Engineering scaffolds for restorative tissue repair of tendon via polycaprolactone microfiber and polycaprolactone membrane

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**ENGINEERING SCAFFOLDS FOR RESTORATIVE
TISSUE REPAIR OF TENDON VIA
POLYCAPROLACTONE MICROFIBER AND
POLYCAPROLACTONE MEMBRANE**

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SUMMARY

In tendon repair surgeries, intra-operative graft replacement and post-operative adhesion prevention are two significant but challenging issues hindering the success of the therapies. Conventional practices involve the use of degradable natural materials (e.g. collagen and hyaluronic acid) or non-degradable synthetic materials (e.g. polyethylene terephthalate and polytetrafluoroethylene). However, these materials either mismatch the required mechanical strength or lack a preferred degradability in the body. Emerging medical solutions involve the use of scaffolds or degradable synthetic materials, which can demonstrate both required strength and desired biodegradability and serve a dual function. In this doctoral thesis, the two issues in tendon repair mentioned above will be addressed using this emerging approach.

The issue of tendon graft is addressed by using a three dimensionally aligned polycaprolactone microfiber bundle, in which the fiber diameter is as low as 10 μm , largely resembling the collagen fibers found in native tendon tissues. An original and novel method, named microfiber melt drawing, has been developed to enable the fabrication of these microfibers. A mathematical model has been developed to understand this method and help optimize the fabrication process. Characterization experiments show that these microfibers have a fiber diameter of about 10 μm and are highly aligned in three dimensions. Mechanical testing shows that the ultimate tensile strength for a single microfiber bundle is 6.8 ± 1.84 N and that the breaking force of a microfiber bundle can be adjusted by the number of microfibers and tailored for different tendons. *In vitro* study shows that the microfibers are able to support the

growth of fibroblasts over 7 days and align cells along the channels formed between them. *In vivo* study in a rabbit model shows that the microfiber bundle can be thoroughly infiltrated when implanted in the defect of the Achilles tendon, forming a tendon-like substance in as early as one month. This promising result suggests that microfiber bundle repair may be a better option than the existing therapies.

The issue of tendon adhesion is addressed by using a low porosity polycaprolactone membrane. An original and novel method, named surface tension melt, has been developed to enable the fabrication of these membranes. This method relies on water surface tension for fabrication and thus is solvent-free. Effects of temperature, heating time, particle size and air blowing have been investigated. Characterization experiments show that the membrane has a thickness of 20 – 30 μm , an average pore size of 16 μm , and a two dimensional porosity of about 1%. *In vitro* study shows that the membrane is able to support the proliferation of fibroblasts over 7 days and suitable for animal implantation. *In vivo* study in a rabbit model shows that the membrane preserves the barrier function even after two months and can effectively prevent the adhesion formation.

In conclusion, the two innovative forms of polycaprolactone, three dimensionally aligned polycaprolactone microfiber bundles and low porosity polycaprolactone membranes, fabricated from two innovative methods, are promising to be used in tendon repair. Future work of this project includes further development of these two novel processes and the investigation of *in vitro* and *in vivo* degradation behaviors of polycaprolactone microfibers and membranes

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LIST OF SYMBOLS

ρ : Material density

d : Orifice diameter

v : Velocity

$\bar{v}$: Average velocity

σ : Stress

h : Melt height

φ : Final fiber diameter

V : Take-up velocity

m : Mass

$\dot{m}$: Mass flow rate

T : Melt temperature

T_r : Reference temperature

g : Gravitational constant

LIST OF ABBREVIATIONS

ECM: Extracellular matrix

H&E: Hematoxylin & eosin

IGF: Insulin-like growth factor

MSC: Mesenchymal stem cells

MTT: Methylthiazol tetrazolium

PBS: Phosphate buffered saline

PCL: Poly(ϵ -caprolactone)

PET: Polyethylene terephthalate

PGA: Polyglycolic acid

pHEMA: Poly(2-hydroxyethyl methacrylate)

PLGA: Poly(lactic-co-glycolic acid)

PTFE: Polytetrafluoroethylene

PUU: Polyurethane

SIS: Small intestine submucosa

TCP: Tissue culture plate

WLF: Williams-Landel-Ferry

CHAPTER 1 INTRODUCTION

1.1 Background

Tendons play an important mechanical function in the human body. They transmit forces from muscles to bones, enabling the directed motions, e.g. in the upper limbs, wrists, arms and toes. When isolated to the naked eyes, they appear silvery and shiny, as tendons are primarily composed of densely packed bunches of collagen fibers which are sophisticatedly organized into several hierarchical levels. The shapes of tendons vary with their location on the body, depending on the specific mechanical environment each resides in. Some appear cord-like, while others look flattened or even branched. The longer tendons are found at the extremities of the body such as hands and feet, and the largest and strongest tendons are the Achilles tendons, which reside behind the ankles.

Injuries to tendons are common, especially in sports activities and amongst the aged group. Commonly injured tendons are listed in Table 1.1 [1]. If the injury is not severe, recovery can be completed in several weeks by spontaneous healing. In severe cases such as acute ruptures, suture repair is needed. However, if the case is neglected or in chronic rupture [2, 3], in which the gap is usually widened by the retraction of two ruptured ends due to a delay of appropriate treatment, suture repair may no longer be possible, and reconstructive repair has to be performed. Reconstructive surgeries are also needed when tendons are partially or entirely severed in trauma [4, 5].

Table 1.1: Common injured tendons (adapted from [1]).

Area	Type of tendon	Injuries
Shoulder	Rotator cuff and biceps brachi tendons	Partially torn Acute rupture Chronic rupture
Forearm	Forearm extensor and flexor tendons	
Knee	Patella and quadriceps tendons	
Lower leg	Achilles tendon	
Foot and ankle	Tibialis posterior tendon	

A majority of tendon injuries results from rigorous sports activities or in the workplace with unsafe environment. For Achilles tendon alone, the incidence of rupture in European and Canadian communities ranges from a few to nearly forty per 10,000 people [6-13]. Using this frequency for estimation, the number of tendon surgeries performed in Singapore would be between 1,500 – 20,000 annually. In fact, as the population ages, the number of incidences of tendon rupture is expected to increase. The desire to remain healthy by being active in sporting activity and the fact of tissue degenerating with age may together contribute to increase the number of incidences in future.

To repair or reconstruct an injured tendon, a number of methods are currently available (Table 1.2): autograft, allograft, xenograft, prosthesis and recently, scaffold. Autograft refers to a piece of tendon tissue taken from a normal site on a person and grafted to a defect site on the same person. Allograft refers to the tissue transferred from one person to another person. Xenograft is the tissue harvested from an animal and grafted to a person. All the methods described above utilize natural tendons with a structure and composition that are highly desired in tendon repair. However there are limitations for these methods. For autograft, the main problem is that, very limited donor sites are suitable and available for grafting purposes. Besides, donor-site morbidity is an additional drawback. For xenograft and allograft, the problem of scarcity is less acute, but both of them are foreign materials to the body of the patient;

thus immunogenicity, risk of disease transmission and long-term stability are of serious concerns [14, 15]. Tendon prostheses are artificial tendons that can be made from a variety of materials, such as plastic fibers, silicone rods [16], collagen fibers [17], carbon fibers [18] and their composites [19]. As tendon replacements, they can fairly imitate and perform the physiological function of a natural tendon, but this is largely mechanical rather than biological, because the regeneration of a new tendon is limited or non-existent [16-19]. The use of a tissue engineering scaffold is the latest concept for tendon repair, and now it is being intensively researched [20]. Distinct from prosthesis, which permanently stays in the body after implantation, scaffold stays in the body only temporarily. It degrades in the body and clears over time as the tendon regenerates. In principle, this is achieved by a cell-seeded biodegradable material, in which the material is able to degrade within the body over time. The seeded cells are able to proliferate and grow to fill the space left by the degraded material. As the scaffold eventually disappears, a new tissue is expected to form and integrate with the host tissues [21].

Table 1.2: Currently available methods for tendon repair.

Autograft	tissue is taken from one site and grafted to another site on the same person
Allograft	tissue is taken from one person (donor) but grafted to another person (receiver)
Xenograft	tissue is taken from animals and grafted to one person
Prosthesis	Artificial permanent replacement of tissue with little consideration on tissue regeneration
Scaffold	Artificial temporary replacement of tissue with focus on tissue regeneration

Native tendons consist of bundles of fine collagen fibers, among which tendon cells are arranged in an aligned manner. This histological observation inspires the development of an aligned scaffold for tendon repair, such as a bundle of microfibers.

It has been shown that carbon microfibers, though non-degradable, could induce good tissue infiltration [22, 23]. Studies have also shown that aligned nanofibers and microfibers were able to align cells in the axial direction along the fibers [24-27], and that aligned cells tended to produce aligned extracellular matrices (ECM) [28, 29]. These suggest the aligned microfibers can play an important role in tendon repair by regulating the organization of cells. Therefore the use of a bunch of aligned microfibers as tendon scaffolds may be a promising strategy for tendon repair.

On the other hand, irrespective of the repair method used, the risk of adhesion formation after surgery is difficult to eliminate. In fact, adhesion formation is a common problem in almost every invasive surgery. After tendon surgery, the surrounding tissues of the repaired tendon can firmly attach to the surgically injured sites, restrain the gliding motion of the repaired tendon, thus limiting joint functions and causing pains. Without taking adequate adhesion prevention, a second invasive surgery may be required to remove the adhesion. In order to avoid adhesion formation, a number of methods such as modification of surgical techniques [30, 31], administration of drugs [32, 33], physical separation barriers or a combination of these have been introduced. Among these methods, the use of a physical barrier is technically simpler than modification of existing surgical techniques; meanwhile its risk of adversely affecting the healing process of tendon is lower in comparison with using drugs. Therefore physical barriers have an edge in preventing adhesion formation. However, a majority of physical barriers are made from hyaluronic acid or its derivatives [34-38], which are natural materials that can rapidly degrade in the body (usually within a month). While the time of tendon healing typically takes about 6 to 12 months. The consequence of this mismatch is that adhesion formation may

occur at any time point once the hyaluronic acid barrier is degraded. Alternatively, synthetic materials such as polyethylene [39], polytetrafluoroethylene [40], and polyhydroxyethyl methacrylate [41] have been considered, but they cannot be degraded by the body.

