecular weight from 3rd week	75
5.3.4	Released IGF-1 maintained bioactivity	76
5.3.5	Released IGF-1 retained functionality by enhancing C2C12 cells proliferation and differentiation	77
5.3.6	Tenocytes proliferated and differentiated on IGF-1 loaded scaffolds	81
5.3.7	Released IGf-1 rescued tenocytes proliferation and differentiation on combined scaffolds	83
5.3.8	Combined scaffolds possess optimal mechanical properties	86

5.4	Discussion	86
5.5	Summary	90
	References	91
 Chapter 6 Medium compatibility study for tri-culture of C2C12 myblasts, tenocytes and TE85 human osteosarcoma		
6.1	Introduction	96
6.2	Materials and Methods	97
6.3	Results	97
6.3.1	C2C12 proliferated on IGF-1 scaffold, regardless of the glucose level of the medium used	98
6.3.2	Glucose level of the medium does not interfere TE85 proliferation on IGF-1 scaffold	99
6.3.3	Tenocytes able to proliferate on IGF-1 scaffolds, regardless of the medium types	100
6.3.4	C2C12 formed myotubes in high glucose conditions	101
6.3.5	Osteogenic supplements are essential for TE85 calcium deposition	103
6.3.6	Tendon specific genes were slightly down-regulated in optimized medium	105
6.3.7	Tri-culture of C2C12/Tenocytes/TE85 on IGF-1 scaffolds	106
6.4	Discussion	108
6.5	Summary	110
	References	111
 Chapter 7 Conclusion and Recommendations		
7.1	Conclusion.....	116

7.2	Recommendations	119
7.2.1	Three dimensional (3D) scaffolds to better represent cellular micro-environment	119
7.2.2	Integration with interface tissue engineering to promote interactions between different cell types	119
7.2.3	In-depth evaluation on the timely-released-NPS scaffolds for prevention of adhesions in animal models	120
7.2.4	Animal studies on dual-release scaffolds to better promote tendon healing	121
	References.....	122
	Appendix.....	125
	Publications and Conferences.....	127

Table Captions

Table 3.1	Primers used for the differentiation determination	38
Table 4.1	Methods used in Chapter 4	44
Table 4.2	Description of different formulations of electrospinning solutions.....	45
Table 4.3	Mean fiber diameter and mechanical properties of the scaffolds.	54
Table 5.1	Methods used in Chapter 5.	70
Table 5.2	Description of different formulations of electrospinning solutions.....	71
Table 5.3	Apparent Young's modulus of NPS scaffolds, IGF-1 scaffolds and combined scaffolds.	86
Table 6.1	Methods used in Chapter 6.	97
Table 6.2	Mediums and their compositions used for tri-culture systems.	97

Figure Captions

- Figure 2.1** Schematic diagram of the structure of tendon14
- Figure 2.2** Schematic diagram of osteotendinous and myotendinous junctions21
- Figure 3.1** Schematic diagram of the multi-layered scaffolds which release IGF-1 and NPS27
- Figure 3.2** Schematic diagram of the set-up for tri-culture experiments, where two 12mm coverslip acted as separator to prevent cells mixing during seeding on IGF-1 scaffolds34
- Figure 4.1** Aligned fibers with beadless morphology was obtained from varying formulations of electrospinning solutions with A) no NPS; and with NPS, (B) 70-30-0 (C) 80-20-0 (D) 90-10-0; (E) 90-10-3; (F) 90-10-5; G) 90-10-7; H) 90-10-10. (scale bar = 10 μ m)46
- Figure 4.2** Graphs showing the difference in fibers diameter when changing (A) polymer composition, i.e. PCL to PLLA ratio, and (B) co-solvent composition, i.e. H₂O to HFIP. When changing PCL percentage, there was no difference in fibers diameter. Decreasing trend in fibers diameter was observed when increasing H₂O percentage (* indicates $p < 0.05$ wrt 90-10-0; # indicates $p < 0.05$ wrt 90-10-3; ** indicates $p < 0.05$ wrt 90-10-5)47
- Figure 4.3** Plots showing cumulative NPS release from the scaffolds with different: A) PLLA to PCL ratio and B) H₂O to HFIP ratio. Scaffolds with higher PCL to PLLA ratio has contributed to faster release of NPS. The addition of 3 vol% H₂O has resulted in scaffolds with slower NPS release profiles, which

lasted for two weeks. However, further increasing H₂O content yielded scaffolds with faster NPS release profiles49

Figure 4.4 Graphs showing the contact angle measurements of different scaffolds with varying: A) PCL to PLLA ratio and B) H₂O to HFIP ratio. 70-30-0 and 80-20-0 scaffolds have significant lower contact angle than that of the 90-10-0. When varying the H₂O to HFIP ratio, contact angle of the scaffolds did not significantly change. (* indicates $p < 0.05$)50

Figure 4.5 Graphs showing water uptake measurements of different scaffolds with varying A) PCL to PLLA ratio and B) H₂O to HFIP ratio, after immersing in PBS for 7 days. Lower water uptake was observed for 90-10-0 scaffolds. While changing the percentage of H₂O as co-solvent, no significant difference in water uptake was observed. (* indicates $p < 0.05$)51

Figure 4.6 Plot showing the XPS analysis of the scaffolds with and without NPS. Na 4s characteristic peak indicates the presence of NPS on scaffold 90-10-10 (dotted line), 90-10-3 (dash-dot line) and 90-10-0 (solid line). There was no peak detected for scaffold without NPS (dash line) at that particular region.52

Figure 4.7 Plots showing mechanical properties of the scaffolds with varying: A) PLLA to PCL ratio and B) H₂O to HFIP ratio. The apparent Young's modulus was similar for the scaffolds with different formulations. Lower PCL to PLLA ratio has yielded scaffolds with lower elasticity. Elasticity of the scaffolds increased with minimal amount of H₂O (3 vol%), however it dropped with increasing H₂O percentage..53

- Figure 4.8** Fluorescence microscopy images of cells on aligned scaffold proliferated and arranged themselves according to the fibers orientation by day 7. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). (scale bar = 50 μ m).55
- Figure 4.9** Graph showing proliferation of tenocytes on NPS scaffolds was slightly prohibited, compared to empty scaffolds where cells proliferated after 3 days of culture. (* indicates $p < 0.05$).....56
- Figure 4.10** Immunostaining images showing more tenocytes were visualized on A) empty scaffolds than on B) NPS scaffolds, where tenocytes showed elongated morphology were immunolabeled with phalloidin (red) to show cytoskeleton of the cells and counterstained with DAPI (blue) to indicate nucleus. (scale bar = 50 μ m).....56
- Figure 4.11** Schematic showing possible NPS distribution within the fibers when increasing the H₂O percentage as the co-solvent in formulations: A) when no water was added, there was an even distribution of the NPS inside the fibers; (B) with the presence of minimal amount of H₂O, more NPS tend to stay inside the fibers; however, (C) further increase the percentage of H₂O resulted in thinner fibers and there was more NPS on the surface of the fibers.59
- Figure 4.12** Schematic showing possible changes in the polymer chains when increasing water percentage as the co-solvent in electrospinning solutions: (A) when no water was introduced, polymer chains have lower molecular orientation; (B) with the presence of small amount of H₂O polymer chains have higher

	molecular orientation; (C) with further addition of H ₂ O polymer chains were highly stretched.	61
Figure 5.1	Graph showing the relative IGF-1 stability in different solvents. HFIP showed relatively better ability in preserving IGF-1 stability as compared to DCM. (* indicates $p < 0.05$, ** indicates $p < 0.001$).....	71
Figure 5.2	FESEM images of IGF-1 loaded scaffolds: A) 8-80/20-0; B) 8-70/30-0; C) 8-70/30-5; D) 5-70/30-5; E) 3-70/30-5. (F) The fiber diameter analysis showed that lowering the concentration decreased the fiber diameter. (* indicates $p < 0.05$).....	72
Figure 5.3	Release profile of IGF-1 under different formulations: A) different polymer ratios; B) with or without PEO; C) different polymer concentration. D) Release of IGF-1 from the optimized scaffold (i.e. 3-70/30-5) lasted for 10 weeks.....	73
Figure 5.4	Plots showing IGF-1 and empty scaffolds degraded from 3rd week onwards, from the evidence of A) mass loss and B) molecular weight change	75
Figure 5.5	Immunoblots showing the expression of A) pAkt Ser473, B) total Akt and C) β -Actin while the cells cultured on IGF-1 scaffolds and empty scaffolds were exposed to 1-day and 7-day preconditioned mediums. D) Densitometry analysis of the blots showed that the up-regulated of Akt phosphorylation indicated that IGF-1 is bioactive after the encapsulation	77

- Figure 5.6** Graphs showing the C2C12 cells proliferated throughout the study as determined by A) AlamarBlue assay and B) PicoGreen assay. Cells on IGF-1 scaffolds proliferated faster compared to empty scaffolds. (* indicates $p < 0.05$)78
- Figure 5.7** Immunostaining images showing A) C2C12 (nuclei was stained blue) reached almost 100% of confluency after culturing for 3 days on IGF-1 scaffolds; however, B) fewer cells were observed on empty scaffolds on day 3, and C) cells only become confluent after 7 days of culture on empty scaffolds. (scale bar = 50 μ m)79
- Figure 5.8** Graphs showing myogenin expression of C2C12 on A) day 0, B) day 1 and C) day 3 of differentiation. The myogenin expression on IGF-1 scaffolds was up-regulated compared to empty scaffolds after 1 day of differentiation but it was leveled off on day 3. (* indicates $p < 0.05$).....80
- Figure 5.9** Immunostaining images showing myotubes formation on A) IGF-1 scaffolds and B) empty scaffolds, where more and larger myotubes were observed on IGF-1 scaffolds. (scale bar = 50 μ m)81
- Figure 5.10** Graph showing tenocytes proliferated on IGF-1 and empty scaffolds, as determined by PicoGreen Assay. Tenocytes proliferated faster on IGF-1 scaffolds compared to empty scaffolds. (* indicates $p < 0.05$).....81
- Figure 5.11** Immunostaining images showing more tenocytes were on a) IGF-1 scaffolds than on b) empty scaffolds, where tenocytes were immunolabeled with phalloidin (red) to show cytoskeleton of the cells and counterstained with DAPI (blue) to indicate nucleus. (scale bar = 50 μ m)82

- Figure 5.12** Graphs showing tendon specific genes: A) scleraxis and B) tenomodulin were up-regulated in tenocytes cultured on IGF-1 scaffolds compared to empty scaffolds; however there was no much difference in C) collagen I gene expression on both types of scaffolds. (* indicates $p < 0.05$)83
- Figure 5.13** Graph showing tenocytes proliferated faster on IGF-1 Scaffold + NPS scaffold than Empty Scaffold + NPS scaffold, as determined by PicoGreen Assay. (*indicates $p < 0.05$).....84
- Figure 5.14** Graphs showing tendon specific genes: a) scleraxis; b) tenomodulin were up-regulated when tenocytes were cultured on IGF-1 scaffold + NPS scaffold; however, there was slightly higher expression of collagen I expressed on IGF-1 scaffold + NPS scaffold. (* indicates $p < 0.05$)85
- Figure 5.15** Schematic diagram showing simplified IGF-1/Akt pathway.....88
- Figure 6.1** Optical microscopic images showing C2C12 cultured on well plates in different growth mediums proliferated and adopted typical spindle shaped morphology after 1 day (1D), 2 days (2D) and 4 days (4D) of culture (scale bar = 100 μ m)98
- Figure 6.2** Graph showing the C2C12 proliferated on IGF-1 scaffolds in both GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)99
- Figure 6.3** Optical microscopic images showing TE85 proliferated and adopted their typical morphology on well plates (scale bar = 100 μ m)100

- Figure 6.4** Graph showing the TE85 proliferated on IGF-1 scaffolds in both GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)100
- Figure 6.5** Graph showing the tenocytes proliferated on IGF-1 scaffolds in GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)101
- Figure 6.6** Immunostaining images showing “myotubes” formation on IGF-1 scaffolds which were immunolabeled by desmin (red) and nucleus (blue) while C2C12 were induced differentiation in A) DM1; D) DM4 and E) DM5; lesser cells were observed while C2C12 were subjected to B) DM2 and C) DM3. (scale bar = 50 μ m).....102
- Figure 6.7** Graph showing myogenin expressions were up-regulated when C2C12 were cultured on IGF-1 scaffolds in DM1, DM4 and DM5.....103
- Figure 6.8** Alizarin red s staining showing the amount of calcium deposition, when TE85 cultured in well plates, was in the order of D) DM3 > C) DM5 > B) DM4 > A) DM1 (scale bar = 50mm for upper deck images, 10 μ m for lower deck images).104
- Figure 6.9** Graph showing Runx2 expression was up-regulated while TE85 were cultured on IGF-1 scaffolds in DM3, DM4 and DM5.....104
- Figure 6.10** Macroscopic images showing calcium deposition which was visualized through alizarin red s staining while TE85 were cultured on IGF-1 scaffolds in (A) DM4, (B) DM5 and (C) DM3 as compared to (D) control which is acellular sample. (scale bar = 50 mm)105

- Figure 6.11** Graphs showing tendon specific genes: A) scleraxis, B) tenomodulin and c) collagen I were slightly down-regulated while tenocytes were cultured on IGF-1 scaffolds in DM5 as compared to in GM3106
- Figure 6.12** Immunostaining images showing the co-existence of TE85 (immunolabeled with RUNX marker, green), tenocytes and C2C12 (immunolabeled with myosin heavy chain, red) and their nucleuses were counterstained by using DAPI. (scale bar = 50 μ m).....107
- Figure 7.1** Schematic showing how proposed solutions addressed the problem statement to improve tendon healing118
- Figure A1** Graphs showing A) TE85 cells proliferated faster and B) Runx2 expression was up-regulated, on IGF-1 scaffolds compared to empty scaffolds. (* indicates $p < 0.05$).....125

Abbreviations

ANOVA	One-way analysis of variance
ASB	L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate
β -Gly	β -Glycerol phosphate disodium salt pentahydrate
BMP- 2	Bone morphogenetic protein-2
DEX	Dexamethasone
DM	Differentiation medium
DMEM	Dulbecco's modified eagle medium
dsDNA	Double stranded deoxyribonucleic acid
ECM	Extracellular matrix
EMEM	Eagle's minimal essential medium
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
GFs	Growth factors
GM	Growth medium
H ₂ O	Water
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HS	Horse serum
IGF-1	Insulin-like growth factor 1
L-Glut	L-glutamine
NaOH	Sodium hydroxide
NPS	Naproxen sodium
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffer saline
PCL	Poly (ϵ -caprolactone)
PEO	Polyethylene oxide
PFA	Paraformaldehyde
PLA	Poly lactide
PLGA	Poly (lactic-co-glycolic acid)
PLLA	Poly (L-lactic acid)

P/S	Penicillin streptomycin
qPCR	Real time quantitative polymerase chain reaction
VEGF	Vascular endothelial growth factor
XPS	X-ray photon spectrometry

Chapter 1

Introduction

This chapter introduces the current state of the art of tissue engineering (TE) in promoting tendon regeneration and the encountered issues during the tendon healing via TE approach. Issues were identified and the solutions were proposed, as the motivation of this developmental dissertation. Key activities of each proposed works are listed out to achieve a specific objective and therefore prove the hypotheses. Finally, the outline of this dissertation is presented, followed by the findings of the dissertation in the last section of this chapter.

.

1.1 Background and problem statement

There are millions of musculoskeletal injuries involving the impairment of tendon and ligament reported annually ¹. Due to low vascularity and cellularity ², spontaneous tendon healings usually results in reduced functional tissues ³. This could be devastating especially for athletes, in addition to the pain and discomfort that need to be endured during the healing process.

Sufficient rest and medical intervention are necessary when the tendon injury is mild. Generally patients are advised not to conduct aggressive activities and sometimes medicines such as non-steroidal anti-inflammatory drug (NSAID) ⁴ or corticosteroids ⁵ will be prescribed to relieve the symptoms of the injury. For severe tendon damage, besides medicine prescription, surgical intervention has become the gold standard for tendinopathies ⁶. Nonetheless, there are insufficient successful cases in which grafts could fully heal injured tendons, as arising issues such as increased inflammatory response, antigenic reactions and lack of long term biocompatibility have limited their application in treating tendinopathies widely ⁷⁻⁹. Notably, **the postoperative adhesion formation often results in interference of tendon gliding and might eventually lead to partial loss of function of the repaired tissue and recurrent injury** ¹⁰. Adhesion formation refers to abnormal adherence between the sheath and tendon ¹¹. One of the promising solutions to avoid adhesion formation is the use of NSAIDs ¹². In particular, naproxen sodium (NPS) has been widely prescribed to tendonitis patients due to its analgesic effects ¹³ and its ability to prevent adhesion formation ¹⁴. It should be noted that sustained releasing systems for NPS are always desirable in order to eliminate its adverse effects e.g. nausea, diarrhea and drowsiness ¹⁵.

For proper tendon healing, tendon tissue engineering (TE) is a promising field which seeks to generate solutions for tendon regeneration. Biodegradable synthetic polymers such as poly-lactide (PLA), poly-glycolide (PGA) and poly(lactide-co-glycolide) (PLGA) are widely used in scaffold fabrication for their ease of processing, reproducibility and better mechanical properties ¹⁶⁻¹⁸. In processing these polymers into scaffolds for TE,

electrospinning stands out as a relatively simple technique to produce continuous fibers such that the resulting scaffold possess the desired interconnected pores to allow tissue integration and diffusion of nutrients¹⁹⁻²¹. However, these electrospun bio-mimicking fibrous structures often lack of bioactivity which is required to promote adequate tendon regeneration²². To address this issue, current research has been focusing on the use of chemical stimulation which involves using molecules or growth factors (GFs) to enhance tendogenesis²¹. For instance, platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF1) have been widely utilized to promote proliferation of tenocytes and trigger the synthesis of collagen^{23,24}. **Despite the beneficial roles of GFs in promoting tendon healing, their susceptibility towards degradations *in vivo* (including denaturation, oxidation and proteolysis) has limited their usage in actual medical practices²⁵.** Therefore, as a whole, a tendon regenerative platform should have a good combination of several criteria such as appropriate morphological environment and sufficient biochemical cues.

In addition, despite numerous researches have been emphasized on the repair of the tendon tissue, the restoration of the intricate structure between tendon and bone (osteotendinous junctions) or tendon and muscle (myotendinous junction) has always been neglected. Being structurally different, this translational part, which serves as the stress concentration at the interface, is always prone to severe or overuse injuries in sports, such as tennis elbow and jumper's knee²⁶. Therefore, in the attempts of re-attaching the tendon to the bone or muscle part, there is high probability of **failures at these fixation sites due to the fact that the unique transitional tissue between the muscle, tendon and bone generally fail to regenerate during healing²⁷⁻²⁹.**

In view of the complex considerations in tendon healing, it is desirable that a scaffold not only possesses the TE criteria as mentioned earlier, but also the ability to release NSAIDs and GFs sustainably. Therefore, it would be useful to investigate the possibility of fabricating a scaffold which combines both tissue engineering and drug delivery of NPS and GFs, and its potential for tendon healing, as well as the regeneration of the unique translational tissue between muscle-tendon-bone.

1.2 Objectives and scope

The general objective of this dissertation is to address the following issues which might defer the success of tendon healing through TE approach:

1) Issue 1: **“The postoperative adhesion formation often results in interference of tendon gliding and might eventually lead to recurrent injury or partial function loss of repaired tissue.”**

In the attempts of tendon healing, other than the necessary TE criteria as mentioned earlier, it is essential to consider the issue of adhesion formations during the healing process. NPS, which can relieve the pain symptoms and prevent fibrous adhesions, has been identified as a promising solution. However, the attempts were not entirely successful in releasing NPS sustainably in hydrophobic matrices³⁰⁻³² because of its low molecular weight and the hydrophilic nature. The first part of this dissertation therefore proposes to fill this gap through a combination of obtaining sustained release of NPS and using of TE approach to better heal tendinopathies. It is postulated that different solvent systems could affect drugs distributions within electrospun fibers and thus affecting their release. It is therefore of interest to investigate the possibility of releasing NPS sustainably by optimizing the solvent system and polymer ratios of the electrospun fibers, and eventually evaluate the ability of the scaffold for tendon regeneration.

Therefore, for this part the specific objective is to achieve sustained release of NPS in a fibrous scaffold which possesses tendon TE characteristics such as aligned morphology and optimal mechanical properties.

It is hypothesized that sustained release of NPS could be achieved in electrospun fibrous system through affecting the drug distribution in the fibers via modulating the formulations of the electrospinning solutions

Key activities:

- Fabricate NPS electrospun scaffolds with tendon TE characteristics by variation of electrospinning controlling parameters;
- Achieve sustained release of NPS through optimizing the 1) polymer compositions; and 2) co-solvent percentage;
- Study the factors affecting the NPS release through analyzing fibers diameter, scaffold hydrophilicity and drug distribution;
- Evaluate the characteristics of scaffolds for tendon healing from their mechanical properties and cell compliance study.

2) Issue 2: **“Despite the beneficial roles of GFs in promoting tendon healing, their susceptibility towards degradations *in vivo* (including denaturation, oxidation and proteolysis) has limited their usage in the actual medical practices”**

Owing to the beneficial roles of GFs in promoting tendon healing and the detrimental side effects for uncontrolled GFs delivery, numerous efforts have been done to incorporate GFs into matrices to achieve controlled release of GFs for tendon regeneration³³⁻³⁶. One of the main challenges is that the entrapment processes which involve the use of organic solvents would deteriorate the protein stability^{37,38}. Recent reports on the feasibility in persevering the ordered peptides secondary structure in hexafluoroisopropanol (HFIP)-based solution has highlighted the possibility of using HFIP as the solvent in electrospinning GFs³⁹, in addition to the fact that HFIP-based solutions can minimize the notorious protein denaturation which occurs at the organic-inorganic interfaces⁴⁰⁻⁴². The second part of this dissertation would thus investigate the feasibility of using HFIP as the electrospinning solvent for GFs delivery in tendon TE.

The specific objectives of this part is thus to incorporate GFs (insulin-like growth factor 1, IGF-1 as identified in this dissertation) in an electrospun fibrous system and achieve controlled release of IGF-1 to promote cellular responses.

It is hypothesized that the bioactivity of entrapped IGF-1 in hydrophobic polymer electrospun matrices could be better preserved through the use of HFIP as the solvent for electrospinning.

Key activities:

- Fabricate the IGF-1 aligned scaffolds with tendon TE characteristics;
- Correlate the relationships between the polymer concentration/compositions with the release of IGF-1 from the electrospun fibrous scaffolds;
- Examine the biological activity of the IGF-1 released from electrospun scaffolds with the use of western blotting technique;
- Evaluate the effect of released IGF-1 on the downstream cellular responses, such as proliferation and differentiation

3) Issue 3: “Failures at these fixation sites due to the fact that the unique transitional tissue between the muscle, tendon and bone generally fail to regenerate during healing”

In order to improve the improper regeneration at the intricate junctions between muscle-tendon-bone, many efforts such as improving suturing techniques⁴³⁻⁴⁵, use of GFs⁴⁶⁻⁴⁹ and mesenchymal stem cells⁵⁰ have been attempted. However in interface TE, most studies focused on scaffolds designs⁵¹; while the culture conditions for growing different tissue types have been inadequately addressed. In view of the fact that *in vitro* models were much more preferred in order to better manipulate cell fate prior to implantation⁵², the last part of this dissertation hence proposes the use of GFs-loaded scaffolds to optimize the culture environments for growing different tissue types, namely myoblasts, tendon cells and osteoblasts.

The specific objective of this part is to optimize the culture conditions for growing three types of cell (namely C2C12, tendon cells and TE85) in one common platform, which is the use of IGf-1 scaffolds.

Key activities:

- Develop a same culture condition where C2C12, TE85 and tenocytes could survive and proliferate;
- Ensure the C2C12, TE85 and tenocytes could undergo differentiation properly;
- Tri-culture the cells on IGF-1 scaffolds

1.3 Dissertation outline

The issues encountered during the tendon healing have been discussed in Chapter 1 to further support the rationale and objectives of the dissertation. In Chapter 2, a review on the tendon structures, current treatments and how tissue engineering could help in tendon regeneration have been discussed. Elaboration of materials and methods utilized in this work will be presented in Chapter 3. For Chapter 4, the feasibility of achieving sustained release of NPS from electrospun fibrous scaffolds were explored through modulating the polymer compositions and solvent ratios. Afterwhich, IGF-1 was incorporated into fibrous scaffolds and the studies regarding the bioactivity of IGf-1 and downstream cellular responses were explored in Chapter 5. In Chapter 6, culture medium compatibility optimization was carried out to tri-culture the three types of cells on the IGF-1 scaffolds, in anticipating to achieve an *in vitro* musculoskeletal model. Finally, Chapter 8 concluded the work in this dissertation with recommendations on future work.

1.4 Findings and outcomes/originality

This research led to several novel outcomes:

1. Achieving sustained release of NPS in electrospun fibrous scaffolds with tendon TE characteristics by affecting the drug distributions via modulating the polymer compositions and the percentage of co-solvent (i.e. water) used.
2. Preserving IGF-1 bioactivity in an electrospun hydrophobic scaffold and successfully promoting tendon cells proliferation and differentiation under the influence of released IGF-1. In addition, the released IGF-1 has rescued the proliferation of tendon cells on combined scaffolds, where the released NPS could have some inhibitory effects on cellular response.
3. Establishing an *in vitro* tri-culture system where three types of cell, namely C2C12, tenocytes and TE85 could proliferate and differentiate, through the culture medium compatibility optimization.

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Chapter 2

Literature Review

This chapter comprises of 4 sections. The first section introduces the structure and function of the tendon, followed by tendinopathies and the healing process involved. The second section would summarize the current methods in treating tendon injuries and the associated issues. These bring out the third section which discusses how tissue engineering contributes to the tendon healing via four approaches, namely scaffolds, cells, chemical stimulation and mechanotransduction. Lastly, the fourth section highlighted the importance in enhancing osteotendinous and myotendinous junctions repair in order to achieve better tendon healing.

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2.1 Tendon

2.1.1 Structure and function

A tendon (or sinew) is a fibrous connective tissue in a form of tough band which links bone to muscle. Healthy tendons are made up of arrays of collagen fibers (mainly type I collagen) parallel to each other, along with 1–5% proteoglycans, 2% elastin, and 0.2% inorganic components such as manganese, copper and calcium¹. Being encased in dense irregular connective tissue sheath, tendon is assembled hierarchically from collagen molecules to form fibrils, fiber bundles, fascicles and tendon units eventually². There are two main types of cells in tendon: tenoblasts and tenocytes. Tenoblasts have spindle shaped morphology and many cytoplasmic organelles. They mature into tenocytes, which are morphologically identical to fibroblasts^{3,4}. Tenocytes detect and respond to mechanical loading through gap junctions formed between them⁵.

Figure 2.1: Schematic diagram of the structure of tendon

The closely packed collagen fibrils which show a wave-like appearance or “crimp pattern” play a pivotal role in their mechanical properties². During tendon stretching, due to crimps development in the collagen fibrils, tendons behave like soft tissue and are more stretchable than ligaments. They allow tendon to withstand tensile stress, while proteoglycans are able to withstand compressive stress⁶. Other than simply transmitting forces, tendons also provide additional stability during locomotion as well as storing and recovering energy.

2.1.2 Sports injuries, tendinopathies and healing process

Regular exercise could bring benefits to the body, however, even regular careful exercise, does come with risks. Most people only experience minor sports-related injuries such as strained muscles and blisters. A few will experience more serious injuries such as torn cartilages or tendon sports injuries. 1 of every 10 people and 1 of every 2 runners are afflicted with tendon injuries. In addition, tendons are also susceptible to various kinds of injuries or tendinopathies due to intrinsic factors such as age, nutrition and body weight or extrinsic factors such as excessive loads and environmental conditions. Tendinopathies can be classified into four types. Paratenonitis, as the first type, is considered when the inflammation occurs at paratenon or paratendinous sheet ⁷. At the cellular level, if the tendon injury is non-inflammatory, it is classified as tendinosis ⁸. Third type would be paratenonitis with tendinosis, which refers to combination of tendon degeneration and paratenon inflammation while for the fourth type, tendinitis refers to vascular disruption besides degeneration with inflammation of the tendon ⁹. Different injuries result in various degrees of inflammation and degeneration of tissues, which may eventually lead to tendon rupture.

For tendon healing, there are three main stages which include inflammation, proliferation or repair, and remodeling. In the inflammation stage (which lasts for few days), inflammatory cells like neutrophils, erythrocytes, monocytes and macrophages are firstly recruited, followed by angiogenesis as well as proliferation and migration of tenocytes to injury site to produce collagen III ^{8,10}. At the second stage, tenocytes continue secreting collagen, proteoglycans and GAGs at the injury site. Associating with collagen, proteoglycans (e.g. decorin) and GAGs (e.g. chondroitin sulfate and dermatan sulfate) are responsible for tendon development by playing important roles in the fibril assembly process ². At remodeling stage, production of collagen and GAGs will be reduced. This part will take about six to ten weeks and the tissue becomes more fibrous. During the second part of the remodeling stage, aligned collagen fibrils start to crosslink, and forming stiffer and scar-like tissue ⁸.

2.2 Current treatments

Spontaneous tendon healing is possible. However, the formation of scar tissue which has smaller crimp pattern with different orientation results in mechanically weaker tissue ¹¹. Furthermore, the imperfect integration at tendon-tendon or tendon-bone interfaces due to fibrous adhesions would have reduced the functionality of repaired tendon through limiting tendon gliding and excursion ^{12,13}. In addition to the long term pain and discomfort which have to be endured by the patients, several issues such as degenerative changes, compromised functionality, reduced joint stability and possibility of re-rupture are often reported on self-healing tendon ¹⁴.

2.2.1 Medicinal intervention

There are two conventional ways for tendinopathies management: conservative and surgical. Conservative management refers to sufficient rest and medicinal intervention, e.g. corticosteroids injections. During the healing process, collagen will be produced to recover the continuity of injured tendon fibrils. However, there will be a surge of inflammatory cells present at the injury site at the same time. This leads to scar tissue formation due to undesirable integration with the fibrils between the sheath and tendon. As a consequent, the scar tissue will interfere with the tendon gliding function and thus leads to partial loss of tendon function and recurrent injury ¹⁰. The use of non-steroidal anti-inflammatory drugs (NSAID), like naproxen sodium, was found to be effective in preventing the adhesion formation during tendon healing.

2.2.2 Surgical intervention

Surgical intervention has been the alternative for tendon treatment when the damage is extensive. Various kinds of therapeutic options have been attempted, including autografts, allografts and prosthetic devices. However, allografts are expensive, limited and possess the risk of immune rejection ¹⁵ while autologous grafts are often associated with certain degree of donor morbidity ¹⁶. Although artificial implants are relatively successful, the

issues like antigenic reactions, the lack of long term biocompatibility, increased inflammatory response and failure at fixation sites have limited their application in treating tendinopathies¹⁷⁻¹⁹. Hence, there is an increasing focus on tendon tissue engineering which aims to induce tendon self-regeneration.

2.3 Tendon tissue engineering (TE)

2.3.1 Scaffolds

A three dimensional scaffold housing the appropriate cells is essential to produce a construct with the aim of regenerating new tendon tissue. Besides, scaffolds must not elicit cytotoxic response and should be biocompatible. In addition, the degradation rate should match the rate of neotissue formation while the materials used has to be bioresorbable such that the by-products could be expelled out naturally from the human body through metabolic pathways²⁰⁻²³. Other than proper surface chemistry to allow attachment of cells and also porous structure to facilitate tissue integration and nutrients diffusion²⁴⁻²⁶, the mechanical properties of scaffold should be able to support loading and match with functional tissue²⁷. In tendon TE, scaffolds are generally made up of natural materials and synthetic polymers. Giving examples of using natural scaffolds, mesenchymal stem cells (MSCs)-seeded collagen gel in Achilles tendon defect of rabbits has demonstrated improvement in biomechanics and structure²⁸. In another study, fibroblast-seeded chitosan-based hyaluronan composite fiber scaffolds has achieved better mechanical properties as well as collagen I production compared to acellular controls²⁹. However, natural scaffolds lack of adequate mechanical properties for long term *in vivo* usage and hence it is relatively hard to reconstruct the complex architectural structure of the native tendon³⁰.

Unlike natural polymers, synthetic biodegradable polymers especially those with saturated poly- α -hydroxy esters such as poly-lactide (PLA), poly-glycolide (PGA) and poly(lactide-co-glycolide) (PLGA) are much more preferred in tendon TE, due to their ease of processing into desired shape with appropriate architectural structure, pore

frequency and better mechanical properties. Among various processing techniques, electrospinning, which uses electrical charge to draw fibers from polymer solutions, stands out for its relatively simple setup to obtain scaffolds with continuous submicron to millimeter fibers with high surface-area-to-volume ratio and interconnected pores ³¹. These advantages have shown the potential of electrospinning scaffold for tendon healing. Sahoo et al. demonstrated bone marrow stromal cells seeded onto a combination of knitted PLGA scaffolds with PLGA electrospun nanofibers proliferated faster and having higher expression of decorin, collagen I and biglycan genes compared to co-knitted PLGA scaffolds ³². Notably, aligned fibers which could be easily modulated in electrospinning have shown potential tendogenesis in human tendon stem/progenitor cells ³³. A recent dual scaffolding system has shown the potential of using co-electrospun poly(L-lactide)/collagen and poly(ϵ -caprolactone)/collagen scaffold to regenerate muscle-tendon junctions for tendon TE ³⁴. Though 80% recovery in mechanical properties of the repaired tendon could be achieved by using tenocytes-seeded PGA fibers, this sub-optimal functionality is not perfect especially for athletes who usually hope for a full recovery ³⁵. This has suggested that produced bio-mimicking structures are not sufficient in promoting tendogenesis, and other factors such as appropriate cell types should also be taken into consideration in tendon TE.

2.3.2 Cells

In tendon TE, using autologous or allogenic tendon cells for therapeutic repair could be quite challenging. The yield of tendon cells extracted is very low and tenocytes tend to de-differentiate *in vitro* ³⁶. Recently, tendon stem/progenitor cells which have been recently isolated by Bi et al. have shed a new light on using autologous or allogenic cells for tendon TE ³⁷. Besides autologous cells, much research has been carried out to investigate the possibility for pluripotent embryonic stem cells (ESCs), multipotent mesenchymal stem cells (MSCs) and pluripotent cord blood stem cells (SCSCs) to differentiate into tenocytes. For example, autologous MSCs have been utilized to enhance tendon tissue repair ³⁸. However, separate study showed that autologous bone marrow-derived MSCs were not adequate to regenerate fully functional tendon tissue in rabbit's

model³⁹. In view of these, stimulations have been used to enhance tendogenesis.

2.3.3 Chemical stimulations

Chemical stimulation has been the most effective method to enhance tendogenesis because a variety of molecules or growth factors (GFs) are involved actively in tendon healing. During the early stage of healing process, insulin-like growth factor 1 (IGF-I) could promote the collagen synthesis and proteoglycans secretions while PDGF could enhance DNA synthesis and tendon cells proliferation². Basic fibroblast growth factor (bFGF) was found to have enhanced collagen III production and cell proliferation in rat patellar tendon model⁴⁰. In addition, as members of transforming growth factor beta (TGF- β) families⁴¹, bone morphogenetic proteins (BMPs) have also been widely accessed and shown promising results. For instance, addition of BMP-12 influenced fibrogenesis which might have regulated the ECM deposition. Interestingly, other than up-regulating the specific growth factors, there are numerous efforts have been done to suppress TGF- β 1 which causes adhesion formation in tendons and reduces the functionality of the repaired tissue.