1.2 Objectives

The trend in tendon repair and adhesion prevention is towards the use of biodegradable materials that can fulfill the intended repair and adhesion prevention purposes yet making only a temporary stay in the body. Therefore, the main objectives of this project are as follows:

- To develop a novel biodegradable tendon scaffold mimicking the collagen fiber bundles found in native tendons for tendon repair, preferably in a bundle of linear, unfused and biodegradable microfibers.
- To develop a novel biodegradable separation membrane using a material with a slow degradation rate for tendon adhesion prevention, with consideration of sufficient porosity on the membrane for nutrient and oxygen diffusion in addition to their primary barrier function.
- To evaluate the suitability of the microfibers and separation membranes to be used as tissue engineering scaffolds via *in vitro* studies.
- To evaluate the potential of the scaffolds and separation membranes for tendon repair and adhesion prevention through *in vivo* studies.

1.3 Scope

This project focuses on creating novel scaffolds for tendon repair and adhesion prevention and evaluating these scaffolds *in vitro* and *in vivo*; therefore the scope of this project is as follows:

- To develop a novel method for fabricating a bundle of linear, unfused and biodegradable microfibers that structurally resemble the collagen fibers in native tendons as tendon repair scaffolds.
- To construct and develop a mathematical model to understand and control the novel microfiber fabrication process.
- To design and build a prototype of a microfiber fabrication device.
- To fabricate microfibers and validate the mathematical model.
- To characterize the microfibers in morphology, mechanical property and thermal property.
- To evaluate the microfibers via *in vitro* cell culture for their capability of supporting cell growth.
- To evaluate the microfibers via *in vivo* study for host tissue responses generated to them.
- To develop a novel concept for fabricating porous biodegradable membranes with a slow degradation rate for prevention of tendon adhesion formation.
- To identify the fabrication controls and optimize the process parameters.
- To characterize the membranes in morphology, porosity and mechanical property.
- To evaluate the membranes via *in vitro* cell culture for their capability of supporting cell growth.

- To evaluate the membranes via *in vivo* study for their effectiveness in preventing tendon adhesion formation.

1.4 Organization

Chapter 1 introduces the background of this project, states the objectives and defines the scope of research.

Chapter 2 critically reviews the literature relevant to this project, including structure and functions of tendon, commercial products available and current research for tendon repair, strategies for tendon adhesion prevention, biodegradable materials, methods for fabricating microfibers, and methods for fabricating membranes.

Chapter 3 describes an original method, named microfiber melt drawing, to fabricate a bundle of linear, unfused and biodegradable microfibers, including the development of this concept, modeling of the fabrication process, design and prototyping of the microfiber fabrication device and validation of producing the desired microfibers.

Chapter 4 documents another novel method, named surface tension melt, for rapidly fabricating low porosity polycaprolactone membranes without using solvent. Discussions on the fabrication controls and on alternative applications of the low porosity membranes are also included.

Chapter 5 details the material, methods and protocols used in experiments characterizing the microfiber bundles and membranes, investigating the *in vitro* cell proliferation on microfibers and membranes prior to their implantation in animals, and

evaluating the *in vivo* tissue response to microfibers in tendon repair as well as the response to membranes in tendon adhesion prevention.

Chapter 6 presents the results obtained from the experiments in Chapter 5 and discusses these results and their implications to the development of tendon tissue engineering.

Chapter 7 concludes the thesis and proposes future work and research directions.

CHAPTER 2 LITERATURE REVIEW

In this chapter, six topics relevant to this project are critically reviewed. Section 2.1 focuses on the basic physiology and function of a normal tendon with two contexts being discussed, a mature tendon and a developing tendon. The emphasis is to gain an understanding on the development of tendon from the structural, mechanical and histological perspectives. Section 2.2 reviews the most recent tendon repair method - tendon tissue engineering. It covers cell source, scaffold, and significant research in this field. Section 2.3 discusses the issue of post-operative adhesion and prevention methods. Section 2.4 discusses biodegradable materials for tendon repair. Section 2.5 reviews currently available methods of fabricating polymer microfibers. Finally, Section 2.6 reviews the methods of fabricating polymer membranes.

2.1 Tendon

2.1.1 The mature tendon

Mature tendons are dense connective tissues that have complex compositions, highly organized structure and viscoelastic mechanical behavior. The hierarchical structure of a mature tendon is shown in Figure 2.1 [20]. Collagen molecules are the building blocks of collagen fibrils, from which collagen fibers are formed and organized into collagen bundles. But a real tendon has many more components and a much more complex structure at the molecular level [42, 43]. For example, an adult mature tendon consists of multiple structural proteins, such as collagen type I, II, III, V, decorin, biglycan, fibromodulin, lumican and aggrecan, which are distributed differently in the middle and the end of tendon [43], with collagen type I proteins concentrating at the mid-tendon or the force transmitting region. Besides collagen,

tenocytes (or fibroblasts) are another major component in tendon tissue [42, 44]. They reside among the collagen fibers and form a three dimensionally interconnected cellular network [45].

Figure 2.1: Structure of tendon [20].

Owing to the complex composition and the hierarchical structure, tendons display a viscoelastic behavior. A typical stress-strain curve [46-48] of native tendons is shown in Figure 2.2. The curve can be divided into three regions: the toe region, the linear region and the failure region. This can be understood from the microscopic structural changes of collagen fibers during tensile loading. At rest, collagen fibers are crimped, creating a buffer to dampen the pulling of fibers. As collagen fibers straightened when loaded, their resistance to loading increases, giving rise to a convex downwards curve at the toe region. When the collagen fibers are completely straightened, they start to undergo an elastic deformation, resulting in the change of stress being linearly proportional to the change of strain, and the modulus of a tendon can be derived from

the slope of the linear portion. Finally, upon reaching their limit, collagen fibers break abruptly at the failure region.

Figure 2.2: Stress-strain curve of a mature tendon (adapted from [48]).

2.1.2 The developing tendon

Tendons are not naturally strong in a newborn. Strength in tendons develops slowly as the newborn grows. Changes in biochemistry, microstructure and mechanics of a tendon can be found in its developing process. It is believed that these changes have underlying interrelations, because composition, structure and mechanical behavior are usually correlated.

One example of biochemical changes is the gene expression pattern of two fibrous proteins: collagen type I and collagen type III. Collagen type I can form thick bundles of fibers, while collagen type III form thin and isolated fibers or even heterotypic fibers of type I and type III [49, 50]. Collagen type I is tough, accounting for the

strength of tendon tissue, whereas type III collagen, being more pliable, enriches tissues with elasticity. At the early stage of tendon development, collagen type I and type III are co-expressed and co-distributed throughout tendon fascicles, but at later stages the expression of collagen type III is reduced and only found rich in the endotendon [51]. On the other hand, an increased presence of collagen type III could delay the maturation of a healing tissue [52], and the expression of collagen type III is found up-regulated during the early wound healing process at the rupture site of human Achilles tendon [53], which suggests that reduction in the expression of collagen type III may be associated with tendon maturation.

The microstructure in a developing tendon also changes over time and is greatly dissimilar to what is in a mature tendon. Specifically, the microstructure in a developing tendon transforms from an immature state to a fully mature state (see Figure 2.3), as evidenced by gradually decreased cell density, more and more organized collagen fibers, and increasingly aligned and elongated tenocytes (indicated by the dark dots in the figures) [54]. This change in microstructure is because of the force required for a growing body to perform normal activities having been increased over the entire developing period of tendon.

Figure 2.3: Change of microstructure in a developing tendon [54].
Animal model: white New Zealand rabbit. A. 5-7 days before birth. B. Newborn. C. 7 days old. D. 112 days old. (Scale bar: 20 μm .)

Owing to the changes of biochemistry and microstructure, mechanical behavior of a developing tendon changes accordingly. As shown in Figure 2.4, over time the nonlinear stress-strain curve gradually shifts up [55]. The reason for the upward shifting of the stress-strain curve is complicated, because multiple factors such as total amount of collagen, organization of collagen, collagen type I/III ratio, collagen fibril diameter and length, may influence at the same time.

Figure 2.4: Mechanical change in a developing tendon [55].

Animal model: chick tendon. 43: post-fertilization 17 days. H0: post-fertilization 21 days. H2: post-fertilization 23 days.

In a developing tendon, biochemical, microstructural and mechanical changes are significant, more importantly, they may have interplays and affect each other. For examples, elongated cell shape was found to be able to enhance the expression of collagen type I [56], and mechanical stimulation could change gene expression and orientation of cells [57, 58]. Therefore, to improve the tissue mechanical property, manipulations on the biochemical and microstructural sides could be an alternative strategy. Also, a dynamic view of the developing tendon should be given more attention in future.

2.1.3 Mechanical properties of human tendons

Because direct measurement of *in vivo* mechanical properties of tendons is difficult, most of data on the strength of human tendons are obtained from measuring human cadaver tendons. Table 2.1 shows the ultimate stress and ultimate strain of various types of human cadaver tendons [59-63]. It can be seen that the strength of a human tendon is site-dependent. At the site such as Achilles tendon that needs to withstand

strong and abrupt forces in daily activities, a higher failure stress is observed. At the site such as stapedial tendon where less force is experienced, the failure stress is lower. Moreover, the strength of tendon tends to correlate with age. In the case of patellar tendon, older people who are above 50 years ago have weaker tendons than the young.

Table 2.1: Strength of human cadaver tendons. (Old: >50; Young: <50)

Site	Population	Ultimate Stress (MPa)	Ultimate Strain (%)	Ref
Achilles tendon	Old	79 ± 22	8.8 ± 1.9	[61]
	Young	64.7 ± 15.0	14 ± 6	[59]
Patellar tendon	Old	53.6 ± 10.0	15 ± 5	[59]
Quadriceps tendon	Young	33.6 ± 8.1	14.7 ± 3.7	[60]
Semitendinosus Tendon	Young	37.2 ± 17.8 (Proximal) 48.5 ± 11.8 (Digital)	13.4 ± 6.3 (Proximal) 14.1 ± 2.0 (Digital)	[63]
Gracilis tendon	Young	34.7 ± 12.0 (Proximal) 63.0 ± 13.3 (Digital)	11.3 ± 5.8 (Proximal) 13.6 ± 2.1 (Digital)	[63]
Stapedial Tendon	Old	4.04 ± 1.95	65 ± 0.13	[62]

2.2 Tendon Repair

To repair injured tendons and restore the normal function, biomaterials are usually used as an augmentation device to strengthen the repaired tendon and aid its healing process. In practice, a few biomaterials are already commercially available for wound healing purposes as well as tendon repair [64]. In academic, a broader spectrum of biomaterials is being under rigorous investigation. This section will discuss tendon in greater details.

2.2.1 Commercial products

Almost all the commercial products for tendon repair are made in developed countries such as United Kingdom and United States (Table 2.2), and none of them has included a component of cellular materials for tendon repair. Based on the materials they are made from, these products can be classified into four categories: polyethylene terephthalate (PET)-based, polytetrafluoroethylene (PTFE)-based, polyurethane urea (PUU)-based and collagen-based. Among this comprehensive range of products, only those based on collagen can be completely degraded *in vivo*, the rest have either very limited or no degradability. Collagen-based products are usually derived from dermal extra cellular matrices (skin) of porcine or human cadavers, but the microenvironment provided by this type of collagen may not be ideal for tenocytes in the tendon tissue as there is a distinct difference in the tissue structure of skin and tendon. Also, the strength of collagen, whether cross-linked or otherwise, is still low compared to synthetic repair materials [20].

Table 2.2: Commercial products for tendon repair.

Material	Products	Company	Country
Polyethylene terephthalate (PET)	LARS®	LARS Ligament Augmentation & Reconstruction System	France
	Neoligaments™	Neoligaments™ A division of Xiros	UK
Polytetrafluoroethylene (PTFE) or ePTFE	Gore-Tex®	W. L. Gore & Associates, Inc.	USA
Polyurethane urea (PUU)	Artelon®	Artimplant AB	Sweden
	SportMesh™	Biomet, Inc	USA
Collagen	Restore™	DePuy Orthopaedics, Inc.	USA
	Permacol™	Covidien	USA
	Graftjacket ®	Wright Medical Technology, Inc.	USA
	Zimmer®	Zimmer, Inc.	USA
	TissueMend®	Stryker	USA
	CuffPatch®	Organogenesis Inc.	USA
	Medeor™ Matrix Meso Biomatrix™	Kensey Nash Corporation	USA
	Allopatch HD™	Musculoskeletal Transplant Foundation	USA
	OthoADAPT Bioimplant	Synovis Orthopedic and Woundcare, Inc.	USA
	No react® Patches	Shelhigh Inc.	USA

2.2.2 Current research

Tissue engineering is an emerging research field involving multiple disciplines such as cell biology, developmental biology, biochemistry, material science, and engineering [21]. The ultimate goal of tissue engineering is to solve the worldwide problem of tissue and organ shortage. A general strategy of tissue engineering involves a combination of cells, biodegradable scaffolds and growth factors to regenerate tissues *in vivo*. In contrast to artificial implants, which are non-degradable and stay in the body permanently, tissue engineering scaffolds are temporary and are eventually to be replaced by the regenerated tissues. Tendon tissue engineering is a subfield of tissue engineering that deals with the problem of tendon tissue shortage in tendon reconstructive surgeries and restorative repair. A majority of research have

been focusing on developing an engineered tendon using various types of cells and biomaterials that are less explored but promising.

To date, four types of cells have been investigated in tendon tissue engineering: tenocytes, tendon stem cells, mesenchymal stem cells (MSCs) and dermal fibroblasts. Tenocytes are fibroblast-like, because when cultured *in vitro*, they display an elongated spindle shape like fibroblasts. The expression of collagen type I but not type III is a unique characteristic of tenocytes, which can be used to distinguish them from other fibroblasts such as dermal fibroblasts. Alternatively, tenocytes can be identified with scleraxis [65-67], a specific marker gene that is predominantly expressed in tendon. Moreover, tenocytes respond differently to different culture conditions. A combination of growth factors such as insulin-like growth factor-I, platelet-derived growth factor-BB and basic fibroblast growth factor could show a synergistic effect on tenocyte proliferation [68]. However, supplement of transforming growth factor β -1, 2, 3 decreased cell number and increased collagen production only [69]. Recently, low oxygen (2% rather than 21% in atmosphere) culture was shown to be able to enhance tenocyte proliferation by three to five folds and down-regulate the expression of matrix metalloproteinase-1 and interleukin-6 [70]. Furthermore, tenocytes can respond to topographical guidance. Microgrooved surfaces could promote both the alignment of tenocytes and the alignment of extracellular matrices secreted by tenocytes. However, whether microgrooves affect the level of gene expression is uncertain [71-73]. In tendon tissue engineering, though tenocytes are favorable cell source due to being autologous, the drawback is that they are not abundantly available because of a low cell density. In addition, autologous tenocytes can risk losing their phenotype when cultured *in vitro* [55, 74].

Another cell source is tendon stem/progenitors cells, which reside within a niche in tendon tissue that comprises biglycan, fibromodulin and other molecules [65]. Studies have shown that alignment of nanofibers was able to induce tenogenic differentiation of these stem cells [75], and that adequate cyclic loading could be a way of controlling their differentiation as well [76]. However the conditions for their differentiation into tenocytes are still poorly understood. More efforts need to be directed to establish controllable conditions that yield consistent results. Moreover, the number of tendon stem cells is limited and may cause the problem of identification and hence isolation in practice.

Mesenchymal stem cells (MSCs), usually isolated from bone marrow or adipose tissue, can be differentiated into tenocytes as well. For example, when transfected with bone morphogenic protein-12, bone marrow-derived mesenchymal stem cells could differentiate into tenocytes which expressed collagen type I but not collagen type III [77]. When treated with growth differentiation factor-5, adipose-derived mesenchymal stem cells could also differentiate into tenocytes [78]. Other than that, studies showed that MSCs had a faster proliferation in culture than tenocytes [79, 80] and that MSCs themselves could act as a multi-drug carrier to enhance repair and regeneration [81]. These suggest that MSCs may be a better candidate for tendon tissue engineering. However, the control of differentiation is still difficult because they are multi-potent and can differentiate into other tissues such as bone [82].

A surprising but interesting report is the use of dermal fibroblasts for tendon repair [83]. Skin and tendon are two distinct tissues, but this work demonstrated that dermal

fibroblasts could be successfully engineered into a tendon. Besides, in another study, autologous skin was transplanted to repair a tendon gap in a canine model, the preliminary result also suggested a transformation from skin tissue to tendon tissue is possible [84].

With these four types of cells, a number of research groups have been attempting to combine them on different biomaterial scaffolds. Cao *et al.* first conducted *in vivo* tendon engineering in a hen model [85]. In their experiment, tenocytes were seeded onto unwoven polyglycolic acid fibers, and then the cell-scaffold construct was wrapped with intestinal submucosa and cultured *in vitro* for one week before implantation for 14 weeks. The engineered tendon reached 83 percent of normal tendon breaking strength. In another experiment, using the same cell type and scaffold material, they attempted to engineer a tendon in an *in vitro* environment. This preliminary study showed that the engineered tendon was significantly weaker than a natural tendon as the engineered tendon was only under a passive strain [86]. In a recent experiment in which a dynamic loading was applied [87], they found that *in vivo* loaded group exhibited a bigger tissue volume and better aligned collagen fibers than *in vivo* non-loaded group and *in vitro* loaded group. These works indicate that an *in vivo* environment is better than an *in vitro* environment in terms of engineering a tendon, and that the effect of mechanical loading is significant, despite the fact that the engineered tendons are still weaker than a natural tendon. Beyond these works, Cao *et al.* also pioneered the use of dermal fibroblasts for tendon tissue engineering [83]. Interestingly, implanted dermal fibroblast did not express type III collagen at 26 weeks, suggesting a possible phenotype change.

Butler *et al.* attempted to engineer a functional tendon by using collagen and MSCs [88]. They first demonstrated that the MSC-collagen construct could significantly improve the biomechanical properties of tendon repair tissues [89-91]. They showed then that in a rabbit model, MSCs did not lose their repair potential as rabbits aged [92]. Next, studies on the effect of cell-to-collagen ratio found that a higher cell-to-collagen ratio could damage the construct due to excessive cell contraction and that a lower ratio made no significant difference in histological appearance from the control group [93, 94]. Another study on the effect of mechanical stimulation found that biomechanics and cellular alignment could be significantly improved [95], owing to an increased total content of collagen type I and type III [57]. They also investigated the effect of scaffold materials (collagen gel versus collagen sponge) [96], the effect of construct length [97], and a study including these two factors found that the highest *in vitro* linear stiffness was achieved in a longer, stimulated cell-sponge construct [98]. Though Butler's group had considered various factors that could enhance the mechanical outcome of cell-collagen constructs, the goal of achieving a functional tendon is still some distance away. In fact, it is challenging to obtain a cell-collagen construct with a highly organized microstructure during *in vitro* culture, even if mechanical loading is applied. Figure 2.5 highlights schematically the difference between a mechanically loaded cell-collagen construct and a native tendon. *In vitro* seeded cells are randomly distributed in the collagen scaffold and only individually aligned, which deviates from what is seen in a native tendon in which cells are in three-dimensional cellular rows. As a result, *in vivo* implantation of a poorly organized construct, perhaps, delays or even impairs the regeneration of a mature tendon.