Recent advancements have led to the development of scaffolds which combine good structural characteristics with capabilities to release GFs sustainably. The current approach to load GFs can be categorized as attachment or encapsulation techniques. To attach GFs on the polymeric scaffolds, various ways such as physical adsorption or chemical crosslinking have been extensively researched. For instance, GFs which have with heparin binding sequences will covalently crosslink to fibrin and the release could be realized passively through simultaneous degradation of fibrin/heparin. As for entrapment method, which is more preferential due to its simplicity, slower release of entrapped GFs could be achieved when the “polymer capsule” degrades, either by surface eroding or bulk eroding. Among various systems such as hydrogels, microspheres and porous solid scaffolds, electrospinning has predominantly been focused in GFs releasing because it simply allows direct loading of GFs into fibers and could achieve high drug loading efficiency⁴². However, rapid release and denaturation of GFs at the oil/water

interface ⁴³ are even inevitable although different kinds of electrospinning have been attempted, like blending electrospinning, emulsion electrospinning or coaxial electrospinning ⁴⁴. To rectify these issues, different approaches have been attempted such as preloading of GFs into micro/nanospheres or proteins in gentle environment prior to adding into electrospun fibers. For example, bFGF-loaded dextran glassy nanoparticles inside electrospun poly-l-lactic acid fibers were shown to have longer release for nearly 30 days while maintaining the bioactivity of bFGF at the same time ⁴⁵.

2.3.4 Mechanotransduction

As an alternative, several researches have been accessing the possibility of mechanical stimulation in enhancing tendon healing. Tendon cells, which are mechanosensitive, can respond to mechanical stimulations and alter their cell phenotype, genes expression and proteins synthesis ⁴⁶. It has been shown that there was higher secretion of TGF- β , bFGF and PDGF when human tendon fibroblasts were 5% stretched at a frequency of 1 Hz ⁴⁷. In contrast, immobilization of rabbit tendons have shown reduction of water, proteoglycans, and collagen crosslinks ¹⁰ and decrease in mechanical properties and total weight ⁴⁸. Proliferation of fibroblast as well as synthesis and realignment of collagen fibrils could also be stimulated when cyclic tension was applied ⁴⁹. Although the optimal magnitude, frequency, duration and type of mechanical stimulation still remain unclear, mechanotransduction of tendon has sparked a lot of interests among researchers.

2.4 Osteotendinous and myotendinous junctions

Other than tendon itself, its connection to the bone (osteotendinous junction) and to the muscle (myotendinous junction) are of clinical importance as well. Myotendinous junction is generally more defined with tendon fibers connected to the endomysium fibers whereas osteotendinous junctions are usually featured into four zones of increasing mechanical stiffness: tendon, fibrocartilage, mineralized fibrocartilage and bone ⁵⁰⁻⁵². These junctions are always subjected to great mechanical stress during the movement, hence making these junctions vulnerable to acute or overuse injuries ⁵³.

Figure 2.2: Schematic diagram of osteotendinous and myotendinous junctions

To repair these regions, they are usually sutured back to their anchorage sites. However some reports indicated the poor quality of repairs, slow healing process and even failure in re-establishing the translational junctions, eventually leading to recurrent injury⁵⁴⁻⁵⁶. To address these, numerous efforts have been done, such as applying growth factors^{57,58}, improving suturing techniques⁵⁹, employing stem cells¹ or multi-lineage cells on biomimetic scaffolds⁶⁰⁻⁶³, and promising results have been demonstrated.

Although different approaches have been employed in order to improve tendon healing, there is a need to combine several approaches together, in order to better tackle on the issues which might hinder the tendon regeneration. Hence in this dissertation, we have utilized “bench-to-bedside” approach by employing the knowledge obtained from clinical observance and applying engineering approach to create a sustained dual-drug therapy to address the issues in tendon TE.

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Chapter 3

Experimental Methodology

This chapter summarizes the materials and methodologies used in this work. First section reveals the materials used, followed by the fabrication of different types of drug-loaded scaffolds in second section. In vitro drug release studies were then described in third section and the scaffolds were characterized as described in fourth section. The resulting scaffolds were then subjected to cell studies in fifth section and being evaluated for their efficacy in inducing relevant cellular responses in sixth section. Statistical analysis was done whenever necessary in this work to demonstrate whether there were significant differences among and between groups of specimens. The methods used for statistical analysis were described in the last section.

3.1 Materials selection

Biodegradable polymers such as poly (L-lactic acid) (PLLA; intrinsic viscosity=6.5 dL/g; Purac Biomaterials), poly (lactic-co-glycolic acid, 80:20) (PLGA, Evonik Industries, intrinsic viscosity= 1.7 - 2.6 dL/g), poly (ϵ -caprolactone) (PCL; M_w =70 000–90 000; Sigma Aldrich), which are FDA approved, were used as received.

3.2 Materials fabrication

In view of the different requirements while incorporating different therapeutic agents, two types of electrospun scaffolds were fabricated.

3.2.1 Preparation of electrospinning solutions

3.2.1.1 Solutions for NPS scaffold

To address the adhesion formation issue, PLLA and PCL were used as the materials for scaffolds fabrication. Electrospinning solutions were prepared at a total polymer concentration of 8w/v% PLLA–PCL blends with 0.4 w/v% naproxen sodium (NPS, Sigma Aldrich) (relative to 1,1,1,3,3,3-hexafluoro-2-propanol, HFIP, Sigma Aldrich). Solutions with varying PLLA:PCL ratio (i.e. 70:30, 80:20 and 90:10) was prepared to understand the effect of polymer compositions. Subsequently, 3%, 5%, 7% and 10% v/v deionized water was added into HFIP to investigate the effect of co-solvent (i.e. water). Scaffolds were named according to their PLLA ratio, followed by PCL ratio and its percentage water volume. For example, 90-10-3 indicates 90wt% PLLA blended with 10wt % PCL, coupled with 3% (v/v) water in HFIP.

3.2.1.2 Solutions for IGF-1 scaffold

For the fabrication of insulin-like growth factor 1 (IGF-1, Sigma Aldrich) scaffolds, PLGA and PCL were used instead. 8w/v% of PLGA-PCL blends (relative to HFIP) with

different ratios (i.e. 70:30 and 80:20) were prepared to understand the effect of polymer compositions on IGF-1 release. 5wt% of poly (ethylene oxide) (PEO, $M_v = 4 \times 10^6$ Da, Sigma Aldrich) (relative to total polymer mass) was added to facilitate the IGF-1 release. In addition, different concentrations (i.e. 8w/v%, 5w/v% and 3w/v%) of PLGA-PCL blends at the ratio of 70:30 (with PEO) were also prepared to obtain different release profiles. Scaffolds were named according to their concentration, followed by PLGA-PCL ratio and whether with or without PEO in the formulations. For example, 8-70/30-0 indicates 8 w/v% of polymer with the composition of 70wt% PLGA blended with 30wt % PCL, and 0 wt% of PEO in the formulation.

3.2.2 Fabrication of electrospun scaffolds

Electrospinning is a fiber production method which uses electric force to draw polymer jets into nano to micron range fibers ¹. Electrospinning was performed to fabricate scaffolds by using the MECC Nanon-01A Electrospinning Set-up. A single-nozzle spinneret (22 gauges) was used and different solutions were dispensed at an accelerating voltage of 15 kV and constant feed rate of 1 mL/h. To obtain scaffolds with aligned morphology, fibers were electrospun and collected by using a metallic mandrel set-up, which was rotated at 1500 rpm and fixed at 14cm away from the tip of the spinneret.

For combined scaffolds, NPS electrospinning solution was electrospun under the aforementioned conditions then followed by electrospinning of equal amount of either IGF-1 containing or pure PLGA/PCL/PEO solutions (empty scaffolds) to obtain IGf-1 scaffold + NPS scaffold or Empty scaffold + NPS scaffold respectively.

Figure 3.1: Schematic diagram of the multi-layered scaffolds which release IGF-1 and NPS.

3.3 *In vitro* drug release studies

Scaffolds were weighed accurately in triplicate, placed in vials containing 5mL phosphate-buffered saline (PBS; pH7.4; OHME Scientific) and incubated in a shaking incubator at 37°C. PBS, which possesses a physiologically relevant concentration of ions similar to the human body, was used in the study.

3.3.1 Release of naproxen sodium (NPS)

At predetermined time intervals, 1 mL PBS solution was removed completely and replenished with fresh PBS solution from each vial. The concentration of released NPS was analyzed using a Shimadzu UV-2501 ultraviolet–visible (UV-vis) spectrophotometer at 331nm. A UV-vis spectrophotometer can be used for quantitative determination of different analytes. Briefly, a monochromatic beam passes through a cuvette containing a solution of the samples. Through absorbing energy in the form of visible light or ultraviolet, sample molecules which contain non-bonding electrons (n-electrons) or π -electrons excite their electrons to higher anti-bonding molecular orbitals, resulting in light absorption and the intensity of the sample beam is defined as I . The intensity of the reference beam, which passes through the cuvette containing only the solvent, is defined as I_0 . Absorbance (A) is defined as $\log I_0/I$. The molar concentration of the sample (c) could be calculated from the formula, $c = A / \epsilon l$ (where A = absorbance, ϵ = molar attenuation coefficient of the sample and l = length of light path through the cuvette)².

3.3.2 Release of insulin-like growth factor 1 (IGF-1)

Released medium (in PBS) was removed completely daily and replenished with fresh PBS solution. The released IGF-1 amount was characterized by using IGF-1 ELISA Kit (Abcam). ELISA (enzyme-linked immunosorbent assay) is a technique that uses antibodies and color change to identify and quantify the amount of a substance³. Briefly, released IGF-1 was absorbed to a pre-coated surface. Then, IGF-1 specific biotinylated

detection antibody was applied over the surface so it can bind to the attached IGF-1. This antibody was linked to Streptavidin-Peroxidase Conjugate, and, in the final step, TMB was added to visualize Streptavidin-Peroxidase enzymatic reaction. The concentration of IGF-1 could be determined by measuring the absorbance using Infinite200 microplate reader (Tecan Inc) at wavelength of 450 nm.

The total IGF-1 amount inside the polymers was quantified by using microBCA assay (Thermo Fisher Scientific). It is a biochemical assay for determining the total concentration of protein in a solution (in the range of 0.5 μ g/mL to 20 μ g/mL)⁴. The IGF-1 scaffolds were firstly weighed and then being degraded in 1M NaOH for 2 weeks. Once scaffolds degraded, solutions were diluted 10 times in PBS to yield concentration at 0.1M NaOH and mixed with equal amount of microBCA working solution (through mixing the reagent A, reagent B and reagent C at ratio of 25:24:1) and underwent incubation at 37°C for two hours, prior to the measurement of the absorbance at 562nm. The background readings (the values of empty scaffolds) were deducted and the amounts of IGF-1 were obtained from the plot of the calibration curve.

3.3.3 Determination of IGF-1 stability in different solvents

Same amount of IGF-1 solution (reconstituted in water) was added into different solvents, namely dichloromethane (DCM, Tedia), HFIP, water and HFIP + PEO. The solutions were mixed and stirred for 30 min then being freeze-dried. The freeze-dried samples were then reconstituted in ELISA buffer and checked for their concentration. Control was the freshly prepared IGF-1 solution, which was set at 100% stability.

3.4 Materials Characterization

3.4.1 Morphological assessment of electrospun scaffolds

The microstructures of the prepared scaffolds were observed under a field emission scanning electron microscopy (FESEM; JSM-6340F, Jeol Co.). In principle, FESEM

scans a sample with a focused beam of electrons, producing secondary electrons from the sample which are detected by the detector and to give out information such as surface topography and composition ⁵. The scaffolds were sputtered with platinum for 60s at 20mA and analyzed at an accelerating voltage of 5 kV. A total of 50 independent fibers were used to determine the mean fiber diameter using ImageJ software (n=50).

3.4.2 Contact angle and wetting tension

Contact angle could provide information about the wettability of a solid surface by a liquid ⁶. A static sessile drop technique was utilized to evaluate the contact angles of the scaffolds on a contact angle goniometer. Distilled water was pumped out at a rate of 5 μ L/s onto the scaffolds and the droplet was allowed to relax for 10 s prior to taking the images for analysis using FTA32 software. The measurements were conducted at room temperature in triplicate.

3.4.3 Surface properties of electrospun NPS scaffolds

X-ray photon spectrometry (XPS) was utilized to determine the distribution of NPS within the electrospun fibers. XPS analyzes the number and kinetic energy of the electrons escape from the top 10nm of the materials after being irradiated with a beam of X-rays ⁷. Kratos AXIS UltraDLD spectrometer (Kratos Analytical Ltd) with a mono Al K α radiation source (hv=1486.71 eV) was performed at 5mA and 15 kV (n=3) to give out the information regarding the surface properties of the NPS scaffolds.

3.4.4 Mechanical properties of electrospun scaffolds

Tensile testing was performed on Instron tensile machine 5567 to measure the mechanical properties of the scaffolds. A load cell of 500 N with a load generation of 5mm/min was used to pull the scaffolds which were cut into a standard dumbbell shape (according to ASTM D 638-08) and tested at room temperature. Three samples for each formulation were used to calculate tensile strength (σ), strain at break (ϵ) and Young's modulus (E) of

the scaffolds (n=3). Generally, the tensile strength of a material is the maximum amount of tensile stress that it can take before failure⁸. The Young's modulus was determined by calculating the gradient of the initial linear slope of the stress-strain curve⁹. Elongation at break is the ratio between initial length and changed length after the test sample breaks. It indicates the ability of a material to resist shape changes without crack generation¹⁰.

3.4.5 Hydrolytic degradation of electrospun scaffolds

Three samples (1×1 cm²) from each formulation were first weighed (W₀) before PBS incubation at 37°C. At fixed time points, the electrospun scaffolds were washed with deionized water and excess water were blotted off prior to measurement of the wet weight (W_{wet}) (n=3). The samples were then thoroughly dried in a vacuum oven for at least 1 week before measuring the dry mass (W_{dry}) gravimetrically. The water uptake was determined according to the following equation:

$$\text{Water uptake (\%)} = [(W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}}] \times 100\%$$

These samples were then dissolved in 1 mL of chloroform for at least an hour and filtered through 0.2 µm cellulose filters into vials and immediately capped for size exclusion chromatography analysis. Size-exclusion chromatography (SEC) is a technique which separates molecules according to their size molecular weight¹¹ and information about the molecular weight change could be obtained accordingly.

3.5 Cell Studies

3.5.1 Cell culture

Four types of cells have been utilized in the work:

3.5.1.1 L929 on NPS scaffold

L929 murine fibroblasts have been utilized to evaluate NPS loaded aligned scaffolds' biocompatibility. L929 murine fibroblasts (CCL-1TM; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratory) with 10% fetal bovine serum (FBS; PAA Laboratory), 1% L-glutamine (200mM; PAA Laboratory) and 1% penicillin–streptomycin (Gibco) at 5% CO₂ and 37°C. Scaffolds were cut into circular discs of 2.15cm diameter, sterilized for 30 min with UV and washed three times with PBS. Cells were then seeded at a density of 5000 cells/cm² onto the samples. The samples were subsequently incubated for 3 h to allow cell attachment at 37°C and 5% CO₂. Afterwhich, 1 mL complete culture medium was added into each well. Tissue culture polystyrene (TCPS) was used as the control. The medium was replenished every 3 days.

3.5.1.2 C2C12 on IGF-1 scaffold

C2C12 murine skeletal myoblasts (Sigma) were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratory) with 20% fetal bovine serum (FBS; PAA Laboratory) and 1% penicillin–streptomycin (Gibco) at 5% CO₂ and 37°C. Scaffolds were cut into 1cm x 1cm square shapes, sterilized for 30 min with UV and washed three times with PBS. Cells were then seeded at a density of 100000 cells/cm² onto the samples. The samples were subsequently incubated for 3 h to allow cell attachment at 37°C and 5% CO₂. Afterwhich, 1 mL complete culture medium was added into each well. Empty scaffolds were used as the control. The medium was replenished every 3 days. Once the cells become confluent, the growth medium was changed into differentiation medium which consisted of DMEM supplemented with 2% horse serum (Sigma) and 1%

penicillin–streptomycin (Gibco).

3.5.1.3 Tenocytes on IGF-1 scaffold

Tenocytes (ZenBio inc.) were cultured not more than passage 4 in the Tenocyte Culture Medium (ZenBio inc.) which composed of FBS and Penicillin / Streptomycin / Amphotericin B at 37°C and 5% CO₂. IGF-1 scaffolds were cut into 1cm x 1cm square shapes, sterilized for 30 min with UV and washed three times with PBS. Cells were then seeded at a density of 50000 cells/cm² onto the samples. The samples were subsequently incubated for 3 h to allow cell attachment at 37°C and 5% CO₂. Afterwhich, 1 mL complete culture medium was added into each well. Empty scaffolds were used as the control. The medium was replenished every 3 days.

3.5.1.4 TE85 on IGF-1 scaffold

TE85 human osteosarcoma (Sigma) were grown in culture medium of Minimum Essential Medium (MEM, Gibco), 10% FBS (FBS; PAA Laboratory), 1% L-glutamine (200mM; PAA Laboratory) and 1% penicillin–streptomycin (Gibco) at 37°C and 5% CO₂. Scaffolds were cut into 1cm x 1cm square shapes, sterilized for 30 min with UV and washed three times with PBS. Cells were then seeded at a density of 100000 cells/cm² onto the samples. The samples were subsequently incubated for 3 h to allow cell attachment at 37°C and 5% CO₂. Afterwhich, 1 mL complete culture medium was added into each well. Empty scaffolds were used as the control. The medium was replenished every 3 days. Once the cells become confluent, the growth medium was changed into differentiation medium which consisted of DMEM (Gibco), 10% FBS (PAA Lab), 1% penicillin–streptomycin (Gibco), 10 mM β-Glycerol phosphate disodium salt pentahydrate (β-Gly, Sigma), 0.05mM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ASB, Sigma) and 0.1μM dexamethasone (DEX, Sigma).

3.5.1.5 Tri-culture of C2C12/Tenocytes/TE85 on IGF-1 scaffolds

Figure 3.2: Schematic diagram of the set-up for tri-culture experiments, where two 12mm coverslip acted as separator to prevent cells mixing during seeding on IGF-1 scaffolds.