Figure 2.5: Microstructure in engineered and native tendons.

Goh *et al.* focused on cellular infiltration, an important aspect of tendon tissue engineering [99-102]. A knitted poly(lactic-co-glycolic acid) (PLGA) scaffold, which was more porous than a tightly-packed braided one, was evaluated in a rabbit model. Eighty seven percent (87%) tensile stiffness and 62.6% modulus of normal tendon were achieved [99]. Another study found that cells were viable for at least 8 weeks after implantation and that cell morphology changed from a round shape to a spindle shape after 5 weeks [100]. However the challenge is to retain a vast number of cells within the knitted structure. To overcome this problem, a fibrin gel system was used to deliver up to ten million cells. Also, there was an attempt to hybridize a cell sheet to the knitted scaffold [101]. Recently, a nanofibrous layer was electrospun onto the knitted scaffold, preventing cells from falling off [102]. Despite these efforts, the issue of cell alignment was not addressed during *in vitro* culture, and the engineered tendons still show significant differences from native tendons in terms of biochemical, microstructural and mechanical characteristics.

Natural extracellular matrix such as small intestine submucosa (SIS) is believed to be attractive scaffold materials in engineering a tendon. However, Derwin *et al.* did not recommend the direct use of SIS for tendon repair due to the unsatisfactory clinical outcomes such as mechanical weakness and post-operative adhesion [103, 104]. In order to enhance the *in vivo* results, they conducted *in vitro* mechanical pre-

conditioning of tenocyte-SIS constructs. Improved structural property was observed, but the mechanical property of these pre-conditioned constructs were significantly lesser than those of a native tendon [105], indicating that the change of microstructure is minor. The problem encountered here seemed much similar to Butler's group, a simple combination of cells, collagen and mechanical loading *in vitro* did not result in a construct with comparable mechanical strength, although mechanical loading indeed played a positive role. Derwin *et al.* also compared four commercial products of natural extracellular matrices (ECMs), a similar biochemical composition to tendon was observed, but all the elastic moduli were lower than that of a normal tendon (Figure 2.6) [106]. Recently, they attempted to estimate the critical thickness of the *in vitro* cultured cell-scaffold construct by modeling oxygen diffusion through three different natural scaffolds [107]. They found that the critical value ranges from 1 mm to 3.5 mm depending on the scaffold material and oxygen concentration.

Figure 2.6: Comparison of commercial ECMs with a normal tendon [106].

Chang *et al.* have been investigating flexor tendon wound healing factors [108]. They improved tenocyte culture by optimizing the supplementation of growth factors [68],

in which they found a synergist effect on cell proliferation could be observed when 100 ng/ml of insulin-like growth factor-1 (IGF-1), 50 ng/ml of platelet-derived growth factor-BB and 5ng/ml of basic fibroblast growth factor were used. They also compared tenocytes and mesenchymal stem cells seeding on acellularized tendon scaffolds and concluded that both could be successfully used for tendon tissue engineering [109]. Recently, they investigated three patterns of cyclic mechanical loading on tenocytes and mesenchymal stem cells. The results showed that continuous cyclic loading inhibited cell proliferation whereas intermittent cyclic loading increased cell proliferation [110].

Table 2.3 summarizes the current research work on tendon tissue engineering. There are also other works on tendon tissue engineering, but they focused mainly on the materials for tendon repair, for example, chitosan-based [111-114] and silk-based [115-117] materials. Earlier, there was also an approach that does not rely on scaffold, in which a premature tendon was engineered simply in a two-poled petri dish [118]. Cultured tenocytes self-assembled and contracted against the poles, resulting in the formation of a premature tendon. However, *in vitro* cell manipulation is a routine of their experiments. To date, no group has reported that a structure of three-dimensional cellular rows has been obtained.

Table 2.3: Current research work on tendon tissue engineering.

Group	Cell type	Scaffold	Findings	Ref
Cao <i>et al.</i> (China)	Tenocytes & dermal fibroblasts	Unwoven PGA fibers	Dermal fibroblasts can be used to engineer a tendon. Tendon engineered <i>in vitro</i> is significantly weaker than that engineered <i>in vivo</i> .	[83, 85-87, 119-121]
Butler <i>et al.</i> (USA)	MSCs	Collagen-based	The effects of scaffold length, scaffold material and mechanical loading all are significant on strength enhancement. <i>In vitro</i> seeded MSCs are isolated and poorly organized before <i>in vivo</i> implantation.	[57, 82, 88-91, 93, 94]
Goh <i>et al.</i> (Singapore)	MSCs	Knitted PLGA fibers	Porosity needs to be addressed in tendon tissue engineering for cellular infiltration. <i>In vitro</i> attachment of a vast number of cells is difficult.	[99-102]
Derwin <i>et al.</i> (USA)	Tenocytes	SIS	The effect of mechanical loading is significant on strength enhancement. <i>In vitro</i> oxygen diffusion limits the size of an engineered tendon.	[103-105, 107]
Chang <i>et al.</i> (USA)	Tenocytes & MSCs	Acellularized tendon	Different patterns of cyclic mechanical loading may have different effects on cell proliferation. <i>In vitro</i> optimized cell expansion requires addition of multiple growth factors involved in tendon healing.	[122-128]
Calve <i>et al.</i>	Tenocytes	Scaffold free	Cultured cells naturally contract against two poles in petri dish. The <i>in vitro</i> self-assembled tendon is even weaker than an embryonic tendon of chicken.	[118]
Others	Tenocytes or fibroblasts	Chitosan-based or silk-based	Biomaterials can be engineered to mimic collagen fibers in tendon. It is challenging to arrange cells in three dimensional rows.	[111-114, 129]

Ideally, an engineered or regenerated tendon should be identical to the native mature tendon. At present, there are substantial differences between them. Mechanically, the engineered tendons show only fractional strengths of the native mature tendon [83, 85-87, 89-91, 93, 94, 99, 105, 118, 129-132]. Histologically, the distribution of aligned collagen fibers is not uniform and the alternative rows of cells and collagen fibers are not as clearly distinguishable as that in native mature tendon [83, 85-87, 89-91, 93, 94, 99, 105, 118, 129-132]. In ultra-microstructure, collagen fibril diameters are much smaller than that of the native mature tendon [83, 85-87]. Biochemically, over-expression of collagen type III is observed [83, 93, 94, 99, 131, 132], which is

however absent in a mature tendon. These differences indicate that an engineered tendon is usually immature.

Many differences between an engineered tendon and a native tendon are observed though, why and how these differences occur in a consistent manner in these studies remains unresolved. One possibility is that multiple strategies (e.g. contact guidance, growth factors, mechanical stimulation, and bioreactor) have not been synergized to achieve the best outcome. This approach requires integration of various resources and technologies, but so far convincing evidence that a mature tendon that is a direct result of such synergy and optimization is still lacking. Moreover, due to the complexity in tissue regeneration involving numerous chemical and physical factors, synergy and optimization are already a great challenge. A second possibility for the inferiority of engineered tissues is, as reviewed by Liu *et al* [20], that there is lack of a suitable scaffold that favors cell adhesion and guides cell alignment (a critical feature found in native mature tendon). Current tendon scaffolds can seldom perform these dual functions. Another possibility is that, in past studies, a cell-scaffold construct is cultured *in vitro*, which is an inferior environment for tissue regeneration as compared to an *in vivo* environment. After implantation, the *in vivo* regeneration is impaired by the earlier *in vitro* culture. As a result, an immature tendon is formed instead of a mature one.

In summary, there have been several groups actively working on tendon repair based on tissue engineering principles. Though their approaches are different, what is common is the use of biocompatible and biodegradable materials and they play an essential part to repair injured tendons. Natural materials such as collagen and its

derivatives lack adequate mechanical strength, while synthetic materials are unfavorable for cell adhesion. Research will progress toward exploration of new materials and new structures for tendon repair, as well as how these new materials and structures will influence cellular behaviors. Besides, attention will also be drawn toward tendon-muscle junctions [64, 133], whose roles on tendon healing remain almost unknown, and have been largely missed in previous studies.

2.3 Tendon Adhesion

After repair, a tendon heals by a combination of intrinsic and extrinsic processes [134]. Intrinsic healing refers to the proliferation and matrix remodeling activities by tenocytes within the tendon tissue. Extrinsic healing refers to the activities of fibroblasts that originally reside in the ends of tendon sheath but migrate to the defect site later. During the healing process, tenocytes and fibroblasts are unlikely to be capable of distinguishing tendon tissue and surrounding tissue. Instead, they are believed to secrete proteins to bridge whatever gaps present. Therefore, tendon adhesion is usually associated with the healing process after tendon repair. Adhesion formation is a highly undesirable result. It can hinder the gliding function of tendon and result in pain and stiffness of joint. Early mobilization is usually suggested to prevent adhesion formation and restore tendon function. Besides early mobilization, a number of other measures, such as modification of surgical techniques, administration of drugs and application of separation barriers, can be taken to further reduce the risk of adhesion formation.

2.3.1 Modification of surgical techniques

Increased suture strands and increased bulk at the repair site are thought to promote adhesion formation, because they increase the frequency of contacts between the

inside and outside of tendon tissue. However, by comparing 2-strand repair with 4-strand repair, the adhesion formation and gliding resistance of tendons were not significantly different [135]. This suggests that simply increasing the number of strands does not necessarily result in more adhesions or resistance. In fact, increasing the number of strands can proportionally increase the amount of suture materials exposed outside the tendon tissue, acting as a separation barrier. However, whether more strands will result in reduced adhesion formation is still unclear. In another study, it is found that high friction (modified Becker) suture technique could cause more adhesion formation than low friction (modified Kessler) technique [31], which suggests a relationship between suture technique, friction and adhesion formation. In addition, bleeding in dissection is also considered a cause of tendon adhesion. Techniques such as use of coagulators or surgical laser that can limit bleeding are thought to be able to reduce adhesion formation. In a chicken model, however, flexor tendons dissected with sharp scalpel with coagulation and carbon dioxide laser were associated with significantly increased adhesion formation in contrast to those dissected with sharp scalpel without coagulation [30]. Therefore it is unnecessary to introduce additional handling to aid or replace conventional sharp dissection technique which actually does no harm.