IGF-1 scaffolds were cut into 5 cm x 1cm rectangular shapes sterilized for 30 min with UV and washed three times with PBS. Prior to the cells seeding, two pieces of 12mm coverslips were placed on top of the scaffolds as the separators to facilitate the seeding of different cell types, as illustrated in Scheme 3.1. Three types of cells, include TE85, tenocytes and C2C12, were then seeded in the uncovered regions, without mixing, respectively. The cell-seeded samples were incubated for 3 h at 37°C and 5% CO₂ to allow cell attachment in petri dish. Subsequently, each well was filled with 5 mL culture medium which consisted of DMEM (Gibco) supplemented with 20% FBS (PAA Laboratory) and 1% penicillin–streptomycin (Gibco). The medium was changed to differentiation medium which consisted of DMEM (Gibco), 2% horse serum (Sigma) and 1% penicillin–streptomycin (Gibco), 10 mM β -Glycerol phosphate disodium salt pentahydrate (β -Gly, Sigma), 0.05mM L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (ASB, Sigma) and 0.1 μ M dexamethasone (DEX, Sigma) after culturing for 3 days.

3.5.2 Cell quantification

3.5.2.1 PicoGreen assay

Cells were lysed with 0.25% trypsin and 0.1% Triton X-100 at predetermined time points. Cell lysates were mixed with equal amount of PicoGreen working solution. PicoGreen

(Invitrogen) has been commonly used to quantify the amount of double stranded deoxyribonucleic acid present within the cells. The fluorescence detected at the excitation and emission wavelengths of 480nm and 520nm respectively, which directly indicates the cells number and tells the information regarding the proliferation of the cells ¹².

3.5.2.2 AlamarBlue assay

The principle of AlamarBlue is through measuring the reduction of resazurin, active ingredient of AlamarBlue reagent, to resorufin by the viable cells and this could tell the information about the cell metabolism ¹³. To measure the metabolic activity, cell culture medium was changed to DMEM without phenol red (Gibco) supplemented with 10% AlamarBlue reagent. The cells were incubated in the dark at 37°C and 5% CO₂ for 4 h before the medium was checked for the absorbance at wavelengths of 570nm and 600nm.

3.5.3 Cell morphology analysis

Live/Dead staining assay has been employed to measure the cell viability and distribution. Samples were washed with PBS and incubated in a solution of 4 µM ethidium homodimer-1 (Invitrogen) and 2 µM Calcein-AM (Invitrogen) for 15 min at 37 °C. Samples were then washed with PBS and observed under a fluorescence microscope (Nikon 80i) at 10x magnification.

3.5.4 Immunocytochemistry

Immunocytochemistry was conducted to visualize the cell morphology, internal microstructure or to indicate the differentiation tendency. Briefly, cells were fixed using 4% paraformaldehyde in PBS at room temperature for 15 min and permeabilized with 0.1% TritonX-100 in PBS at room temperature for 5 min. Subsequently, blocking of samples was done by adding 10% goat serum (Dako) in PBS for 1 h at room temperature, followed by incubation of primary antibody solution for overnight at 4°C. The primary antibody solutions were prepared by diluting mouse monoclonal anti-desmin antibody

(1:400, Sigma), rabbit polyclonal anti-RUNX2 antibody (1:50, Santa Cruz) in blocking solution for detection of desmin and RUNX2 respectively. Detection of primary antibodies were done by incubation of goat anti mouse Alexa Fluor 546 (1:400, Invitrogen) and goat anti rabbit Alexa Fluor 488 (1:400, Invitrogen) in blocking solution for 1h at room temperature, respectively. Cells cytoskeleton was visualized through the staining of phalloidin (1:400, Invitrogen) in PBS for 1 h at room temperature. The nuclei were counterstained by DAPI (1:1000, Invitrogen) at room temperature for 5 min. Samples were washed with PBST (PBS with 0.05% tween 20) thrice after every incubation step. Finally, samples were transferred onto glass coverslips and mounted with fluorescent mounting medium (Dako) for taking images under a fluorescence microscope (Nikon 80i) at 10x magnification. Negative staining (without secondary antibody) was done to confirm the signal obtained was not non-specific staining

3.5.5 Western blotting for determination of IGF-1 bioactivity

To determine the bioactivity of encapsulated IGF-1, IGF-1 scaffolds were soaked into culture medium for 1 day and 7 days under 37°C and 5% CO₂. The conditioned mediums were then added into wells with confluent C2C12 cells and treated the cells for 30 min prior to cell lysis.

Cells were lysed in a 10:1 (v:w) ratio of ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 0.5% sodium deoxycholate, 150mM sodium chloride, 0.5 mM EDTA, 1% NP-40, 1% SDS and protease inhibitor cocktail (100X). Cell lysates were then centrifuged at 13 000g at 4°C for 10 min and subsequently the protein concentration was determined by using Bradford Assay (Bio-Rad). 5 µg proteins were then loaded into 10% acrylamide gels and electrophoresis was conducted at 100 V for 20 min and then at 200 V for 40 min. After transferring the proteins to polyvinylidene difluoride (PVDF) membranes, 5% non-fat milk powder in Tris Buffered Saline with 0.05% Tween (TBS-T) was used for the blocking process. After 1 h of blocking, primary antibodies from Cell Signaling Technology such as Phospho-Akt Ser473 antibody (no. 9271), total Akt antibody (no. 9272) and β-Actin antibody (no. 4967) were used to stain the membranes at 4°C

overnight. The PVDF membranes were then washed thrice prior to the incubation of secondary antibody, horseradish peroxidase-linked anti-rabbit immunoglobulin G (no. 7072; New England Biolabs), at room temperature for 1 h. The membranes were then washed thrice and exposed to chemiluminescence detection reagent (RPN 2106; Amersham Biosciences). The membranes were scanned using Molecular Imager® ChemiDoc™ XRS System (Bio-Rad) and the blot density was determined by using Quantity One 1-D Analysis software (Bio-Rad).

3.5.6 Real time quantitative polymerase chain reaction

Real time quantitative polymerase chain reaction (qPCR) was used to determine expression of specific mRNA levels on the constructs. qPCR uses the DNA amplification concept to amplify a certain sequence and determine the expression of certain genes¹⁴. Primer sequences (see Table 3.1), were checked for specificity by performing BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and melt curve analysis. All primers were ideally designed to span exon-exon boundaries to prevent amplification of genomic DNA and with 40-60% GC content. RNA was isolated using TRIzol (Sigma) according to manufacturer's instructions and then re-suspended in RNA storage solution (Sigma).

To conduct qPCR, Applied Biosystems 7300 Real Time PCR System and the associated sequence detection software (Version 1.4) was employed. SYBR Green gene expression assays and PCR mastermixes (Applied Biosystems) were used to evaluate the expression of the endogenous control (RPII β) and the target genes. Fluorescence was detected after every cycle and data was analyzed using the $2^{-\Delta\Delta C_T}$ method using RPII β as a housekeeping gene. ΔC_T 1 corresponds to the difference in C_T between target genes and endogenous control, and ΔC_T 2 corresponds to the difference in C_T between target genes and endogenous control in the calibrating samples.

Table 3.1: Primers used for the differentiation determination

Gene target	Sequence (5'-3')
Scleraxis	F: CAGCGGCACACGGCGAAC
	R: CGTTGCCCCAGGTGCGAGATG
Tenomodulin	F: CCATGCTGGATGAGAGAGGTT
	R: TTGGTAGCAGTATGGATATGGGT
Collagen I α 1	F: TAAAGGGTCACCGTGGCT
	R: CGAACCACATTGGCATCA
RUNX2	F: TGGTTACTGTCATGGCGGGTA
	R: TCTCAGATCGTTGAACCTTGCTA
Myogenin	F: GCAGGCTCAAGAAAGTGAATGA
	R: TAGGCGCTCAATGTACTGGAT
RPII β	F: ACATAACGAAGACGGTCAT
	R: TAAGCCATTCAACAAGCAATA

3.5.7 Assessment of mineralization by Alizarin Red S staining

Calcium deposition is commonly used to indicate osteogenesis was determined by using Alizarin Red S staining ¹⁵. Briefly, constructs were rinsed with PBS (without Ca^{2+}) thrice and fixed with 4% paraformaldehyde in PBS for 15 min. 10% ammonium hydroxide was used to adjust the pH of 2% (w/v) Alizarin Red S (Sigma) in distilled water to 4.2. The constructs were stained for 15 min and followed by thorough washing before being imaged digitally or using optical microscopy at 10x magnification.

3.6 Statistical analysis

One-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test, on SPSS v. 11.5 software were conducted to compare between different groups. One-way ANOVA is a statistical technique used to determine if the means are different between samples (using F distribution). If the variance between groups is larger than the variance within the group, it's likely that the mean differs ¹⁶. Tukey's HSD post hoc test is a

statistical test for executing all pairwise comparisons. It is fundamentally similar to t-test, with the correction of type I error when multiple comparisons are made. For conducting Tukey's test, the groups should be normally distributed, independent and the within-group variance should be equal¹⁷. Differences were considered statistically significant at $p < 0.05$.

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Chapter 4

Sustained-release of Naproxen Sodium from Electrospun-aligned PLLA–PCL Scaffolds

The unfavorable adhesion formations, due to the inflammation response after surgery, restrict the tendon healing. It has been demonstrated that NPS can inhibit inflammatory response and hence prevent adhesion formation. Thus, this work aims to incorporate a non-steroidal anti-inflammatory drug, naproxen sodium (NPS) into aligned electrospun scaffolds to address this issue. Factors such as polymer composition (i.e. poly-L-lactic acid (PLLA): poly (ϵ -caprolactone) (PCL) ratios) and solvent composition (i.e. hexafluoroisopropanol:water ratios) were explored to gain knowledge on how these factors can affect NPS release from electrospun scaffolds and thus be optimized to achieve NPS sustained release. Results showed that NPS could be released sustainably for as long as 2 weeks by changing the amount of water as the co-solvent. Besides, scaffold breaking strength increased with the addition of water as the co-solvent. Tenocytes cultured on the NPS scaffolds adhered well to the scaffolds and exhibited their typical elongated morphology. Besides, tenocytes could respond to the topographical cues given by the aligned fibrous scaffolds, creating a network of cells which mimic the native tendon tissue arrangement. These scaffolds are therefore potential candidates in enhancing tendon regeneration in vivo due to their desirable tendon TE characteristics.

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4.1 Introduction

It has been reported that there are approximately 15 million musculoskeletal injuries relating to the impairment of ligament and tendon annually ¹. Due to the low vascularity and cellularity of these organs especially for tendon ², unaided natural healing usually results in deteriorated tissue functionality ³. Tissue engineering (TE) thus came forth as a potential field that helps to provide viable solutions to tendon tissue regeneration. Enormous efforts were done with the aim to mimic the tendon microenvironment through investigating different materials as the scaffold ⁴⁻⁹, selecting appropriate cell types ^{9,10} and evoking the healing of tendon tissues, either chemically ¹¹⁻¹³ or mechanically ¹⁴⁻¹⁷. Although promising results were demonstrated through TE approach, the problem of post-surgical adhesion formation, which might ultimately impede the success of tendon regeneration, is still insufficiently addressed.

Adhesion formation, which is the abnormal adherence of the undesirable fibrous tissue to the surrounding structures, could happen as a result of huge inflammatory feedback at the surgical site ¹⁸. During the natural tendon-healing process, to recover the continuity of damaged tendon fibrils, collagen will be secreted ¹⁹. However, inflammatory cells will also be recruited to the injury site at the same time. The unfavorable integration of these inflammatory cells with the formed collagen fibrils results in scar tissue formations and adherence to the surrounding tissues. Such kind of adherence could interfere with the tendon gliding function and ultimately lead to recurrent injury ³. To reduce these peritendinous adhesions, numerous efforts have been attempted. For example, improving on the surgical techniques ²⁰, tendon sheath closure ²¹ and post-operative mobilization ²²⁻²⁴. Besides, researchers have also utilized materials such as hyaluronic acid ¹⁶, polyethylene tubes ²⁵, silastic ²⁶ and hydroxyapatite ²⁷ to serve as a confinement between the repaired tendon and its surrounding tissues in a form of pseudo-sheath to address the adhesion formation issue. Nevertheless, certain extent of adhesion tissue formation was still observed in the later phases of the experiments ²⁸.

Another approach in preventing adhesion formation would be the prescription of nonsteroidal anti-inflammatory drugs (NSAIDs), in particular naproxen sodium (NPS). NPS is commonly prescribed to relieve pain and treat inflammation. Through the mechanism of decreasing the by-products of arachidonic acid and metabolites via competitive inhibition of cyclo-oxygenase, NPS can effectively reduce adhesion formation^{29,30}. However, the side effects of NPS include nausea, diarrhea and abdominal pain if administrating orally³¹. In view of this, it is essential to design a system that can release NPS sustainably and locally so as to minimize adhesion formation and at the same time avoid the systemic body response during tendon healing. Currently, direct compressing technique³² and the use of hydrophilic matrices³³ have been employed to for the development of NPS-loaded products. However, drawbacks, such as rapid release of NPS from the hydrophilic matrices and inadequate mechanical properties^{34,35}, have limited their application in clinical practices. Existing studies that reported the release of NPS from hydrophobic matrices³⁶⁻³⁸ yielded little successful, mainly because of the low molecular weight and hydrophilicity of NPS. Therefore, **this chapter will address the adhesion formation issue in tendon TE by designing a scaffold that is capable of releasing NPS sustainably and able to provide aligned bio-mimicking environment at the same time.** It is postulated that the distribution of NPS in the electrospun fibers, and thus its release, can be controlled by manipulating the formulations of the electrospinning solution, i.e. co-solvent ratios and polymer ratios. These scaffolds, electrospun from different formulations, were evaluated and characterized in terms of their surface morphology, mechanical behavior, *in vitro* drug-releasing properties and cell compliance.

4.2 Materials and Methods

For the information regarding the materials and methodology used in this chapter, please refer to Chapter 3.

Table 4.1: Methods used in Chapter 4

Description		Section
Scaffold fabrication	Solution preparation	3.2.1.1
	Electrospinning process	3.2.2
<i>In vitro</i> drug release		3.3.1
Materials characterizations	Scaffold morphology	3.4.1
	Scaffold hydrophobicity	3.4.2
	Surface properties	3.4.3
	Mechanical properties	3.4.4
Cell studies	Cell seeding	3.5.1
	Cell proliferation	3.6.2.1
	Cell morphology	3.6.3 / 3.6.4

4.3 Results

Different ratios of 8 w/v% PLLA/PCL blends (90:10; 80:20; 70:30), together with 0.4 w/v% NPS, were dissolved into HFIP and electrospun to look into the effects of polymer compositions on the drug releasing and structural properties. PLLA/PCL blend with ratio of 90:10 was then used to investigate the co-solvent (i.e. H₂O) effect of HFIP/H₂O solvent systems. For the co-solvent effect study, the scaffolds were prepared by adding 0%, 3%, 5%, 7% and 10% of H₂O into HFIP. Scaffolds were labelled with reference to their PLLA ratio, followed by PCL ratio and the water volume percentage. For example, 90-10-3 indicated 90 wt% of PLLA blended with 10 wt% PCL, coupled with 3 % of H₂O in HFIP (shown in Table 4.2).

Table 4.2: Description of different formulations of electrospinning solutions.

Samples	PLLA (wt%)	PCL (wt%)	NPS (wt%)	HFIP (vol%)	H₂O (vol%)
70-30-0	5.6%	2.4%	0.4%	100%	0%
80-20-0	6.4%	1.6%	0.4%	100%	0%
90-10-0	7.2%	0.8%	0.4%	100%	0%
90-10-3	7.2%	0.8%	0.4%	97%	3%
90-10-5	7.2%	0.8%	0.4%	95%	5%
90-10-7	7.2%	0.8%	0.4%	93%	7%
90-10-10	7.2%	0.8%	0.4%	90%	10%

4.3.1 Aligned fibrous scaffolds to mimic tendon micro-environment

FESEM was conducted to observe the morphology of the scaffolds. All formulations of the electrospinning solution (with or without NPS) could be electrospun into well-formed microstructure of aligned fibers with negligible beads, as shown in Figure 4.1. Figure 4.2A shows that changing the PLLA and PCL ratio has no significant effect in terms of fibers diameter. All the formulations yielded fibers with submicron range diameter: $1.071 \pm 0.153 \mu\text{m}$, $1.090 \pm 0.233 \mu\text{m}$ and $0.944 \pm 0.096 \mu\text{m}$ for 90-10-0 80-20-0 and 70-30-0 respectively.

Figure 4.1: Aligned fibers with beadless morphology was obtained from varying formulations of electrospinning solutions with A) no NPS; and with NPS, (B) 70-30-0 (C) 80-20-0 (D) 90-10-0; (E) 90-10-3; (F) 90-10-5; G) 90-10-7; H) 90-10-10. (scale bar = 10μm)

With regards to the co-solvent effects, as shown in Figure 4.2B, there is a decreasing trend of the fibers diameter with the addition of water. The average fibers diameter decreases ($1.071 \pm 0.153 \mu\text{m} > 0.995 \pm 0.221 \mu\text{m} > 0.954 \pm 0.197 \mu\text{m} > 0.836 \pm 0.171 \mu\text{m} > 0.760 \pm 0.176 \mu\text{m}$) while increasing water content from 0%, 3%, 5%, 7% to 10%.

Figure 4.2: Graphs showing the difference in fibers diameter when changing (A) polymer composition, i.e. PCL to PLLA ratio, and (B) co-solvent composition, i.e. H_2O to HFIP. When changing PCL percentage, there was no difference in fibers diameter. Decreasing trend in fibers diameter was observed when increasing H_2O percentage (* indicates $p < 0.05$ wrt 90-10-0; # indicates $p < 0.05$ wrt 90-10-3; ** indicates $p < 0.05$ wrt 90-10-5).

4.3.2 NPS released sustainably up to two weeks

Figure 4.3A shows the *in vitro* NPS release profiles when different PCL to PLLA ratios were used. For the formulation of 90-10-0, the NPS release could be tremendously slowed down, with the first hour burst release recorded at about 25% and NPS released sustainably up to 3 days. However, relatively faster NPS release was observed on 80-20-0 and 70-30-0 formulations, which can only last for 1 day.

Figure 4.3B shows the effect of co-solvent (i.e. H₂O) on NPS. When 3% of H₂O was added, there was a significant reduce of NPS release rate. Release of NPS could last for two weeks with low amount of NPS burst release. However, with the increasing of water content, it was found that release became faster, where formulation 90-10-5 could continue to release NPS for about ten days but 90-10-7 and 90-10-10 scaffolds could only release NPS for less than seven days. Throughout the experiment, the NPS spectrum was similar with the spectrum on the first day of release, indicating that there is no denaturation of the NPS.

Figure 4.3: Plots showing cumulative NPS release from the scaffolds with different: A) PLLA to PCL ratio and B) H₂O to HFIP ratio. Scaffolds with higher PCL to PLLA ratio has contributed to faster release of NPS. The addition of 3 vol% H₂O has resulted in scaffolds with slower NPS release profiles, which lasted for two weeks. However, further increasing H₂O content yielded scaffolds with faster NPS release profiles.

4.3.3 Scaffold hydrophilicities affected NPS release profiles

Scaffolds' hydrophilicities, which have influence in affecting the associated release profiles, were determined by contact angle and water uptake measurements. Figure 4.4 demonstrates the contact angle of various scaffolds. 90-10-0 scaffold showed lower hydrophilicity, as evidenced from the higher contact angle compared to that of 80-20-0 and 70-30-0, as shown in Figure 4.4A. Figure 4.4B shows that the contact angle has no significant change when changing the amount of the H₂O as the co-solvent.

Figure 4.4: Graphs showing the contact angle measurements of different scaffolds with varying: A) PCL to PLLA ratio and B) H₂O to HFIP ratio. 70-30-0 and 80-20-0 scaffolds have significant lower contact angle than that of the 90-10-0. When varying the H₂O to HFIP ratio, contact angle of the scaffolds did not significantly change. (* indicates $p < 0.05$)

Figure 4.5 shows that water uptake measurements of different scaffolds after immersing in PBS for 7 days. As shown in Figure 4.5A, 90-10-0 scaffolds have significantly lower water uptake than that of 70-30-0 and 80-20-0 scaffolds ($p < 0.05$). Meanwhile the water uptake of the scaffolds has no difference while varying the percentage of H₂O as co-solvent (Figure 4.5B).

Figure 4.5: Graphs showing water uptake measurements of different scaffolds with varying A) PCL to PLLA ratio and B) H₂O to HFIP ratio, after immersing in PBS for 7 days. Lower water uptake was observed for 90-10-0 scaffolds. While changing the percentage of H₂O as co-solvent, no significant difference in water uptake was observed. (* indicates $p < 0.05$)

4.3.4 Co-solvent affected NPS distribution inside the fibers

X-ray photon spectrometry (XPS) has been employed to determine the presence of NPS at the binding energy of 1071 eV, which is the Na 4s characteristic peak. Figure 4.6 demonstrates the XPS analysis of scaffolds with and without NPS. The presence of NPS on scaffold 90-10-10, 90-10-3 and 90-10-0 could be confirmed with the peak observed,

while there was no peak detected on 90-10 (No NPS) scaffold at that particular region. The area under the spectra could be interpreted as the amount of NPS present on the surface of the fibers (depth of 0-10nm). Figure 4.6 shows that relatively lower NPS amount was detected on 90-10-3 scaffolds (Figure 4.6, dash-dot line). Whereas, when no water was added as the co-solvent (90-10-0, Figure 4.6, solid line), higher amount of NPS was detected, while. However, a spectrum with an intermediate area that lies between 90-10-3 and 90-10-0 when increasing the percentage of co-solvent to 10 vol%.

Figure 4.6: Plot showing the XPS analysis of the scaffolds with and without NPS. Na 4s characteristic peak indicates the presence of NPS on scaffold 90-10-10 (dotted line), 90-10-3 (dash-dot line) and 90-10-0 (solid line). There was no peak detected for scaffold without NPS (dash line) at that particular region.

4.3.5 Scaffolds yielded optimal mechanical properties

The mechanical properties of various fibrous scaffolds could be seen in Figure 4.7 and Table 4.3. Apparent Young's modulus (E_{app} , Figure 7A solid line) and apparent tensile strength (σ_{app} , Table 4.3) of the scaffolds were not significantly altered while changing the PLLA to PCL ratio, which were of average 300 MPa and 25 MPa respectively. However, as shown in Figure 6A (dotted line), the elasticity of the scaffolds has been improved with increasing the PCL to PLLA ratio. When more PCL was added, the apparent strain at break (ϵ_{app}) improved: 70-30-0 ~ 80-20-0 > 90-10-0.

Figure 4.7: Plots showing mechanical properties of the scaffolds with varying: A) PLLA to PCL ratio and B) H₂O to HFIP ratio. The apparent Young's modulus was similar for the scaffolds with different formulations. Lower PCL to PLLA ratio has yielded scaffolds with lower elasticity. Elasticity of the scaffolds increased with minimal amount of H₂O (3 vol%), however it dropped with increasing H₂O percentage.

As shown in Figure 4.7B, the addition of the H₂O did not significantly alter the σ_{app} and E_{app} of the scaffolds, which were on average 15 MPa and 250 MPa respectively. However, formulation 90-10-3 has resulted in the scaffolds with maximum ϵ_{app} value, which was at $51.29 \pm 5.56 \%$ as shown in Figure 7B (dotted line).

Table 4.3: Mean fiber diameter and mechanical properties of the scaffolds

Samples	Mean Fiber Diameter (μm)	Apparent Young's Modulus (MPa)	Apparent Tensile Strength (MPa)	Apparent Strain at Break (%)
70-30-0	0.944 ± 0.096	290.31 ± 31.36	24.99 ± 1.13	51.33 ± 6.06
80-20-0	1.090 ± 0.233	325.03 ± 9.38	27.18 ± 0.39	60.69 ± 11.25
90-10-0	1.071 ± 0.153	284.18 ± 28.58	20.04 ± 1.80	28.44 ± 9.87
90-10-3	0.995 ± 0.221	224.41 ± 36.44	14.99 ± 2.70	51.29 ± 5.56
90-10-5	0.954 ± 0.197	272.15 ± 8.38	18.87 ± 0.37	37.29 ± 3.03
90-10-7	0.836 ± 0.171	244.37 ± 1.78	16.76 ± 1.01	28.51 ± 9.27
90-10-10	0.760 ± 0.176	256.26 ± 26.77	14.57 ± 1.23	11.72 ± 7.34

4.3.6 NPS scaffolds exhibited no cytotoxicity on L929

According to ASTM standard (ASTM F813-07: Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices), cytotoxicity of the scaffolds were evaluated by assessing the L929 murine fibroblasts viability through using the Live/Dead staining assay on the NPS scaffold (90-10-3). Figure 4.8 shows the fluorescence images of L929 cultured samples. On day 1 of culture, viable cells were stained green on NPS scaffolds as well as TCPS, which served as the positive control. By day 7, cells on the NPS scaffolds arranged themselves according to the orientation of the scaffolds and exhibited high degree of spreading while the cells on TCPS were quite confluent and some were rounded.

Figure 4.8: Fluorescence microscopy images of cells on aligned scaffold proliferated and arranged themselves according to the fibers orientation by day 7. Calcein-AM was used to stain viable cells (green) while ethidium homodimer-1 was used to stain the nuclei of non-viable cells (red). (scale bar = 50 μ m)

4.3.7 Tenocytes exhibited elongated morphology but proliferation was slightly inhibited under NPS influence

Tenocytes were seeded onto NPS scaffolds (90-10-3) and their proliferation was evaluated by using PicoGreen Assay. As shown in Figure 4.9, under the influence of NPS, cells cultured on NPS scaffolds showed relatively slower proliferation rate compared to empty scaffold (same formulation but no NPS was added). Nevertheless, immunostained images on Figure 4.9 show that tenocytes cultured on both scaffolds exhibited their typical elongated morphology.

Figure 4.9: Graph showing proliferation of tenocytes on NPS scaffolds was slightly prohibited, compared to empty scaffolds where cells proliferated after 3 days of culture. (* indicates $p < 0.05$)

Figure 4.10: Immunostaining images showing more tenocytes were visualized on A) empty scaffolds than on B) NPS scaffolds, where tenocytes showed elongated morphology were immunolabeled with phalloidin (red) to show cytoskeleton of the cells and counterstained with DAPI (blue) to indicate nucleus. (scale bar = 50 μm)

4.4 Discussion

In order to enhance the healing process of tendon, not only is it necessary to consider TE approach in which a biocompatible material suitable for tendon-related cells to grow and function can be implanted to the injury site, it is vital to also consider the solution to the issue of undesirable post-surgical adhesion formation during tendon healing. Sustained releasing of NPS has been identified as one of the promising solutions to prevent adhesion formation^{33,34}. In this preliminary work, the possibility to incorporate NPS into a TE scaffold and achieve sustained release of NPS was shown by exploring different formulations. These formulations fixed with 0.4 wt% NPS, were varied in their polymer compositions (PCL to PLLA ratio) and percentage compositions of H₂O as the co-solvent in the main solvent HFIP.

Foremost, a scaffold which mimics the tendon's extracellular matrix (ECM) was fabricated by using electrospinning as a technique to process a mixture of polymers, PLLA and PCL loaded with NPS. Tendon ECM possesses well aligned closely packed collagen fibers. Hence, there are numerous efforts in fabricating fibrous scaffolds with aligned morphology. For example, Yin et al. demonstrated that human tendon stem/progenitor cells (hTSPCs) exhibited tendency of tendogenesis when they were cultured on nanofibers with aligned morphology rather than random morphology⁷. The aligned morphology is hence deemed as an essential criterion in producing scaffolds with biomimetic structural properties for tendon regeneration. Scaffolds with better fibers alignment could be obtained from electrospinning conducted on mandrel set up at higher speed (1500rpm)³⁹. Aligned NPS-loaded PLLA/PCL scaffolds were therefore successfully fabricated from all formulations of the electrospinning solutions. Other than fibers orientation, fibers diameter was also an important consideration in tendon TE as tendon cellular responses could be affected by the fibers diameter. In this study, fibers diameter of the scaffolds was similar when changing the PCL to PLLA ratio, though lower viscosities of the electrospinning solution were observed⁴⁰. However, there was a decreasing trend of the fibers diameter when increasing the co-solvent (i.e. H₂O) percentage. This could be understood as lower actual concentration of polymer solutions

were being pushed out at the tip of the spinneret due to increasing H₂O portions ⁴¹. Another reason could be that the dielectric constant of the HFIP/H₂O solutions had increased with the addition of H₂O (dielectric constant = 80) into HFIP (dielectric constant = 18.7) ^{42,43}. Overall, the fibers diameter obtained from all formulations in this study ranged from 0.7 μ m to 1.1 μ m. This submicron feature highlighted the potential of these scaffolds for tendon TE because cell proliferation as well as production of collagen was promoted as the cells cultured on scaffolds with smaller fibers ⁴⁴.

Although scaffolds with desirable tendon TE morphological properties were successfully fabricated, the major concern was still the drug release profiles of NPS to prevent adhesion formations as mentioned earlier. Several attempts in loading of NPS into Eudragit S-100 coated sodium alginate microspheres, PLGA microspheres, lipid-like carnauba wax microparticles and electrospun cellulose acetate were reported ^{37,45,46}. However, short NPS release profiles was achieved from a few hours to few days because of NPS's hydrophilic nature and low molecular weight. This relatively short NPS release profile is not completely sufficient for tendon healing as the inflammation process which can cause adhesion formation may sometimes last for as long as two weeks. In this study, different formulations of electrospinning solutions, which involve variations in polymer compositions and H₂O percentage in HFIP, were investigated on their effects in drug releasing properties. Undesirable increase in NPS release was observed with higher PCL to PLLA ratio of the fibers. This is because PCL chains during the drug release study are in a flexible rubbery and highly mobile state with sufficient free volume^{47,48}. This would allow more NPS to be diffused towards the fibers surface and thus resulting in a relatively faster release. As for the variation of the H₂O percentage in HFIP, it was found that to a desirable slow release of NPS was achieved when H₂O was introduced at 3% into the formulation as a co-solvent. It is hypothesized that the formation of two different phases within the fiber was formed with the presence of H₂O as co-solvent, due to a change in the solvent evaporation rate. HFIP evaporates faster than H₂O during the electrospinning process and this might have led to the formation of polymer shells trapping the H₂O phase within the fiber. Being hydrophilic in nature, NPS hence prefers to reside inside the fiber with the H₂O phase. As evidenced in Figure 4.6, lower amount

of NPS was detected by XPS on the fiber surface when H₂O was added in the formulation, indicating that there are more NPS resided in the core region of the fiber (Figure 4.10).

Figure 4.11: Schematic showing possible NPS distribution within the fibers when increasing the H₂O percentage as the co-solvent in formulations: A) when no water was added, there was an even distribution of the NPS inside the fibers; (B) with the presence of minimal amount of H₂O, more NPS tend to stay inside the fibers; however, (C) further increase the percentage of H₂O resulted in thinner fibers and there was more NPS on the surface of the fibers.

However, when increasing the percentage of H₂O in the electrospinning solutions, this effect would be countered. Fibers with smaller diameter would be produced when increasing the H₂O content, due to higher stretching experienced by the fiber. A higher release rate was observed due to the higher surface area to volume ratio in thinner fibers⁴⁹. Overall, this NPS release study has identified that the formulations with the least PCL to PLLA ratio and minimal H₂O content (up to 3%) as the co-solvent gave the most optimal NPS release profile. It is applaudable that this formulation could release NPS sustainably as long as two weeks which was considered the longest sustained release of NPS ever reported compared to other published studies which reported three days as the highest. This long term release of NPS could have sufficiently alleviated the inflammation symptoms which might sometimes last for two weeks.

The scaffolds were investigated further to understand its potential as a TE scaffold for tendon regeneration. A natural tendon exhibits a very high Young's modulus of about 1.2-1.6 GPa⁵⁰. Adequate mechanical property is thus an important criterion for TE scaffolds especially those for tendon regeneration. Due to the high modulus of PLLA, it was considered as the candidate polymer in order to match the Young's modulus of a tendon. However, PLLA is very brittle and hence PCL was introduced into the formulations to improve the elasticity of the resulting scaffolds. This is because the crystallinity of PLLA could be disrupted with the addition of PCL and thus resulting in improved elasticity⁵¹. Scaffolds fabricated from all formulations possessed relatively good mechanical properties ranging from 250 to 300 MPa and 15 to 25 MPa for their apparent Young's modulus (E_{app}) and apparent tensile strength (σ_{app}) respectively.

To investigate the influence of co-solvent (i.e. H₂O) on the mechanical properties of the resulting scaffolds, only formulations with 90:10 PLLA/PCL composition were chosen as they possessed the most optimal NPS release profile. Results showed that highest elasticity was exhibited on the scaffold when 3% H₂O was used in the electrospinning solutions. This might be because of the formation of solvent mixtures with higher boiling point when there is minimal amount of H₂O in HFIP, leading to higher degree of molecular orientation of the polymer chains within the fiber (Figure 4.11), and hence an enhancement of elasticity^{51,52}. However, when increasing the H₂O content, scaffolds exhibited lower elasticity as a result of the highly stretched polymer chains⁵³.

Figure 4.12: Schematic showing possible changes in the polymer chains when increasing water percentage as the co-solvent in electrospinning solutions: (A) when no water was introduced, polymer chains have lower molecular orientation; (B) with the presence of small amount of H₂O polymer chains have higher molecular orientation; (C) with further addition of H₂O polymer chains were highly stretched.

Last but not least, these scaffolds were also tested for their biocompatibility. Preliminary cell study on L929 indicated that scaffolds exhibited no cytotoxicity and they were able to proliferate healthily and arrange themselves according to the fiber orientation. Culture of tenocytes as depicted on Figure 4.10 also showed that the cells could respond to the orientation of the fibrous morphology, and adopting their typical elongated morphology, mimicking the actual tendon tissues. As less proliferation of tenocytes was observed on NPS scaffolds due to the inhibitory effect of NPS on tenocytes proliferation, this scaffold will be coupled with the growth factors (GFs) releasing scaffolds in the next chapter to further enhance its potential in promoting tendon healing. The integration of NPS scaffold with GFs scaffold for enhanced tendon regeneration will be discussed in the next chapter.

4.5 Summary

In this chapter we have demonstrated the feasibility to load NPS, which is a kind of hydrophilic anti-inflammation drug, into electrospun PLLA/PCL scaffolds with continuous, beadless and aligned fibrous morphology. Different portions of PCL inside the fibers were found to have less effect in affecting fibers diameter, though faster NPS release rate and higher strain at break were observed with increasing PCL content. Through further adjusting the H₂O percentage which served as the co-solvent in HFIP, NPS was released sustainably as long as 2 weeks in a scaffold which possessed optimal mechanical properties. *In vitro* cell studies have shown that tenocytes could survive, exhibiting their typical elongated morphology and arranging themselves according to the fibers orientation. Although lower proliferation rate was observed under the influence of NPS, this optimized scaffold shall be coupled with scaffold which possesses GFs releasing ability as discussed in the next chapter, in order to further enhance its potential in promoting tendon healing

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Chapter 5

Controlled release of IGF-1 from Electrospun Scaffolds Enhanced Tenocytes Proliferation and Differentiation

Slow tendon healing is devastating. Growth factors (GFs) are prescribed for tendon repair but they degrade easily in vivo, thus necessitating the need for delivery vehicles to release them timely. In this chapter, hexafluoroisopropanol was used as electrospinning solvent for its capability in better preserving insulin-like growth factor 1 (IGF-1) stability. IGF-1 was subsequently loaded into electrospun poly (lactic-co-glycolic acid)/ poly (ϵ -caprolactone) (PLGA/PCL) aligned scaffold. Through adjusting the polymer composition (i.e. PLGA:PCL ratio and inclusion of poly (ethylene oxide)) and concentration of the electrospinning solution, a 10-week release of IGF-1 was achieved. The released IGF-1 showed preservation of bioactivity and they were able to trigger C2C12 downstream cellular response. Tenocytes cultured on the scaffolds adopted their typical elongated aligned morphology while proliferation and differentiation were enhanced with the stimulation of IGF-1. The released IGF-1 has compensated the inhibitory effect of naproxen sodium on tenocytes proliferation, via culturing tenocytes on combined scaffolds of NPS fibers and IGF-1 fibers. It is hence concluded that the scaffolds that further developed in this chapter could be better candidates in promoting tendon healing.