2.3.2 Application of drugs

Intra-operative application of drugs has been proven successful in some cases [136, 137], but with what mechanism the drug works is poorly understood. Tendons treated with 5-fluorouracil solution showed significantly reduced adhesion formation in comparison with buffered saline treated control group [136]. However, 5-fluorouracil is an anti-cancer drug that can inhibit fibroblasts from producing extracellular matrix proteins and growth factors. This actually raises the question on its effect on the

healing process during adhesion prevention. A drug that is able to effectively prevent adhesion yet that has not compromised the wound healing has yet to be reported so far. In another study involving hyaluronic acid and nonsteroidal anti-inflammatory drugs, it was found that the combined gel could prevent adhesion formation by reducing inflammatory response and maintaining the separation between tendons and surrounding structures [137]. However, the mechanism of how hyaluronic acid prevents adhesion still remains largely undiscovered.

2.3.3 Physical barriers

Another way to prevent tendon adhesion is to apply a membrane to the repair site as a separation barrier preventing tendon tissues from interacting with surrounding structures. Membranes made of various materials have been developed and investigated (Table 2.4). It is found that synthetic polytetrafluoroethylene (PTFE) membranes could result in better tendon gliding compared to patches made of natural materials such as collagen patch , and was not associated with a significant inflammatory reaction at six weeks [40]. Although synthetic and natural patches were equally effective in the end, synthetic PTFE membrane was biomechanically and histologically superior to natural patches during the early healing period [40]. A similar result was found when e-PTFE membranes were used [138]. Chondroitin sulfate coated pHEMA membrane showed a superior result in reducing adhesion formation in the first 6 weeks, but the effectiveness faded afterward [41], perhaps due to uncontrolled coating thickness. In another study, amniotic membrane combined with hyaluronic acid injection resulted in the least adhesions compared to either treatment alone [139]. Seprafilm and Interceed are two commercial products currently available for prevention of adhesion. The former contains sodium hyaluronate and carboxymethyl cellulose and the latter contains oxidised cellulose. When used as

adhesion barriers for tendon repairs, both can lower the rate or degree of adhesion formation [37, 140], but no clinical trials have been carried out to demonstrate their efficacy in humans.

Table 2.4: Membranes for tendon adhesion prevention.

Type of membrane	Composition	Model	Duration	Ref.
PTFE or e-PTFE membrane	Polytetrafluoroethylen	Rabbit	12 weeks	[40, 138]
pHEMA membrane	Coated on one side with chondroitin sulfate	Rabbit	12 weeks	[41]
Amniotic membrane	Injected with hyaluronic acid	Rabbit	20 weeks	[139]
Septrafilm	Sodium hyaluronate and carboxymethyl cellulose	Rabbit	12 weeks	[37]
Interceed	Oxidised cellulose	Rat	4 weeks	[140]

Despite these advancements in research, an optimal method for the prevention of post-operative adhesions is still lacking. Compared to improving surgical techniques and injecting drugs with poorly understood mechanism, applying a separation barrier appears to be a simple and effective preference. Indeed there has been a significant growing interest in the development of biodegradable barriers due to their ability to be absorbed by the body after performing the preventive function. Looking into the future, along with advocating early post-operative mobilization, more research work will be directed toward the mechanistic understanding of current treatments and the development of effective and biodegradable separation barriers.

2.4 Biodegradable Materials

Polymeric materials, especially those having biodegradability, such as polyglycolic acid (PGA), poly(lactic-co-glycolic) acid (PLGA) and polycaprolactone (PCL), are very important material in biomedical science and engineering. PGA and PLGA are

fast-degrading materials, and their degradation times can be as short as one month. PCL degrades more slowly, and the degradation time is usually beyond one year. The degradation time of PCL also differs when placed *in vitro* and *in vivo*. This is thought to be due to the presence of lipases *in vivo* which can catalyze and accelerate the degradation [141-143]. Another difference between PGA, PLGA and PCL is that their degradation products create different acidic environment [144]. As PCL generates less acidic environment, it is thus more favorable for *in vivo* implantation. Moreover, PCL is much easier to process, because the melting point of PCL is around 60°C, whereas the melting point of PGA exceeds 220 °C. In terms of cost, PCL is significantly cheaper than the other two. Furthermore, PCL has a variety of proven applications. It has been used to build tissue engineering scaffolds [145-148]. It has also been studied as drug delivery devices for controlled release [149-151]. Other applications of this material include absorbable sutures [152] and wound dressings [153].

Unlike the healing of other tissues, tendon takes a longer time to heal due to their low cell density and limited vasculature. Therefore, to match this slow healing rate, biodegradable polymer with slow degradation rate is preferred. As illustrated in Figure 2.7, scaffold with slow degradation property can take the strength over a longer period to wait for the full regeneration of tendon. Another consideration is the effect of stress shielding, in which too strong a material will bear a majority of the load and weakens the regenerating tissue. In order to reduce the effect of stress shielding which can impair the strength of the regenerated tissue, a low modulus material such as PCL is preferred.

Figure 2.7: Fast degradation versus slow degradation.

2.5 *Methods for Fabrication of Microfibers*

The history of man-made fibers is less than 100 years old and there are not many fiber-forming techniques. The number of microfiber generation techniques is even fewer. Following is a review of the existing microfiber generation techniques based on the strategy used to reduce the diameter of fibers.

2.5.1 **Melt- and jet- blowing**

Melt-spinning is a common method to process polymeric materials into the form of filaments. It involves extruding and cooling of multiple jets of polymer melt from a spinneret, as well as winding the jets onto a high speed roller. Melt-blowing is derived from melt-spinning. Compared with conventional melt-spinning, the extruded jet is attenuated by the downward high velocity air stream which closely surrounds the nozzle rather than directly stretched and thinned by a high speed take-up roller. Due to the weak strength of each individual microfiber, only non-woven fiber meshes can be easily collected. The distribution of the fiber diameters is usually wide, from several hundred nanometers to tens of micrometers. The average fiber diameter is 1-2 μm in a commercial process [154]. However, a recent study demonstrated that fibers with average diameter less than 500 nm were successfully produced under

commercially viable processing conditions [155], which opened up the possibility of rapid production of nanofibers.

Another technique is jet blowing, in which a highly pressurized gas-particle mixture is forced through a capillary nozzle at an elevated temperature. Due to the sudden contraction of the flow, particles are melted and stretched by the gas stream, resulting in multiple fibers within the nozzle. The technique is effective for at least polytetrafluoroethylene (PTFE) and polycaprolactone (PCL) [156]. However, like melt blowing, the end products are in the form of random meshes as well.

2.5.2 Splitting

Bicomponent melt-blowing is a process that fabricates fibers comprising two types of polymers. Separation or splitting of the two polymers is one of the strategies to reduce fiber diameter. Christine and Zhang investigated the splitting of bicomponent melt-blown fibers by hydro-entanglement [157] and water treatment [158], respectively. However, pressurized steam caused more fiber breakage than splitting, and water treatment resulted in a tendency for the distribution curve of fiber diameter to shift, though the shift was not thought to be significant. A commercially available bicomponent melt-blowing system designed by Hills, Inc. is able to produce fibers with a diameter of approximately 2 μm [154].

Another technique using the splitting approach is “islands in the sea”, which is a configuration of a cross-section of a single filament extruded by melt spinning. The “islands” and the “sea” are two different polymers. By dissolving the “sea” component, the “islands” or microfibers can be made at a diameter of approximately 300 nm [154]. In contrast to “islands in the sea”, the cross-section of a “multi-

component fiber” is pie-like, in which sections are bound together by their interfacial energy. After mechanical, chemical or hydro treatment, the sections can be separated, giving rise to finer fibers. However, due to the limited number of sections, the dimensions of the fibers usually do not reach the submicron range [154].

Not only can a fiber be split, so can a film. A highly crystallized polymeric film is strong in the oriented direction but weak in the perpendicular direction. By imparting fluid energy onto the surface of the film, fibrillated microfibers can be generated. In a US patent [59], the fiber diameters have a skewed distribution from 0.4 μm to 2 μm while concentrated at 0.4 – 0.8 μm .

2.5.3 Laser thinning

Suzuki *et al.* made microfibers from a spool of spun fibers rather than polymer melt [159-161]. In their method, a carbon dioxide laser was used to melt and thin the fiber when the fiber was constantly supplied downward. The thinned fibers were cooled by air. Both microfiber spools [160] and nonwoven webs [159] were able to be produced. Recently, nanofibers were obtained by enclosing the “laser thinning process” in vacuum [161]. Nakata applied a laser thinning method to conjugate spun fibers [162]. After removing the sea component, fibers with diameter as low as 39 nm were obtained.

2.5.4 Electrospinning

Electrospinning is similar to dry spinning, but the formation of a continuous jet is driven by a high voltage applied at the nozzle tip [163, 164]. The high voltage breaks up the surface tension of a droplet, causing an extensional flow of the polymer solution. As the charged jet whips in the electric field, solvent evaporates and the

fiber-like jet is stretched and thinned until it contacts with the ground collector. Nonwoven mat, aligned fibers and nanofiber bundles can be obtained as end products, but fiber-fiber contacts are usually fused together. The average final fiber diameter can be controlled, and the range of control spans from several nanometers to several micrometers. However, two drawbacks associated with electrospinning are low production rate and the use of toxic solvent.

Recently, there are few studies on “melt electrospinning”, in which the high voltage is applied to polymer melt rather than solution. Lyons *et al.* studied the effects of molecular weight, electric field strength and spinning volume on fiber diameter in melt electrospinning process [165, 166]. Although submicron fiber was obtained by tuning the parameter, majority of the fiber diameters ranged from 1 μm to tens of microns [165]. Dalton *et al.* found that by supplementing viscosity-reducing additives, submicron polypropylene fibers could be obtained [166]. Also, the “gap method of alignment” was discovered to be effective in reducing the fiber diameter in melt electrospinning though it was not the case in solution electrospinning [166]. This is likely because of the stretch-thinning action between the contact points and the whipping fibers.

Yarin and Zussman reported a method based on needleless electrospinning [167]. In this process, the polymer solution is placed on top of a magnetic suspension. The magnetic field destabilizes the surface of magnetizable fluid, which gives rise to standing conical spikes. When an electric field was applied additionally, these spikes served as the sites of upward whipping jets of polymer solution. Up to about 1000 jets were reported, and this approach could enhance the output of fiber production. Instead

of using magnetic field, He *et al.* made bubbles on the surface of the polymer solution through a gas tube, then an electric field was applied, in small bubbles whose surface tension is lower and could be easily overcome and the bubble is deformed to become an upward solution jet [168].

2.5.5 Microfluidic chip-based

A recent approach to fabricate biomaterial microfibers involves a microfluidic chip [169]. In this method, PLGA solution was made to flow in the micro channels. On exit, the solution evaporated, leaving solid PLGA microfibers. The fiber diameter, as low as 10 μm – 20 μm , could be controlled by flow rate. This method is, in principle, similar to dry spinning.

2.5.6 Drawing

The drawing or pulling approach has been experimented much earlier than extrusion and spinning technique. In 1908, “fano flow” was discovered and paved the way to today’s industry of man-made fibers. In 1967, a similar phenomenon, “tubeless siphon” was reported in Nature [170]. In 1987, Mackley proposed a method called “die-free spinning”, in which a rod was inserted into a polyethylene hot gel and then lifted up and wound onto a take-up spool [171]. However, at that time the fiber diameter was not the focus, rather, the spinnability of materials and high production rate had gathered more attention.

Recently, several studies on the drawing approach have been reported. Direct drawing of nanofibers from polymer solution was reported by Ondarcuhu and Joachim in 1998 [172]. Later, Harfenist *et al.* demonstrated the fabrication of suspended micro/nanofibers by direct drawing [173]. However, in their work, pre-deposition of

liquid polymer droplet on the substrate pillars were critical to form a network of suspended fibers. Nain *et al.* further evolved to continuous drawing, in which polymer solution is constantly supplied to a microglass pipette and drawing is initiated by attachment and detachment of the polymer droplet on the substrate [174], thus eliminating the need of pre-deposition.

Besides drawing from a solution, drawing from a polymer melt can also produce microfibers [175]. A simple way to do it is to use a fine needle to break the surface of the polymer melt and then lift it up, and the drawn part solidifies in air and forms a fiber spontaneously. The advantages of using the drawing approach are: (1) microfibers are easily obtainable; (2) a complex die design is not required, thus the problem of clogging is avoided; (3) it is simple and usually inexpensive.

Other methods that can fabricate microfibers include self-assembly [176], template synthesis [177], phase separation [178], stirring [179], and ultrasonic exaction [180]. However, these methods mainly fabricate microfibrils or disintegrated silks rather than continuous synthetic microfibers.

2.6 Methods for Fabrication of Membranes

Fabricating membranes that are porous, biodegradable and free of toxicity is critical toward successful prevention of tendon adhesion. The few common methods to fabricate membranes are described below.

2.6.1 Solution casting

The most common method of fabricating membrane is solution casting or solvent casting [181, 182]. In this method, a volatile organic solvent is used to dissolve a

polymeric material and then the mixture is poured into a glass container where the dissolved material precipitates into a solid layer after the solvent fully evaporates. Solution casting is simple and versatile, but the major drawback is that solution-casted films are usually uneven in thickness and contain residues of the solvent, and seldom are they porous. In addition, the process usually takes a few days to finish.

2.6.2 Spin casting

Similar to solution casting, preparation of a polymer solution is essential in this method. The difference is that the polymer solution is first deposited on a rotating metal sheet and then spread into a thin film by a strong centrifuging force. Polycaprolactone films of thickness approximately 6 μm could be produced by this method [182]. However, it has the same drawbacks as solution casting, that is, use of organic solvent and the resulting film is dense without pores.

2.6.3 Roll milling

In roll milling, materials are simply fed directly in-between the heated rolls to be milled, scraped off with a blade and left to solidify into a uniform piece [182]. This method typically produces thick membranes without pores.

2.6.4 Electrospinning

Electrospinning is another method to fabricate membranes. A detailed discussion on this method can be found in Section 2.5.4. This method can produce thin membranes with fine pores. Similar to solution casting, the process requires the use of an organic solvent.

2.6.5 Biaxial drawing

Biaxial-drawing is a method to make thin films by stretching a pre-form in perpendicular directions, so that an ultrathin film can be formed by enlarging the pre-form into a larger area [181, 183, 184]. These stretched thin films usually do not have pores. In order to create pores on the film, additional steps have to be taken.

In summary, most of the methods reviewed above fabricate a dense film rather than a membrane, and are unable to create a porous structure in a single step. The only exception is electrospinning which can create a nanofiber membrane. However, the use of an organic solvent is unavoidable when using electrospinning, meaning that toxic materials are likely to be left trapped in the membrane after fabrication.

2.7 *Summary*

The use of biodegradable materials is an emerging approach to repair an injured tendon. Current research on tendon repair focuses on fast-degrading materials, such as PGA, PLGA and collagen, while slow degrading materials have been largely ignored. Besides, there are limited methods to process slow degrading materials into microfiber bundles and low porosity membranes for tendon reconstruction and tendon adhesion prevention. In order to bridge this gap, new methods of fabricating repair devices, such as microfiber bundles and low porosity membranes using slow degrading materials, have to be developed.

CHAPTER 3 MICROFIBER MELT DRAWING

3.1 Introduction

In this project, the first objective is to develop a microfiber fabrication device for tendon repair. A bundle of unfused microfibers has been proposed to mimic a bundle of collagen fibers in the native tendon. After reviewing the processes for microfiber fabrication, including melt blowing, biocomponent splitting, carbon dioxide laser thinning, electrospinning, etc., it is found that at present, it remains to be a challenge to obtain a bundle of unfused biomaterial microfiber bundle. For each of the available techniques, not all materials can be processed into the form of microfibers. Even if the material can be processed, toxic additives may be an integral part of the process, which is highly undesirable for fabricating devices for biomedical applications. In addition, ease of processing, cost effectiveness and complexity, all make it ever harder to fabricate a microfiber bundle from the available techniques.

In order to proceed with the project a new process has to be developed. In this new process, the following specifications must be met. First, the diameter of microfiber is targeted at 10 μm , or at least, have a narrow range of 5 – 15 μm . This is because the size of a collagen fiber in natural tendon tissue measures around 10 μm . Another reason for this dimension selection is that cell alignment is critical for tendon tissue, and cells on microfibers with a diameter larger than 10 μm tend to lose their alignment and become disordered [25]. Secondly, as the device is not meant for large-scale manufacturing, only a small batch production of microfiber bundles for laboratory testing is needed. Hence, the device should be minimized in size and remain low in cost. This means that there is no need for high precision instruments or

costly apparatus such as a laser or high voltage involved. Also, the device should be kept simple. Sophistication or high level of complication will make the device difficult to fabricate. Knowing how to operate it simply is highly desirable. Thirdly, the output must be of good quality. Production of microfibers must be within a reasonable time frame, in a practical quantity, and an adequate production rate, such as 1mg/min. The fiber must be continuous and is able to form a bundle. Fibrous mesh, fibrils, or short fibers are not desired. Fourthly, there should be no chemical additives or any post processing. For biomedical applications, cytotoxicity and biocompatibility are important criteria. Any process involving toxic substance that is harmful or of any potential to be harmful in future will be excluded from consideration. Post processing, via either chemical or physical means, should be avoided so as to be free of contamination as well as for the sake of simplicity. Fifthly, there should be no fusion among the fabricated microfibers. The microfibers must not stick to each other and they should be able to spread in a wet environment so that spaces can be created for cell infiltration. Last but not least, portability of the device will be an added advantage. Transfer of the device to any location, like the operating theatre, should be convenient.

Given these criteria, a new process is conceived, designed and developed. Microfiber melt drawing is the name given to a novel microfiber fabrication process developed at Nanyang Technological University. This process is intended to create a bundle of unfused microfiber scaffold for tendon tissue engineering. At the very initial stage, it is implemented manually to generate porous microfiber scaffolds. To overcome the problem of a lack of control, it is later further developed into a benchtop device with a

degree of automation. Effectiveness, simplicity and economy are key features of this technique.

The rationale behind microfiber melt drawing is that polymer with primarily a linear chain structure and little side kinks can be easily stretched into a thin fiber once it becomes a melt, which is a fluid. Flow of molecules within the polymer allows redistribution of the material, resulting in the change in its original shape. Therefore, the fiber in essence is a shape, and microfiber melt drawing is to redistribute the polymer material and change its shape to a thin fiber.