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5.1 Introduction

To tackle on the slow healing issue, growth factors (GFs), a kind of endogenous proteins which are capable of stimulating cellular activities involved in the healing of new tissue ¹, have been extensively researched their potential in promoting tendon repair. They are commonly prescribed for tissue repair by direct injection into the human body. ^{2,3}. However, owing to the nature of GFs which degraded easily *in vivo* (including denaturation, oxidation and proteolysis) ⁴, there is insufficient amount of GFs available to promote tissue healing by the time they reach the specific target sites of the injury. This often results in failure in most phase II clinical trials ⁵. Consequently, large amount of GFs are always injected into the body. However, the initial high doses might also lead to severe side effect. For instance, vascular endothelial growth factor (VEGF) only possesses 30 min half-life while injected intravenously ⁶. Large doses of VEGF were hence frequently used; however this supraphysical concentration could result in catastrophic pathological vessel formation at non-target sites ⁷. Therefore, enormous efforts have been done to incorporate the GFs into the carrier or constructs, such that the GFs could be delivered timely, while their bioactivity could be preserved at the same time before being released out.

The current approaches for GFs delivery can be categorized into attachment or encapsulation techniques ⁸. Despite the relatively easy process to either physically or chemically attach the GFs onto the polymers, GFs are commonly entrapped inside polymer matrices in order to achieve controllable and slower release as well as preserving their biological activities ⁹. Nonetheless, many methods are compromised with burst release ¹⁰, limited duration of release, reduced efficacy ¹¹ and formulation complexity ¹². In view of the fluidity of nanoparticles and low mechanical properties of hydrogel systems, electrospun fibrous systems are generally deemed as the much more preferable GFs delivery vehicles in TE. In fibrous systems, entrapment techniques such as core-shell or emulsion electrospinning have been extensively reviewed in loading GFs as well as preserving their biological activities in fibers. However, the associated set-up is usually very complex and required emulsifying agents in order to stabilize the core or formation

of emulsions in solutions¹³. Besides, several issues such as drug agglomerations on fibers surface or inconsistency in forming a continuous core in core-shell fibers, resulting in compromise of the mechanical properties of the resultant fibers have been reported^{14,15}.

On the other hand, numerous efforts have been done to load GFs into blend electrospinning systems, which require relatively easy set-up and represent higher reproducibility. To avoid harsh organic solvents environment, most of these studies are focusing on hydrophilic based polymers, such as gelatin or poly(ethylene oxide)¹⁶⁻¹⁸. However, release of GFs from these matrices could only inevitably last for a few days due to faster release. Especially for tendon TE which requires constructs to possess longer period of GFs releasing ability¹⁹, the aforementioned systems might not be able to fit into actual medical practices. Hence, **the aim of this chapter is to load GFs into hydrophobic polymeric systems via a blend electrospinning system and fabricate an aligned electrospun scaffold which is essential for tendon TE, at the same time achieving controlled release of GFs.**

IGF-1 has been selected because it can promote the synthesis of collagen and proteoglycans during the early stage of tendon healing²⁰. In order to ensure that GFs loaded into polymers would not lose their native states and ability to function, a suitable solvent system is essentially important. Roccatano et al. has reported that HFIP clusters which interact with the peptide in HFIP-based solutions could promote the formation of ordered secondary structure²¹. It is then postulated that the bioactivity of the IGF-1 could be better preserved after the electrospinning by using HFIP as the solvent and the IGF-1 release could be controlled by manipulating the polymer compositions. These optimized scaffolds were characterized in terms of their morphology, the ability of releasing *in vitro* and potential in inducing tenocytes proliferation and differentiation. The last part of this chapter will explore the feasibility of the combined scaffolds (IGF-1 + naproxen sodium (NPS, please refer to chapter 4)), in terms of tenocytes proliferation and differentiation in promoting tendon regeneration.

5.2 Materials and Methods

Relevant information regarding the materials and methodology used in this chapter please refer to Chapter 3.

Table 5.1: Methods used in Chapter 5

Description		Section
Scaffold fabrication	Solution preparation	3.2.1.2
	Electrospinning process	3.2.2
<i>In vitro</i> drug release		3.3.2
Materials characterizations	Scaffold morphology	3.4.1
	Mechanical properties	3.4.4
	Hydrolytic degradation	3.4.5
Cell studies	Cell seeding	3.5.1.3 / 3.5.1.5
	Cell proliferation	3.6.2.1 / 3.6.2.2
	Immunocytochemistry	3.6.4
	Western Blotting	3.6.5
	Real time quantitative polymerase chain reaction (qPCR)	3.6.6

5.3 Results

5.3.1 HFIP as the solvent for electrospinning

Several solvents have been tested for their capability to preserve IGF-1 stability. Figure 5-1 shows the relative protein stability in different solvents (determined by ELISA) after freeze-drying process. The stability of these samples was compared to the freshly prepared IGF-1 protein solution as the standard set at 100% stability. IGF-1 showed the least stability after loading into dichloromethane (DCM), inside which around 44% of protein was detected. In contrast, IGF-1 which was loaded into the water could maintain its stability up to around 90%. IGF-1 which was diluted in HFIP showed a stability of

about 80%, which could be further enhanced with the inclusion of PEO into the HFIP solution, yielding a stability of about 96%.

Figure 5.1: Graph showing the relative IGF-1 stability in different solvents. HFIP showed relatively better ability in preserving IGF-1 stability as compared to DCM. (* indicates $p < 0.05$, ** indicates $p < 0.001$)

By using HFIP as the solvent, electrospinning of IGF-1-loaded scaffolds were conducted by using mandrel set up, with different formulations as stated in Table 5.2. Scaffolds were named according to their concentration, followed by PLGA-PCL ratio and whether with or without PEO in the formulations. For example, 8-70/30-0 indicates 8 w/v% of polymer with the composition of 70wt% PLGA blended with 30wt % PCL, and 0 wt% of PEO in the formulation.

Table 5.2: Description of different formulations of electrospinning solutions.

Samples		Total Concentration (w/v%)	PLGA (wt %)	PCL (wt %)	PEO (wt %)
A	8-80/20-0	8	80	20	0
B	8-70/30-0	8	70	30	0
C	8-70/30-5	8	66.5	28.5	5
D	5-70/30-5	5	66.5	28.5	5
E	3-70/30-5	3	66.5	28.5	5

Figure 5.2 shows the FESEM images of scaffolds prepared by using different formulations. All the scaffolds possessed aligned morphology to mimic the tendon cells micro-environment, while the fiber diameters were all in the submicron range.

Figure 5.2: FESEM images of IGF-1 loaded scaffolds: A) 8-80/20-0; B) 8-70/30-0; C) 8-70/30-5; D) 5-70/30-5; E) 3-70/30-5. (F) The fiber diameter analysis showed that lowering the concentration decreased the fiber diameter. (* indicates $p < 0.05$)

5.3.2 Controlled IGF-1 release from the electrospun scaffolds

Figure 3 shows that the *in vitro* released amount of bioactive IGF-1 (determined by using ELISA kit) from the scaffolds fabricated from the different formulations. Figure 5.3A shows the amount of IGF-1 being released when the scaffolds were fabricated by using different PLGA:PCL ratios. It was observed that IGF-1 was released faster with higher percentage of PCL content. With the presence of PEO in the fibers, the release of IGF-1 was faster as shown in Figure 5.3B. As depicted in Figure 5.3C, the release of IGF-1 could be further enhanced: from 600pg/week to 1000pg/week to 1700pg/week, while the total polymer concentration of the formulations was decreased from 8w/v% to 5w/v% to 3w/v. By using the scaffold which was fabricated from the formulation E as described in Table 5.2 (i.e. 3w/v% of total polymer concentration, with 66.5wt% of PLGA and 28.5wt% of PCL and 5wt% of PEO as additives), long term release study was conducted and release of IGF-1 was observed to have continued for about 10 weeks (Figure 5.3d). The total IGF-1 loaded inside the polymers was determined by using microBCA assay. After extraction, there was about 76% of IGF-1 encapsulated inside the polymers.

Figure 5.3: Release profile of IGF-1 under different formulations: A) different polymer ratios; B) with or without PEO; C) different polymer concentration. D) Release of IGF-1 from the optimized scaffold (i.e. 3-70/30-5) lasted for 10 weeks.

5.3.3 Mass loss and change in molecular weight from 3rd week

Mass loss measurement and size exclusion chromatography were conducted in PBS solution to understand the degradation of the IGF-1 scaffolds and the empty scaffolds. As shown in Figure 5.4, the inclusion of IGF-1 did not alter the degradation. Both scaffolds started to have significant mass loss (Figure 5.4a) and molecular weight change (Figure 5.4b) from 3rd week of the study onwards. It should be noted that the scaffolds have insignificant morphological change throughout the study.

Figure 5.4: Plots showing IGF-1 and empty scaffolds degraded from 3rd week onwards, from the evidence of A) mass loss and B) molecular weight change.

5.3.4 Released IGF-1 maintained bioactivity

Western Blotting technique was used to visualize the phosphorylation level of protein kinase B, also known as Akt to determine the bioactivity of the release IGF-1. Briefly, IGF-1 scaffolds were immersed into culture medium for 1 day and 7 days respectively. These preconditioned mediums were then exposed to confluent C2C12 cells for 30 min before the cells were lysed and checked for the phosphorylated Akt level by using Western Blot technique. Figure 5.5a, Figure 5.5b and Figure 5.5c show that the immunoblots for phosphorylated AKT (Ser473), total AKT and β -Actin antibodies respectively. The graph of Figure 5.5d shows the densitometry analysis, obtained by normalizing bands intensity of the immunoblots. The densitometry analysis showed that a 7-fold and 17-fold up-regulation of the phosphorylated Akt level in the cells which were treated under 1-day and 7-day IGF-1 scaffold preconditioned mediums, respectively. The expression of 1-day empty scaffold preconditioned medium was set as the standard at 1.

Figure 5.5: Immunoblots showing the expression of A) pAkt Ser473, B) total Akt and C) β-Actin while the cells cultured on IGF-1 scaffolds and empty scaffolds were exposed to 1-day and 7-day preconditioned mediums. D) Densitometry analysis of the blots showed that the up-regulated of Akt phosphorylation indicated that IGF-1 is bioactive after the encapsulation.

5.3.5 Released IGF-1 retained functionality by enhancing C2C12 cells proliferation and differentiation

C2C12 cells were cultured on scaffolds and the proliferation was determined by using AlamarBlue assay and PicoGreen Assay. Figure 5.6a shows that the cells, which were seeded on IGF-1 scaffolds, proliferated faster from day 1 to day 3 as determined by using AlamarBlue assay. Similarly, PicoGreen analysis showed that the cells proliferated faster

on IGF-1 scaffolds compared to empty scaffolds, as shown on Figure 5.6b. Notably, both assays suggested that there were higher number of cells on IGF-1 scaffolds compared to empty scaffolds for every predetermined time points.

Figure 5.6: Graphs showing the C2C12 cells proliferated throughout the study as determined by A) AlamarBlue assay and B) PicoGreen assay. Cells on IGF-1 scaffolds proliferated faster compared to empty scaffolds. (* indicates $p < 0.05$)

Figure 5.7 shows the nucleus staining images of C2C12-seeded scaffolds. From Figure 5.7A, the cells became confluent on IGF-1 scaffolds after culturing for 3 days whereas cells only became confluent after culturing on empty scaffolds for 7 days (Figure 5.7C).

Figure 5.7: Immunostaining images showing A) C2C12 (nuclei was stained blue) reached almost 100% of confluency after culturing for 3 days on IGF-1 scaffolds; however, B) fewer cells were observed on empty scaffolds on day 3, and C) cells only become confluent after 7 days of culture on empty scaffolds. (scale bar = 50μm)

Real time polymerase chain reaction (qPCR) was utilized to evaluate the differentiation tendency of the C2C12 cells on the scaffolds. C2C12 cells were cultured on IGF-1 and empty scaffolds until confluent (i.e. 3 days and 7 days respectively) prior to changing the culture medium to differentiation medium. As shown in Figure 5.8, significant up-regulation of myogenin gene (2-fold change) was observed after 1 day as differentiation of the cells was being induced. The myogenin gene expression was leveled off 3 days after changing the medium into differentiation medium. The differentiation was further ascertained at translational level through the immunostaining of desmin protein which was shown in Figure 5.9. From the images, more and larger multinucleated fibers or “myotubes” which formed through the fusion of cells were observed on IGF-1 scaffold (Figure 5.9A) compared to empty scaffold (Figure 5.9B).

Figure 5.8: Graphs showing myogenin expression of C2C12 on A) day 0, B) day 1 and C) day 3 of differentiation. The myogenin expression on IGF-1 scaffolds was up-regulated compared to empty scaffolds after 1 day of differentiation but it was leveled off on day 3. (* indicates $p < 0.05$)

Figure 5.9: Immunostaining images showing myotubes formation on A) IGF-1 scaffolds and B) empty scaffolds, where more and larger myotubes were observed on IGF-1 scaffolds. (scale bar = 50µm)

5.3.6 Tenocytes proliferated and differentiated on IGF-1 loaded scaffolds

Figure 5.10 shows that the tenocytes proliferated relatively faster on IGF-1 scaffolds compared to empty scaffolds, as determined by using PicoGreen assay ($p < 0.05$). Besides, immunostaining images from Figure 5.11 shows that more cells could be observed on IGF-1 scaffolds (Figure 5.11A) as compared to empty scaffolds (Figure 5.11B).

Figure 5.10: Graph showing tenocytes proliferated on IGF-1 and empty scaffolds, as determined by PicoGreen Assay. Tenocytes proliferated faster on IGF-1 scaffolds compared to empty scaffolds. (* indicates $p < 0.05$)

Figure 5.11: Immunostaining images showing more tenocytes were on a) IGF-1 scaffolds than on b) empty scaffolds, where tenocytes were immunolabeled with phalloidin (red) to show cytoskeleton of the cells and counterstained with DAPI (blue) to indicate nucleus. (scale bar = 50μm)

The differentiation tendency of the tenocytes was determined by qPCR technique. As shown in Figure 5.12, gene expression of scleraxis (Figure 5.12A) showed 3-fold increment on IGF-1 scaffold after culturing for 3 days while the tenomodulin (TNMD, Figure 5.12b) gene expression was up-regulated on IGF-1 scaffolds compared to empty scaffolds on day 3 and day 7. Although the tendon specific gene markers (i.e. Scleraxis and TNMD) were up-regulated, no significant difference was observed in terms of collagen I gene expression (Figure 5.12c) for both IGF-1 scaffolds and empty scaffolds.

Figure 5.12: Graphs showing tendon specific genes: A) scleraxis and B) tenomodulin were up-regulated in tenocytes cultured on IGF-1 scaffolds compared to empty scaffolds; however no much difference was observed in C) collagen I gene expression on both types of scaffolds. (* indicates $p < 0.05$)

5.3.7 Released IGf-1 rescued tenocytes proliferation and differentiation on combined scaffolds

Tenocytes were then seeded onto combined scaffolds (either with or without IGF-1 integrated with NPS scaffolds) and the cell proliferation was determined by using

PicoGreen assay, as shown in Figure 5.13. Proliferation of tenocytes were affected with the presence of NPS, in which less proliferation was observed on empty scaffolds + NPS scaffolds; however, the released IGF-1 has compensated this influence and significant proliferation was observed on IGF-1 scaffold + NPS scaffold from day 1 to day 7. On day 7, there were also more cells on IGF-1 scaffolds + NPS scaffolds compared to empty scaffold + NPS scaffold ($p < 0.05$).

Figure 5.13: Graph showing tenocytes proliferated faster on IGF-1 Scaffold + NPS scaffold than Empty Scaffold + NPS scaffold, as determined by PicoGreen Assay. (*indicates $p < 0.05$)

The differentiation tendency for tenocytes on the combined scaffolds was again determined by checking the scleraxis, tenomodulin and collagen I gene expression by using qPCR (Figure 5.14). There was significant up-regulation of scleraxis and tenomodulin gene expressions when tenocytes were cultured on IGF-1 scaffold + NPS scaffold, compared to that of when cultured on empty scaffold + NPS scaffold.

Figure 5.14: Graphs showing tendon specific genes: A) scleraxis; B) tenomodulin were up-regulated when tenocytes were cultured on IGF-1 scaffold + NPS scaffold; however, there was slightly higher expression of C) collagen I expressed on IGF-1 scaffold + NPS scaffold. (* indicates $p < 0.05$)

5.3.8 Combined scaffolds possess optimal mechanical properties

Table 5.3 summarizes the apparent Young's modulus (E_{app}) of IGF-1 scaffolds, NPS scaffolds and combined scaffolds. Combined scaffolds, which was fabricated through electrospinning of NPS scaffolds followed by IGF-1 scaffolds sequentially, possess a Young's modulus of about 148.75 ± 16.76 MPa, which lies between the Young's modulus of pure NPS scaffolds (i.e. $E_{app} = 272.15 \pm 8.38$ MPa) and pure IGF-1 scaffolds (i.e. $E_{app} = 105.65 \pm 8.37$ MPa).

Table 5.3: Apparent Young's modulus of NPS scaffolds, IGF-1 scaffolds and combined scaffolds

Scaffolds	Apparent Young's Modulus (MPa)
NPS scaffolds	272.15 ± 8.38
IGF-1 scaffolds	105.65 ± 8.37
Combined scaffolds	148.75 ± 16.76

5.4 Discussion

It has been demonstrated that scaffolds which are able to release biomolecules, have therapeutic benefits in encouraging tissue formation either in *in vitro* culture or *in vivo* environment²²⁻²⁵. However, preserving GFs conformation is critical as the loss of conformation of GFs could reduce their intended therapeutic potential and cause unwanted immunogenic effects due to exposure of nonnative peptide epitope^{26,27}. Generally, GFs delivery in fibrous systems could be achieved through chemical immobilization or physical entrapment into the polymer matrices²⁸⁻³². For the latter, it allows better GFs bioactivity stability and protection against the outer environment. Even so, GFs are inevitably susceptible to various risks of degradation, either during fabrication and release^{33,34}. In the present study, electrospinning from blended solution was employed and HFIP was selected to be the solvent for electrospinning. This is because IGF-1 stability in HFIP was higher at 76% compared to IGF-1 stability in DCM at 44%. Unlike DCM, HFIP does not create organic-inorganic interface where proteins tend to aggregate, unfold and lose its nature states^{10,35}. Hence, with the use of HFIP as

the electrospinning solvent, IGF-1 bioactivity could thus be better preserved.