3.2 Concept Development

3.2.1 Discovery

Microfiber melt drawing was initially discovered by a manual approach. The manual method is shown in Figure 3.1. Briefly, a few grams of polycaprolactone (PCL) powder, which is a biocompatible and biodegradable polymer with a melting point around 60 °C, were put at the bottom of a glass beaker, followed by pouring boiled water to the half of the breaker. Upon contact with heat, the PCL powder melted, with some air bubbles caught inside. A glass rod was used to anchor the melt in hot water, while another one was poked into the melt, picked up and pulled to a distance. Solidification occurred at the same time as the continuous elongation and thinning of the melt. The distance being pulled was as long as 4-5 meters. The middle portion of the pulled fiber was light, silk-like and hardly visible when it was hanging in the air. A piece of the long fiber (about 1 cm) was then further stretched by hand from one end until breakage occurred. The broken ends of the fibers spontaneously coiled up

and appeared cotton-like. Fibers on the right hand were collected. After repeating the process many times, a three dimensional porous microfiber scaffold could be obtained.

Figure 3.1: Schematic drawing of the manual method.

When examined by scanning electron microscope, it is shown that the hand-made fibers had a variety of fiber diameters, ranging from several micrometers to a few tens of micrometers (Figure 3.2). For some fibers, the surface was wrinkled as a result of the sudden breakage, but others still remained smooth. The morphology of the fibers was diverse, including cylinders, grooved cylinders, flat ribbons and other irregular shapes. The pores formed by the network of fibers were not closed but open. This open-porous structure is desirable for nutrient diffusion and cell infiltration. However, the lack of control turns out to be a major problem in this simple approach.

Figure 3.2: SEM images of a three dimensional porous microfiber scaffold.

Though the manual method is able to create porous microfiber scaffolds, the end result, being a cotton-like structure, deviates from the requirement of this project – a bundle of unfused microfibers. Also, when pulled, the microfiber maintains a single thread and is too thin to be easily seen by naked eyes, resulting in difficulty in collection. Additionally, the process is extremely tedious as numerous breakages need to be created by hand. Therefore, it is necessary to automate this process to eliminate labor intensive works and ensure the consistency of results.

3.2.2 Wheel-stretch

Wheel-stretch is the first idea for automating this process. In this concept, two identical wheels (Figure 3.3a) are positioned face to face and perpendicular to each

other (Figure 3.3 b). Each has equally-spaced needles mounted on the circular edge. When these two wheels are synchronized to rotate around their axes, the needles on the two wheels follow the synchronized rotation. A needle on one wheel is expected to meet its counterpart on the other wheel when both needles reach the bottom. As the two needles rotate to the top, the longest distance between them is reached. The circular motion of these two synchronized needles can result in a continuous increase in the distance between them. Given an initial mass shared by these two needles when they are at the bottom, this shared material can be stretched into a single microfiber.

Figure 3.3: Schematic of wheel-stretch.

3.2.3 Needle array-swing

This concept involves an array of needles (Figure 3.4 a). This needle array is mounted on a rotating platform and has a semi-circular motion track (Figure 3.4 b). When it is swung from the left to the right, fibers are expected to be drawn out of the melt pool and brought onto a square plate for collection.

Figure 3.4: Schematic of needle array-drawing.

3.2.4 Microfiber melt drawing

In this concept, a semi-automated step is introduced in the collection of the microfiber after it is drawn manually from the melt. As shown in Figure 3.5, a cylindrical cup with an orifice at the bottom is used to hold polymer melt. To initiate the process, a needle is inserted into the melt through the orifice breaking the melt surface, and then

pulled the melt down, creating a single strand of microfiber. The drawn microfiber can be wound onto a rotating mandrel to continue the collection.

Figure 3.5: Schematic of microfiber melt drawing.

3.2.5 Feasibility

One of the aims in this project is to design and develop a novel apparatus to fabricate a bundle of biodegradable microfibers. Of the three concepts, wheel-stretch, needle array-swing and microfiber melt drawing, their feasibilities are summarized in Table 3.1.

Table 3.1: Comparison of three concepts for automation.

Concept	Testing Phase	Result
Wheel-stretch	Radial slots were made at the edge of each acrylic wheel and acupuncture needles were inserted and glued to the slots. Two wheels were synchronized through a bevel gear.	At the bottom, two needle tips occasionally met each other, but not always, because of the challenges in assembling two identical needle-mounted wheels. Thus microfiber can be created but the challenge of getting the needles to meet at the same time is high.
Needle array-swing	Pattern of needles was achieved by placing the needles on a mold. A needle array was created by gluing a small acrylic plate to the patterned needles.	After one round of swing, melt firmly adhered to the tips of needles and proved difficult to remove. In subsequent swings, the tips grew bigger and bigger, resulting in inconsistency and, in some instance, merger of adjacent melt strands. Multiple strands of microfibers can be created but succession is a serious problem.
Microfiber melt drawing	Orifice was manually drilled on a small aluminum cup and a motor shaft was used as the rotating collector.	Microfiber was successfully drawn from the orifice and wound onto the shaft easily.

In wheel-stretch and needle array-swing, serious problems as described in Table 3.1 have been found, albeit microfiber could be generated. These problems cause a great difficulty to obtain a bundle of microfibers. Therefore, melt microfiber melt drawing is selected as the best option among the three to ensure continuous production of microfibers and consistent results.

3.3 Modeling the Microfiber Melt Drawing Process

To have a better understanding of microfiber melt drawing, it is necessary to develop a mathematical model to describe the process so that the critical control parameters can be identified. A schematic illustration of the microfiber melt drawing process is shown in Figure 3.6 and important parameters characterizing the process are defined as shown.

Figure 3.6: A schematic drawing of the microfiber melt drawing process.

3.3.1 Conservation of mass

Assuming a steady state, the process is characterized by applying the conservation of mass concept, that is, the amount of the material passing through the orifice plane is the same as that passing through the take-up plane. Orifice plane is where the melt contacts with the inner bottom of the melt holder. Take-up plane is where the microfiber is tangential to the cylindrical collector. Hence the mass flow rate is:

$$\dot{m} = \rho \cdot \frac{\pi}{4} d^2 \cdot \bar{v} = \rho \cdot \frac{\pi}{4} \phi^2 \cdot V \quad (1)$$

where $\dot{m}$ is mass flow rate, ρ is density, d is the orifice diameter, $\bar{v}$ is average velocity at the orifice plane, ϕ is final fiber diameter and V is take-up velocity of the

spun fiber. The fiber is assumed to have a circular cross-section and the density of the fiber is assumed to be unchanged.

From Equation (1), it can be seen that given the average velocity $\bar{v}$, take-up speed V , and orifice diameter d , the final fiber diameter can be simply calculated by

$$\varphi = \sqrt{\frac{\bar{v}}{V}} \cdot d \quad (2)$$

Take-up velocity V and orifice diameter d are controllable parameters that can be set at a defined value, but $\bar{v}$ is an unknown value. To work out $\bar{v}$, additional equations are needed.

3.3.2 Conservation of energy

It is known that when applied to the situation of an object free falling in air, the conservation of energy is described as gravitational potential energy being converted to kinetic energy and friction loss, or Equation (3).

$$-E_f + mgh = \frac{1}{2}mv^2 \quad (3)$$

where E_f with a minus sign represents friction loss, m is mass, g is gravitational constant, h is height and v is the velocity of the falling object. The air temperature is assumed to be constant and no change in temperature is expected.

By considering the change in potential energy of the polymer melt to be similar to a free fall of the melt within the flow path of the polymer melt, an equation similar to Equation (3) can be written (see Equation 4).

$$-\sigma + \rho gh = \frac{1}{2} \rho v^2 \quad (4)$$

where σ represents shear stress, ρ is material density, g is gravitational constant, h is melt height, and v is velocity when the melt exit the orifice. Although stress is a tensor, in this model, it is simplified to a vector with a magnitude and a downward direction. The energy loss is represented by shear stress. The energy loss is due to frictions between polymer chains as they are sliding along each other. The melt temperature is assumed to be constant and therefore the change in thermal energy is not considered in the equation. The volume term is found in all the terms of the equation and therefore is cancelled on both sides of the equation.

It can be seen that given density ρ , gravitational constant g , and melt height h , the velocity v can be determined by the shear stress σ . However σ is related to viscosity and strain rate, thus it is necessary to develop the equations relating these terms by examining the flow of the polymer melt.

3.3.3 Constitutive equation

Polymer melt is generally considered as a non-Newtonian fluid when the melt is subjected to a high strain rate, whereas at low strain rate, melt viscosity is independent of the strain rate [185]. Before the drawing process is initiated, bulging of the melt drop at the orifice is observed to be slow due to a relatively low processing temperature, a small orifice diameter and a high viscosity of the melt. This slow protrusion suggests a very low strain rate in the melt, however, measurement of this strain rate can be challenging, because it is difficult to accurately define the original length of the melt and accurately measure the change of the melt. Based on this

observation and for simplicity, it is assumed that the melt viscosity is independent of strain rate and the melt behaves like a Newtonian fluid. Hence the stress can be expressed by Newton's law of viscosity [186]:

$$\sigma = \eta \frac{dv}{dx} \quad (5)$$

where σ is shear stress, η is shear viscosity and dv/dx is velocity gradient of shear flow.

However, directly substituting Equation (5) into the shear stress term in Equation (4) is inappropriate, because when the drawing process is initiated, two types of flow are present: shear flow and elongational flow (see Figure 3.7). Shear flow occurs when velocity gradient is perpendicular to the flow direction, while elongational flow occurs when velocity gradient is along the flow direction. The flow above the orifice plane is essentially shear flow, because the velocity increases radially from the inner wall of the ring holder to the center, which is perpendicular to the downward flow direction. In comparison, the flow below the orifice plane is elongational flow, because the velocity increases longitudinally from the orifice plane to the take-up plane, which is along the flow direction. How this elongational flow affects Equation (5) is not yet known.

Figure 3.7: Velocity profiles.