Protein release from nanofibers occurs through a combination of material degradation or passive diffusion across nanopores on the fiber surface^{36,37}. In the attempt of releasing IGF-1 from the scaffolds presented in this chapter, a relatively low amount of IGF-1 was being released when PLGA alone was used as the polymeric vehicle. PCL was thus added into the electrospinning formulation to fasten the IGF-1 release as PCL chains could allow higher water penetration into the fibers to facilitate the IGF-1 release³⁸. The addition of PCL also aided in preventing the possible shrinkage of the scaffolds though obstructing the PLGA chains movement³⁹. PEO was further added into the formulation to accelerate the release of IGF-1. The addition of PEO was believed to have facilitated the release of IGF-1 through enhancing the hydrophilicity of the scaffolds and at the same time, introduced tiny pores to the matrices to facilitate the IGF-1 release^{40,41}. Interestingly, with the inclusion of PEO, IGF-1 stability could also be improved to about 96%. This might be due to the use of hydrophilic additives such as PEO which can minimize the hydrophobic interaction of IGF-1 and the solvent environment and thus further preserve the stability of IGF-1⁴². The total polymer concentration was then further reduced to adjust the daily released of IGF-1 amount and the optimized formulation was set at 3w/v% polymer concentration, with the PLGA:PCL fixed at ratio of 70:30 and 5wt% PEO as additives. This is because lower polymer concentration could yield fibers with smaller diameter hence resulting in higher specific surface area to facilitate the IGF-1 release^{43,44}. However, it should be noted that electrospinning solutions with too low in polymer concentration (i.e. < 3w/v%) would compromise on the electrospinnability due to insufficient polymer entanglements⁴⁵. With this optimized formulation, long term drug release study was conducted and lasted for about 10 weeks. The release profile could be divided into three phases. In phase 1, IGF-1 was released sustainably with daily release of 300pg/mL up to 1 week. It was followed by a relatively slow release (phase 2) and the scaffolds started releasing faster from third week onwards (phase 3) and came to a stop around 10th week of the study. The first phase of the release was believed to be related to the dissolution of PEO and it has contributed to the release of IGF-1 which was resided near to the fiber surface. In phase 2, relatively smaller

amount of IGF-1 was released before the polymer blends started to degrade. After 3 weeks, the scaffolds started to degrade as supported by the evidence of mass loss and slight molecular weight change of the scaffolds (Figure 5.4A and Figure 5.4B). In this third phase, IGF-1 was further released until 10 weeks. It is noted that less amount of bioactive IGF-1 was detected by using ELISA throughout the study, compared to the total loaded amount of IGF-1 (encapsulation efficiency of 76%). This could be due to the possible denaturation either during the electrospinning process or throughout the study^{46,47}. Besides, other possible reasons could be the incomplete detection of released IGF-1, which has been absorbed back to the polymeric surface after releasing out from the matrices^{27,48}.

With controlled release being achieved, the next important factor to consider is that the released IGF-1 should be able to trigger the relevant cellular response to prove their efficacy. Scheme 5-1 depicts the simplified IGF-1/Akt pathway. Generally, binding of IGF-1 to its receptor results in activation of tyrosine kinase and phosphorylation of insulin receptor substrate (IRS), which further activates cascades of phosphorylation and hence leading to Akt activation. Immunoblotting of Akt protein in C2C12 cells has demonstrated that the bioactivity of IGF-1 was maintained after the encapsulation process (Figure 5-4). Akt stimulates protein production via the mammalian target of rapamycin (mTOR), and subsequently contributes to hypertrophy in skeletal muscles⁴⁹.

Figure 5.15: Schematic diagram showing simplified IGF-1/Akt pathway.

It is worth to note that the released IGF-1 was also able to further trigger the downstream cellular response. Figure 5.6 has shown the enhancement of cell proliferation with the culture of C2C12 cells on IGF-1 scaffolds. The differentiation of C2C12 cells was also promoted, as demonstrated by the up-regulation of myogenin gene expression at transcriptional level (Figure 5.8) and immunostained multinucleated cells or “myotubes” formation at translational level (Figure 5.9). Myogenin is a muscle-specific basic-helix-loop-helix transcription factor, which is essential for the development of functional skeletal muscle while the myotube formation indicates myogenesis⁵⁰⁻⁵³. Engert et al. reported similar observation in their IGF-1-stimulated myogenesis study, in which IGF-1 was found to enhance cell proliferation and promoted larger myotubes formation at the later stage of the study⁵⁴. These results have demonstrated that the released IGF-1 was bioactive and able to induce relevant cellular response.

Several *in vitro* studies have reported the successful tendon tissue engineering either by mechanical stimulation^{55,56}, knitted scaffolds⁵⁷, using different cell sources^{58,59} or introducing growth factors^{60,61}. IGF-1 is well known for their ability in stimulating the proliferation and migration of cells. It is also known to subsequently increase the collagens synthesis during the remodeling stages⁶². In the present study, tenocytes were able to proliferate faster under the influence of IGF-1 and adopt their typical elongated aligned morphology by going along with the topographical cues from the scaffolds. Besides, the expression of tendon specific genes such as scleraxis and tenomodulin were also up-regulated with the stimulation of IGF-1 released from the scaffolds. Scleraxis and tenomodulin have been demonstrated their important role in regulating tendon proliferation and maturation⁶³⁻⁶⁵. The IGF-1 scaffolds were then sequentially electrospun with the previously optimized NPS scaffolds. As mentioned in Chapter 4, the purpose of having NPS is to prevent adhesion formation during the healing process, as it might lead to recurrent injury due to improper healing⁶⁶. In spite of its beneficial role in preventing inflammation and providing analgesic effect, it has been shown that NPS could interfere with tendon healing through inhibiting the tenocytes proliferation and associated collagen synthesis^{67,68}. Indeed, the proliferation of the tenocytes in this work was reduced by the

release of NPS as demonstrated in Chapter 4. However, the released IGF-1 recovered the loss in proliferation as shown in Figure 5.13. It should also be noted that the tendon-related genes such as scleraxis and tenomodulin have also been up-regulated to show the tendency of differentiation. In addition, this combined scaffold (IGF-1 scaffold + NPS scaffold) presented optimal mechanical properties with apparent Young's modulus of 148.75 ± 16.76 MPa (Table 5.2), which is sufficient to withstand the cyclic loading normally applied for improving the tendon regeneration^{69,70}. It is therefore believed that the scaffolds, which possess morphological cues to guide cellular growth and the NPS and IGF-1 releasing ability, presented in this study will be a beneficial add-on for the current research to achieve better tendon regeneration.

5.5 Summary

In this chapter, IGF-1 was successfully incorporated into PLGA/PCL aligned electrospun scaffold. Release of IGF-1 lasted for about 10 weeks, and results showed that released IGF-1 preserved their bioactivity and was able to trigger C2C12 downstream cellular response. Tenocytes cultured on the scaffolds adopted their typical elongated aligned morphology while proliferation and differentiation were enhanced with the stimulation of IGF-1. Although NPS has slightly inhibited tenocytes proliferation and differentiation, the released IGF-1 has compensated the loss via culturing on combined scaffolds of NPS fibers and IGF-1 fibers. It is hence concluded that the scaffolds that further developed in this chapter could be better candidates in promoting tendon healing.

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Chapter 6

Medium compatibility study for tri-culture of C2C12, tenocytes and TE85

The musculoskeletal interfaces are susceptible to injuries. However, the regeneration at this translational region is always suboptimal and thus lead to recurrent injury. In view of the necessity of having an in vitro musculoskeletal model which will allow different tissues interaction, three types of cells, namely C2C12, tenocytes and TE85 were cultured on an insulin-like growth factor containing scaffold under the same medium environment. PicoGreen analysis, PCR results and immunolabeling of differentiation markers have shown that these cells survived, proliferated and differentiated under the optimized medium conditions. The co-existence of these three types of cell in a general in vitro environment implies the possibility of re-creating the muscle-tendon-bone configuration. With the knowledge obtained from this in vitro musculoskeletal model, the quality of the biological tools and the approaches for therapeutic agents' discovery could be significantly improved.

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6.1 Introduction

The musculoskeletal interfaces where tendon connects to bone and muscle (i.e. osteotendinous junction and myotendinous junction respectively) are always subjected to frequent large mechanical stress and hence making these junctions vulnerable to acute or overuse injuries¹. To repair these regions, the gold standard is to suture grafts at these anchorage sites². However, due to the lack of graft integration with their attachment sites, these grafts fail easily³⁻⁵. In view of the fact that these regions are generally not homogeneous and have natural functional gradients in their structure⁶, interface tissue engineering thus stands out and emphasizes the necessity of having graded materials to meet the structural and mechanical demand of these translational tissues⁷⁻¹⁰. Although several reports indicated the possibility of improving tissue regeneration at this translational region, most studies in interface tissue engineering focused on the scaffold design¹¹⁻¹⁷; whereas the culture conditions to cultivate the cells *in vitro* so as to regenerate a partially formed constructs have been inadequately addressed. Especially in tendon TE, instead of just having a graded scaffold for implantation, a partially formed tissue constructs is much more preferred for their ability to yield optimal mechanical strength at the time of implantation. This will allow the onset of physical therapy soon after surgery, and thus for functional recovery^{18,19}. Hence, in this chapter, **an *in vitro* musculoskeletal model was proposed**. Three types of cells, namely C2C12 murine myoblast, tenocytes and TE85 human osteosarcoma (to represent muscle-like, tendon and bone-like cells respectively) were seeded onto previously optimized IGF-1 loaded scaffolds, and the ***in vitro* culture condition (i.e. culture medium) was optimized in order to provide the optimal environment for the proliferation and differentiation** of these three cells. This chapter emphasizes on sourcing for the general medium for culturing these three types of cell simultaneously, with the postulation that a graded tissue similar to that at the native structure could be formed via promoting heterotypic interactions between different cells in close proximity.

6.2 Materials and Methods

For the information regarding the materials and methodology used in this chapter, please refer to Chapter 3.

Table 6.1: Methods used in Chapter 6

Description		Section
Scaffold fabrication	Solution preparation	3.2.1.2
	Electrospinning process	3.2.2
Cell studies	Cell seeding	3.5.1
	Cell proliferation	3.6.2.1
	Real time quantitative polymerase chain reaction	3.6.6
	Immunocytochemistry	3.6.4
	Alizarin red S staining	3.6.7

6.3 Results

For the medium compatibility study, different types of growth medium and differentiation medium were used as illustrated in Table 6.2.

Table 6.2: Mediums and their compositions used for tri-culture systems

Medium	Medium Composition
GM1	High Glucose DMEM + 20% FBS + 1% P/S
GM2	Low Glucose EMEM + 10% FBS + 1% L-Glut+ 1% NEAA + 1% P/S
DM1	High Glucose DMEM + 2% HS + 1% P/S
DM2	Mixture of 1:1 of DM1 and DM3
DM3	Low Glucose DMEM + 10% FBS + 1% P/S + 0.05mM ASB + 10 mM β -Gly + 0.1 μ M DEX

DM4	High Glucose DMEM + 2% HS + 1% P/S + 0.05mM ASB + 10 mM β -Gly
DM5	High Glucose DMEM + 2% HS + 1% P/S + 0.05mM ASB + 10 mM β -Gly + 0.1 μ M DEX

6.3.1 C2C12 proliferated on IGF-1 scaffold, regardless of the glucose level of the medium used

Figure 6.1: Optical microscopic images showing C2C12 cultured on well plates in different growth mediums proliferated and adopted typical spindle shaped morphology after 1 day (1D), 2 days (2D) and 4 days (4D) of culture. (scale bar = 100 μ m)

Figure 6.1 shows the optical microscopic images of the C2C12 cells cultured on well plates in GM1 and GM2. In both mediums, the cells adopted their typical spindle shaped morphology and proliferated throughout the study. C2C12 cells were then seeded onto IGF-1 scaffolds and cultivated in both types of medium. As shown in Figure 6.2, PicoGreen assay demonstrated that C2C12 cells proliferated from day 1 to day 3 on IGF-1 scaffolds in both mediums ($p < 0.05$), though proliferation was slightly faster in GM1 than in GM2.

Figure 6.2: Graph showing the C2C12 proliferated on IGF-1 scaffolds in both GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)

6.3.2 Glucose level of the medium does not interfere TE85 proliferation on IGF-1 scaffold

Similarly, TE85 were cultured in the same conditions as C2C12 cells. Figure 6.3 shows the TE85 cells exhibited their typical cell shapes and more cells were observed after culturing for 4 days on well plates. The cells numbers cultured on IGF-1 scaffolds were further quantified by using PicoGreen assay as depicted in Figure 6.4. In both medium, TE85 proliferated throughout the study, while they grew slightly better when cultured in GM2.

Figure 6.3: Optical microscopic images showing TE85 proliferated and adopted their typical morphology on well plates (scale bar = 100μm)

Figure 6.4: Graph showing the TE85 proliferated on IGF-1 scaffolds in both GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)

6.3.3 Tenocytes able to proliferate on IGF-1 scaffolds, regardless of the medium types.

GM1 and GM2 were used to investigate for their compatibility in terms of tenocytes proliferation. As shown in Figure 6.5, tenocytes proliferated throughout the study, although slightly lower proliferation was observed while cultured in GM2.

Figure 6.5: Graph showing the tenocytes proliferated on IGF-1 scaffolds in GM1 and GM2, as determined by PicoGreen Assay. (* indicates $p < 0.05$)

6.3.4 C2C12 formed myotubes in high glucose conditions

C2C12 cells were cultured on IGF1 scaffolds in GM1 until confluent (3 days in growth medium) before changing the culture condition to different types of differentiation mediums. The cells were allowed to differentiate for another 4 days before they were fixed and stained for desmin protein to visualize myotube formation. As illustrated in Figure 6.6, myotubes were only formed and observed when DM1, DM4 and DM5 were used as shown in Figure 6.6A, Figure 6.6D and Figure 6.6E respectively. Whereas, few cells were observed when osteogenic differentiation medium (DM3) or 1:1 mixture of myogenic and osteogenic differentiation medium (DM2) were used, as shown in Figure 6.6C and Figure 6.6B respectively.

For studies using DMs which induced myotubes formation, C2C12 cells were analyzed for their myogenic differentiation tendency. Figure 6.7 shows that myogenin gene was indeed up-regulated even though myogenin gene expression was peaked on day 1 and then leveled off for cells differentiated in DM5 .

Figure 6.6: Immunostaining images showing “myotubes” formation on IGF-1 scaffolds which were immunolabeled by desmin (red) and nucleus (blue) while C2C12 were induced differentiation in A) DM1; D) DM4 and E) DM5; lesser cells were observed while C2C12 were subjected to B) DM2 and C) DM3. (scale bar = 50μm).

Figure 6.7: Graph showing myogenin expressions were up-regulated when C2C12 were cultured on IGF-1 scaffolds in DM1, DM4 and DM5.

6.3.5 Osteogenic supplements are essential for TE85 calcium deposition

Alizarin red s staining has been employed to determine the calcium deposition of TE85 cells, which indicates the mid to late stage of the osteogenic differentiation. TE85 were firstly cultured in GM1 for 3 days until confluent in the well plates, and subsequently changing to different types of differentiation medium to check their capability to induce TE85 osteogenic differentiation. TE85 was also cultured in their typical growth medium (GM2) and changed to osteogenic differentiation medium (DM3) as the control. TE85 were cultured for another 28 days before they were fixed and stained for calcium deposition. Figure 6.8 shows the amount of calcium deposited by TE85 cells cultured in different mediums; with DM3 (Figure 6.8D) resulting the most calcium deposition, followed by DM5 (Figure 6.8C), DM4 (Figure 6.8B) and DM1 (Figure 6.8A) in descending order. The Runx2 markers (early osteogenic differentiation markers) were also checked on the cells cultured on IGF-1 scaffolds and the gene expression was observed to be up-regulated (Figure 6.9). Calcium deposition was visualized by using alizarin red s staining to further confirm their osteogenic lineage commitment (Figure 6.10).

Figure 6.8: Alizarin red staining showing the amount of calcium deposition, when TE85 cultured in well plates, was in the order of D) DM3 > C) DM5 > B) DM4 > A) DM1. (scale bar = 50mm for upper deck images, 10 μ m for lower deck images)

Figure 6.9: Graph showing Runx2 expression was up-regulated while TE85 were cultured on IGF-1 scaffolds in DM3, DM4 and DM5.

Figure 6.10: Macroscopic images showing calcium deposition which was visualized through alizarin red staining while TE85 were cultured on IGF-1 scaffolds in (A) DM4, (B) DM5 and (C) DM3 as compared to (D) control which is acellular sample. (scale bar = 50 mm)

6.3.6 Tendon specific genes were slightly down-regulated in optimized medium

Tenocytes were cultured in GM1 for 3 days and changed to differentiation medium, DM5. After culturing for 4 days, as shown in Figure 6.11, tendon specific genes such as scleraxis and tenomodulin were slightly down-regulated (though not significantly) as compared to culturing tenocytes in the control medium (i.e. tenocytes original growth medium (GM3)).

Figure 6.11: Graphs showing tendon specific genes: A) scleraxis, B) tenomodulin and c) collagen I were slightly down-regulated while tenocytes were cultured on IGF-1 scaffolds in DM5 as compared to in GM3.

6.3.7 Tri-culture of C2C12/Tenocytes/TE85 on IGF-1 scaffolds

In the last part of the study, C2C12, tenocytes and TE85 were seeded onto the IGF-1 scaffolds. After culturing in GM1 for 3 days, constructs were changed into DM5 and cultured for another 4 days. As shown in Figure 6.12, Runx2-immunolabeled TE85 cells

were grown at one end of the scaffolds, while C2C12 cells were immunolabeled by using anti-myogenin antibody resided at the other end of the scaffolds. Tenocytes, which were counterstained with DAPI to visualize the nucleus, grew at the centre of the scaffolds. Three types of cells could survive, proliferate and differentiate in the optimized medium, as shown in Figure 6.12.

Figure 6.12: Immunostaining images showing the co-existence of TE85 (immunolabeled with RUNX marker, green), tenocytes and C2C12 (immunolabeled with myosin heavy chain, red) and their nucleuses were counterstained by using DAPI. (scale bar = 50 μ m)

6.4 Discussion

Tissue regeneration at the interface, especially regions with mechanical mismatch such as osteotendinous junction, is often unsuccessful and hence requires re-surgery. This could be due to the fact that the native feature at this region, which possesses gradients in mineral content and collagen organization, often fails to be re-created during the natural healing process^{20,21}. Thus, TE approach which includes multi-lineage cell culture methods and the fabrication of biomimetic scaffold has been explored to regenerate this specialized transitional region. For example, graded bone-like minerals were deposited on the surface of electrospun scaffold to imitate the composition and mechanical function of the osteotendinous junction^{12,22-25}. Additionally, “aligned-to-random” fibers transition was also created to mimic the collagen fibers organization at the tendon-to-bone insertion site²⁶⁻²⁸. Apart from this, regeneration at the muscle-tendon interface or myotendinous junction (MTJ), which is critical for integrative tendon repair, has been explored to improve the tendon repair. Larkin et al. co-cultured skeletal constructs with engineered tendon constructs and showed that the MTJ formed could sustain tensile loading and paxilin was up-regulated at the neo-interface²⁹. However, studies on tri-culture systems relating to bone-tendon-muscle are still under-explored. Considering that the supplements required to tissue engineer these tissues could be very different and might cause detrimental effects towards other cell types in the *in vitro* system³⁰⁻³³, we have tissue-engineered C2C12 murine myoblast (muscle-like cells), tenocytes (tendon cells) and TE85 human osteosarcoma (bone-like cells) in an *in vitro* tri-culture platform using IGF-1 scaffolds. This study seeks to understand and provide optimal condition for tri-culture of these cells.

Firstly, C2C12 which has been commonly used to study the myoblast differentiation³⁴⁻³⁷, were subjected to high glucose (GM1) and low glucose DMEM (GM2) to check their proliferation in these conditions. As shown in Figure 6.2, the proliferations in both conditions were not affected by the glucose difference and the cells maintained their morphology. However, while C2C12 were subjected to differentiation, the low level of glucose was unable to promote the cell fusion and most of the cells detached in DM2 and

DM3. Similar phenomenon was also observed in Fulco et al.'s work where glucose restriction has inhibited the myogenesis through activation of AMP-activated protein kinase³⁸. For those cells differentiated in high glucose level of medium, myogenin gene expression was up-regulated and myotubes were successfully formed. Notably, myotubes were also successfully formed in DM 5 which consisted of dexamethasone. Generally, dexamethasone is the compound which is known to favor the expression of the osteogenesis³⁹⁻⁴¹. However, the presence of dexamethasone could cause atrophy in skeletal muscle which was correlated with the up-regulation of myostatin promoter activity^{42,43}. The myotube formation in DM5 in the study could be due to the synergistic differentiation which produced hypertrophic myotubes after the treatment of C2C12 cells with the released IGF-1 in combination with dexamethasone⁴⁴⁻⁴⁷. As a summary, the medium type has less effect towards the proliferation of C2C12 cells; however, glucose restriction (i.e. low glucose environment) could inhibit the myogenesis in differentiation phase. With these results, only DM1, DM4 and DM5 would be further evaluated for their capability in inducing mineralization for the TE85 osteogenesis determination.

TE85 human osteosarcoma, which is commonly used for osteoblast differentiation study^{48,49}, has been used to represent the bone-like cells in the proposed musculoskeletal model. In the present study, the medium types have less influence towards the cell proliferation; while the presence of IGF-1 did induce more proliferation⁵⁰, as what have been shown in Figure A1A (in Appendix). Generally, high glucose level could suppress the mineralization (an indication for osteogenesis at late stage) in mesenchymal stem cells through inhibition of osteogenic markers such as ALP, Runx2 and OCN⁵¹⁻⁵³. However, in the present study, mineralization could still be visualized by alizarin red staining, though less calcium deposition and lower Runx2 expression were observed as compared to control (DM3, low glucose DMEM). This phenomenon might be due to the compensatory effect of the presence of IGF-1, which could promote higher Runx2 expression compared to the empty scaffolds⁵⁴⁻⁵⁶, as shown in Figure A1B (in Appendix). In conclusion, TE85 could proliferate well regardless of the medium types used; however, the osteogenic supplements are essential in promoting mineralization when TE85s were cultured in high glucose environment for the subsequent tri-culture experiments.

Lastly, for the culture of tendon cells, tenocytes have shown similar proliferation rate while being cultured in both types of growth medium (i.e. GM1 and GM2). They were further subjected to optimized differentiation medium (i.e. GM5, high glucose DMEM with osteogenic supplements, after optimization from C2C12 and TE85 differentiation studies) and their tendency to differentiate was evaluated by using checking the expression of tendon specific genes, which are scleraxis, tenomodulin and collagen I respectively. Though all three genes were slightly down-regulated (but not significantly) as compared to control (i.e. cultured in tenocytes original medium), the tenocytes were still able to survive, proliferate, maintain their typical elongated morphology on IGF-1 scaffold, as visualized by the cytoskeleton staining. The final experiments showed that C2C12 cells could reside on one end of the scaffold while showing differentiation through the immunolabeling of myogenin; TE85 cells could reside on the other end while showing differentiation through labeling of Runx2 markers and tenocytes survived at the centre part of the scaffold after culturing for 1 week (3 days for proliferation phase and 4 days for differentiation). This is the first study to show that three types of cells could proliferate and differentiate in one general medium, mimicking the actual musculoskeletal tissue arrangement⁵⁷. With such outcome, it would be interesting to use this study as the platform to further explore how cells interact with each other and how to improve the interaction among different types of cell in future experiments.

6.5 Summary

C2C12, tenocytes and TE85 were cultured on the same scaffold and under the same medium environment. PicoGreen analysis, PCR results and immunolabeling of differentiation markers have shown that these three types of cells could survive, proliferate and differentiate under the optimized medium conditions. Although the interaction among the cells was not explored, the co-existence of these three types of cell in one environment implies that the possibility for re-creating the muscle-tendon-bone configuration *in vitro*. With the knowledge obtained from this *in vitro* musculoskeletal model, the quality of the biological tools and the approaches for therapeutic agents'

discovery could be significantly improved.

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Chapter 7

Conclusions and Recommendations

This chapter firstly relates the two hypotheses to the findings in this work and discusses on how objectives were thus achieved. Recommendations on the possible future work as a continuation to the various findings of this work were then suggested at the later sections.

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7.1 Conclusion

The aim of this dissertation was to address the issues which might defer the success of tendon healing via tendon tissue engineering (TE) approach, namely postoperative adhesions, improper healing due to lack of endogenous biochemical cues and failure in recreating the translational junctions between muscle-tendon-bone. Despite the fact that numerous efforts have been attempted, such as improving tendon healing through scaffolds with tendon characteristics (i.e. alignment to provide morphological cues, mechano-stimulation, chemical stimulation or appropriate cell types, these abovementioned issues might pose as threat in deferring the success of tendon healing. Thus, with the knowledge in delivering therapeutics, we thus develop a sustained dual therapy for accelerating tendon healing.

The first objective, which is to obtain sustained release of naproxen sodium (NPS) in an electrospun fibrous scaffold in order to address the postoperative adhesions, was achieved. XPS analysis showed that when minimal amount of water was used at 3v/v% as the co-solvent, less NPS was detected on the fiber surface, indicating that more NPS prefer to reside into the inner region of the fibers and hence slower release was obtained and lasted for two weeks. This supports the first hypothesis of the dissertation that sustained release of NPS was realized through affecting the distribution of the NPS via modulating the polymer compositions and co-solvent (i.e. water) used. However, NPS was found to be released faster with the increase of H₂O content. When increasing the water content, thinner fibers were produced. A higher release rate of NPS was observed with the thinner fibers, which have higher surface area to volume ratio. Therefore, the NPS release rate is relied on a balance between the fiber thickness and solvents evaporation rate. Besides, the scaffolds possessed desirable characteristics for tendon TE, such as aligned fibrous morphology and good mechanical properties. The preliminary cell studies showed that L929 proliferated healthily indicating the scaffolds had no cytotoxicity issues. In addition, tenocytes could respond to the topographical cues given by the aligned fibrous scaffolds, forming a network of cells which mimic the native tendon tissue arrangement. Despite the beneficial role of NPS in preventing adhesions, NPS has inhibitory effects towards

tenocytes proliferation. Nonetheless, the release insulin-like growth factor 1 (IGF-1) from the combined scaffolds in the subsequent study recovered this phenomenon. It would be of further interest if study could be done to examine the effect of released NPS in preventing the adhesion formations in the actual tendon healing. This will be discussed in the recommendation section.

The second objective of this dissertation is to achieve controlled release of IGF-1 to improve the tendon healing. Growth factors (GFs, such as IGF-1) are important in stimulating cellular growth and maturation, but GFs have short half life *in vivo* and hence it is necessary to deliver GFs sustainably. However, current approaches in delivering GFs generally deteriorate the protein stability. In view of this, HFIP has been utilized as it was found that HFIP could better preserve IGF-1 stability (~80%) and western blot analysis has indicated that the released IGF-1 remained bioactive. These have proven the hypothesis that IGF-1 bioactivity could be better preserved through the use of HFIP as electrospinning solutions. Notably, a 10-week controlled release of IGF-1 was achieved via adjusting the electrospinning solution formulations. The released IGF-1 was also able to promote downstream cellular responses, such as enhancing the tenocytes proliferation and differentiation. Although tenocytes proliferated slower in NPS environment, the released IGF-1 could rescue the NPS inhibitory effect by promoting tenocytes proliferation. This dual-release scaffold hence has the potential to enhance the tendon healing, firstly by reducing the adhesion formation and at the same time promoting tendon cells growth under the influence of scaffolds' topographical cues to mimic the native tendon tissue arrangement.

The third objective was to address the difficulty in regenerating the translational region between muscle-tendon-bone connections. In view of the necessity to provide proper environment for the growth and maturation of different tissue types, the culture conditions (in terms of medium glucose level and supplements for inducing differentiation) have been optimized. It was shown that three different cell types could grow and mature on the IGF-1 scaffold to allow the formation of muscle-tendon-bone configuration, similar to how it is arranged in the native human body. This study focused

on the medium compatibility optimization, which proved the concept of cultivating three types of cells *in vitro* under same environment. Further studies, such as improving interactions between cells and the related functional study, could be done to ameliorate this musculoskeletal model. This will be further discussed in the recommendations section.

As a summary, a scaffold which has tendon characteristics, such as aligned morphology and optimal mechanical properties, and the ability to release IGF-1 sustainably for 10 weeks was successfully fabricated. In order to address postoperative adhesion formations, NPS was loaded and 2-week sustained release was achieved. IGF-1 scaffold demonstrates the feasibility to compensate the inhibitory effect posed by NPS, though the latter has beneficial effect in preventing adhesion formation. At the same time, this IGF1 scaffold can be served as a platform for cultivating different cell types to promote the regeneration at the muscle-tendon-bone connections. The proposed approaches can either be applied separately or combined together in order to better promote tendon healing (Figure 7.1).

Figure 7.1: Schematic showing how proposed solutions addressed the problem statement to improve tendon healing

7.2 Recommendations

7.2.1 Three dimensional (3D) scaffolds to better represent cellular micro-environment

Despite the fact that cellular responses in this dissertation demonstrated the great potential of the presented scaffolds (which released NPS and IGF-1 simultaneously) in promoting tendon healing, these results may not be entirely representative to the actual cellular environment. This is because the cells were actually subjected to a semi 3D scaffolds as fabricated by the electrospinning technique, where the extent of cell penetration into the scaffolds is limited^{1,2}. The fabrication of 3D scaffolds arouses a lot of interests as the cellular response (such as cell morphogenesis, cell-cell interactions and migration) from *in vitro* study using 3D scaffolds could hence be better correlated to the actual organs or tissues. Several methods such as multi-layering³⁻⁵, post-processing⁶⁻⁸, porogen-assisted^{9,10}, liquid-assisted^{11 12} or self-assembly^{13,14} methods could be further explored to create 3D scaffolds for tendon TE by using electrospinning technique. Notably, most optimizations relating to electrospinning 3D scaffolds are involved in introducing sufficient spaces to allow cell penetration through additional processes, where the fibers forming step is generally unchanged. This implies that the aforementioned methods will have less effect in affecting the fabrication of previously optimized drugs-loaded scaffolds. By having 3D scaffolds, scientists will be able to regulate the cell cycle or tissue formation more effectively to improve the living expectancy of human beings.

7.2.2 Integration with interface tissue engineering to promote interactions between different cell types

This dissertation demonstrated the feasibility of tri-culture of different cell types via medium compatibility optimization. Although Figure 6.12 showed that three cell types could survive, proliferate, differentiate and the muscle-tendon-bone configuration could be achieved to mimic the actual musculoskeletal arrangement *in vivo*, there was no

interaction study done between different cell types. To properly address the regeneration issue at this intricate, translational region, cells should be allowed to have cross-talk in order to promote integration. Hence, it is suggested that further studies should work closely with interface tissue engineering, which aims to re-establish the continuous interface and to facilitate the integration between different tissue types through tissue engineering approach. From the aspect of scaffold designing approach, the drug-loaded fibers could be produced into a transitional fibrous scaffold with “aligned-to-random” features, to mimic the collagen fibers organization at the tendon-to-bone insertion site^{15,16}. Secondly, it would be of interest if the cells are allowed to have longer time to migrate and interact, or smaller separator could be used to allow more cellular interactions. At the integration site, further analysis such as collagen or calcium depositions should be analyzed to investigate if the unique translational tissue formation could be reproduced at this region *in vitro*¹⁷⁻¹⁹.

7.2.3 In-depth evaluation on the timely-released-NPS scaffolds for prevention of adhesions in animal models

Several studies have shown the adhesion formations could be improved through the use of non-steroidal anti-inflammatory drug, such as NPS in the animal models²⁰. In contrast, other researchers demonstrated that NPS had deleterious effects in terms of tissues healing²¹. This paradox implies that the optimal therapeutic dosage which could address the adhesion formation without inhibiting tissues healing is yet to be confirmed *in vivo*. A controllable and sustained-release system as demonstrated in this dissertation could hence become a platform to understand the optimal continuous dosage needed to prevent adhesion formations by having continuous stimulations to the injury sites.

One of the options to evaluate the efficacy of released NPS could be culturing macrophages as an indication for the possibility of the associated inflammation process. Being inflammatory cells, macrophages are recruited into synovial sheath and epitenon and initialize the subsequent inflammation responses, leading to the adhesion formations in the body^{22,23}. Hence, through understanding the cellular response (such as

proliferation and apoptosis) on the sustained-release system in this dissertation, we can further determine the dosage required for preventing adhesion formations. Besides, for *in vivo* approach, other than evaluating the inflammatory cells related genes expression (such as Cox-2) at the injury site ²⁴, the adhesion coefficient (i.e. to measure the degree of metatarsophalangeal joint range of motion with increasing loads) could be used to justify the extent of adhesion formations ^{25,26}. Besides, biomechanical properties of the regenerated tendons from animal models could be also assessed to understand the efficacy of the released NPS.

7.2.4 Animal studies on dual-release scaffolds to better promote tendon healing

In this dissertation, the possibility of released IGF-1 in rescuing the NPS inhibitory effect on tenocytes proliferation has been demonstrated. This has suggested that this dual-release scaffold could have the advantageous effect in preventing adhesion formations and promoting tendon cell proliferations and maturation simultaneously. Therefore further studies involving *in vivo* work on the dual-release scaffold is recommended, so as to check on their actual effect in promoting tendon healing. The following is some suggested studies to evaluate the proposed *in vivo* work:

- 1) For the evaluation of efficacy of NPS, please refer to animal studies in section 7.2.3;
- 2) To examine the proliferative effect on IGF-1 in the animal models in order to confirm the regenerative response rather than scar formation response, tendon specific genes such as scleraxis and tenomodulin would be analyzed on their expressions and localizations in the injury sites ^{27,28}.
- 3) Type 3 collagen and type I collagen expressions which are associated with the tendon healing would also be determined to confirm the extracellular matrix deposition ^{29,30}.
- 4) The mechanical testing of the harvested tissues could also be done to examine the efficacy of the released IGF-1 ³¹.

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A

APPENDIX

B

Figure A1: Graphs showing A) TE85 cells proliferated faster and B) Runx2 expression was up-regulated, on IGF-1 scaffolds compared to empty scaffolds. (* indicates $p < 0.05$)

Publications

- [1] Y.S. Lui, M.P. Lewis, S.J.C. Loo, Sustained-release of Naproxen Sodium from Electrospun-aligned PLLA/PCL Scaffolds, *Journal of Tissue Engineering and Regenerative Medicine*, DOI: 10.1002/term.2000. (Impact Factor: 4.71)

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- [3] Y.S. Lui, N. Martin, M.P. Lewis, S.J.C Loo, Sustained-release of IGF-1 from Electrospun Scaffold Promoted Human Tenocytes Proliferation and Differentiation -- in preparation

- [4] X. Zhao*, Y.S. Lui*, C.K.C. Choo, W.T. Sow, C.L Huang, K.W. Ng, L.P. Tan, S.J.C. Loo, Calcium phosphate coated Keratin-PCL scaffolds for bone tissue regeneration, *Materials Science and Engineering: C*, 2015, 49, 746–753. (Impact Factor: 3.420)

- [5] X. Zhao, Y.S. Lui, P.W.J. Toh, S.J.C. Loo, Sustained Release of Hydrophilic L-ascorbic acid 2-phosphate Magnesium from Electrospun Polycaprolactone Scaffold—A Study across Blend, Coaxial, and Emulsion Electrospinning Techniques, *Materials*, 2014, 7, 7398-7408. (Impact Factor: 2.728)

- [6] W.T. Sow, Y.S. Lui, K.W. Ng, Electrospun Human Keratin Matrices as Templates for Tissue Regeneration, *Nanomedicine*, 2013, 8, 1-11. (Impact Factor: 4.889)

- [7] H. Yu, Y.S. Lui, S. Xiong, W.S. Leong, F. Wen, H. Nurkafhianto, S. Rana, D.T. Leong, K.W. Ng, L.P. Tan, Insights into the Role of Focal Adhesion Modulation in Myogenic Differentiation of Human Mesenchymal Stem Cells, *Stem Cells and Development*, 2013, 22(1): 136-147. (Impact Factor: 3.777)

Conferences

[1] 26th Annual Conference of the European Society for Biomaterials (ESB)

[2] 1st International Conference in Sports Science and Technology (ICSST)