However, in this process, shear flow is continuous with elongational flow, meaning that is a transition between them. Trouton's ratio states that elongational viscosity is three times of shear viscosity for non-Newtonian fluids [187]. Conforming to this, the viscosity in the transition should gradually increase three-fold. In other words, the velocity profile of shear flow should become flattened during the transition. Due to the presence of elongational flow, the stress at the orifice plane is modified as:

$$\sigma = 3\eta \frac{dv}{dx} \quad (6)$$

By substituting Equation (6) into Equation (4), it becomes

$$\rho gh = \frac{1}{2} \rho v^2 + 3\eta \frac{dv}{dx} \quad (7)$$

Solving Equation (7)

$$v = \frac{(e^{\frac{\rho x}{3\eta} \sqrt{2gh}} - 1)}{e^{\frac{\rho x}{3\eta} \sqrt{2gh}} + 1} \sqrt{2gh} = \sqrt{2gh} \tanh\left(\frac{\rho x}{6\eta} \sqrt{2gh}\right) \quad (8)$$

$$\text{when } x = 0, \quad v_{\min} = 0$$

$$\text{when } x = d/2, \quad v_{\max} = \sqrt{2gh} \tanh\left(\frac{\rho d}{12\eta} \sqrt{2gh}\right) \quad (9)$$

From Equation (9), it seems that velocity is zero at the contact of the melt with the ring holder, and reaches its maximum value at the center of the orifice. To simplify the calculation of the average velocity at the orifice plane, the average of the minimum and maximum velocity is used,

$$\bar{v} = \frac{0 + v_{\max}}{2} = \frac{\sqrt{2gh}}{2} \tanh\left(\frac{\rho d}{12\eta} \sqrt{2gh}\right) \quad (10)$$

By substituting Equation (10) into Equation (2), the final fiber diameter is

$$\phi = \sqrt{\frac{\bar{v}}{V}} = \left(\frac{\sqrt{2gh}}{2V} \tanh\left(\frac{\rho d}{12\eta} \sqrt{2gh}\right) \right)^{\frac{1}{2}} \quad (11)$$

Up to now, the melt is treated as Newtonian fluid based on the assumption of a small strain rate, that is, the melt viscosity at the orifice is independent of strain rate. However, the melt viscosity at the orifice is not independent of temperature. A higher temperature usually lowers melt viscosity. Therefore it is necessary to look at the equation that describes the relationship of melt temperature and melt viscosity.

3.3.4 Williams–Landel–Ferry equation

Williams–Landel–Ferry (WLF) equation is an empirical formula that describes the relationship of temperature and melt viscosity [188], and a complete curve can be plotted by knowing only a single point on the graph. In this model, the WLF equation is used to relate temperature and shear viscosity.

$$\eta = A \cdot \exp\left(\frac{C_1(T - T_r)}{C_2 + T - T_r}\right) \quad (12)$$

where η is shear viscosity, T is melt temperature, T_r is reference temperature, A , C_1 , and C_2 are constants. $C_1 = 17.4$ and $C_2 = 51.6$ if glass transition temperature is used as the reference temperature [188].

By combining Equations (11) and (12), the final fiber diameter can be mathematically expressed as:

$$\varphi = \left(\frac{\sqrt{2gh}}{2V} \tanh \left(\frac{\rho d \sqrt{2gh}}{12A} \cdot \exp \left(\frac{-C_1(T - T_r)}{C_2 + T - T_r} \right) \right) \right)^{\frac{1}{2}} \quad (13)$$

In summary, the conservation of mass, conservation of energy and WLF equation are used to develop a mathematical model for the steady state of the microfiber melt drawing process. By observing the protrusion of a melt at the orifice plane is slow, the model assumes that the viscosity of the melt at the orifice is strain-independent but temperature-dependent. Also, conforming to Trouton's ratio, the model assumes that the presence of elongational flow affects the melt viscosity at the orifice by a three-fold increase. Therefore, the final fiber diameter can be expressed in terms of controllable process parameters including orifice size d , temperature T , and take-up velocity V .

3.4 Design and Prototyping of the Microfiber Melt Drawing Device

After developing a mathematical model to understand the microfiber melt drawing process, a device prototype is designed and built accordingly. In this prototype, five subsystems are designed: the melt holder, the positioning and motion system, the heating system, the collecting system and the framework. Each subsystem is modular and can be developed and improved independently. Moreover, the size has been kept

compact and the complexity low in this prototype, making it handily portable and straight forward to use.

3.4.1 System design

Figure 3.8 shows the physical setup of the microfiber melt drawing device.

Figure 3.8: The microfiber melt drawing device.

3.4.1.1 Melt holder

The holder, made of aluminum, is in the form of a cylindrical cup, and an orifice of 2 mm diameter (or other diameters) is drilled at the center of the bottom of the holder.

The holder is fastened by an appropriate band heater, and the combination is adapted to a Z-axis adjustable stage (Z-stage).

3.4.1.2 Positioning and motion system

The melt holder has three degrees of freedom. Motion along X-axis is done by a slide rail to which the Z-stage attaches. Positioning on Y-axis is achieved by an alignment adjuster which is connected to the slide and moves along the slots of the top plate. Positioning on Z-axis is done by the Z-stage on which the melt holder, band heater and the adapter are fixed.

3.4.1.3 Heating system

The heating system includes a voltage controller, a band heater, a temperature indicator, a thermal couple, and an adapter covered with an insulating board for heat protection. The voltage controller controls the output of the band heater, or the input to the melt holder, and the temperature indicator, placed on the top plate, monitors the real-time temperature of the melt in the holder.

3.4.1.4 Collecting system

The collecting system includes a speed controller, a motor and a mandrel. The mandrel is fixed directly to the shaft of the motor. The front face of the mandrel is tangential to the vertical projection of the orifice. The form of mandrel is versatile and can be changed accordingly. It can be a cylinder, a U-fork, or a circular array of prongs.

3.4.1.5 Framework

The framework consists of four rectangular plates, which form the top, the front, the rear and the base of the device. The base is about twice as long as the top, when viewed in the front. The top plate, to the left of the base, supports the overhang of the

X-axis slide rail by the Y-axis alignment adjuster. An AC motor is mounted and locked to the base and encased by the front, top and rear plates. A speed controller is embedded in the front plate and protrudes to the space above the motor for compactness.

3.4.2 List of components

A detailed list of components used in the design of the microfiber melt drawing device is summarized in Table 3.2. The components are either purchased or fabricated as indicated in the remark column. The total cost for the purchased items is less than 1500 SGD, and the fabricated items are machined at low cost using either aluminum or acrylics. Hence, the device is comparatively inexpensive.

Table 3.2: List of components for the microfiber melt drawing device.

System	Components	Remarks
The melt holder	Melt holder	Fabricated
The positioning and motion system	X-axis: slide rail	MISUMI Slide Guide JKSG24-160
	Y-axis: alignment adjuster	Fabricated
	Z-axis: height adjuster	MISUMI Z-Axis Stage ZLWG100
The heating system	Voltage controller	Variact Variable Transformer, Output 0-250 VAC, Shanghai, China
	Band heater	MISUMI Band Heater MBHS40
	Adapter	Fabricated
	Insulator	MISUMI Heat Insulating Plates HIPLA100-100-3
	Temperature indicator	OMRON, K3MA-L Meter, Temperature 100-240 V
The collecting system	Motor	OrientalMotor 2IK6GN-SW2 AC Induction Motor, 6W Output, Three-Phase 200/220/230 VAC
	Mounting bracket	OrientalMotor SOL2M4
	Speed controller	OrientalMotor FE100C, Inverter, FE series, Max 100W Output, Single-Phase 200-240 VAC
	Connection cable	OrientalMotor CC01AC04
	Acrylic mandrel	Fabricated
The framework	Top plate 160mmx100mmx15mm	Fabricated
	Side plates (2) 300mmx100mmx15mm	Fabricated
	Bottom plate 250mmx160mmx20mm	Fabricated
	Black paper board	Fabricated

3.4.3 Drawings of fabricated parts

For the fabricated parts, detailed drawings are shown in Figure 3.9 –3.16. Except for the cylindrical acrylic mandrel, all the others are made of aluminum. The reason for choosing acrylic for the mandrel is that, the mandrel is fixed to the motor shaft at one end and subject to a rotation up to 2000 rpm. Using a material of a lower density

($\rho_{\text{acrylic}} = 1.2 \text{ g/cm}^3$, $\rho_{\text{aluminum}} = 2.7 \text{ g/cm}^3$) can help reduce wobbling and maintain motor performance.

When the band heater is tightened, the outer surface of the melt holder must be in contact with the inner surface of the band heater for heat transfer, so the size of the melt holder is constrained by the size of the band heater used. The diameter of the melt holder is constrained by the size of the band heater used. The diameter of the orifice machined in the melt holder can be between 2 mm and 4 mm. Figure 3.9 shows a melt holder that has an orifice diameter of 2 mm.

Figure 3.9: The melt holder. Dimension in mm.

The alignment adjuster (Figure 3.10) consists of a thin aluminum plate and 3 socket screws. The center screw in the middle is loosened whenever the alignment of the slide rail requires adjustment. It is used to align the slide rail along the top plate so that the melt holder's orifice can accommodate different mandrel diameter.

Figure 3.11 shows the adapter that is fixed to the Z-stage through the four screws on its back. The band heater can be mounted and adjusted by means of two screws on its sides.

Figure 3.10: Y-axis alignment adjuster. Dimension in mm.

Figure 3.11: Adapter to Z-stage. Dimension in mm.

Figure 3.12: Acrylic mandrel. Dimension in mm.

Figure 3.13: The top plate. Dimension in mm.

Figure 3.14: The base plate. Dimension in mm.

Figure 3.15: The front plate. Dimension in mm.

Figure 3.16: The rear plate. Dimension in mm.

3.4.4 Name of the device

This device is developed based on the concept of microfiber melt drawing and named accordingly. Conventionally, this technique may be classified under the family of melt spinning, simply because it creates fibers from melt. However, it has three features significantly different from other family members such as micro melt spinning, direct melt spinning and electro melt spinning. First, the process is initiated by drawing a fiber from melt while other processes start by forced extrusion. Secondly, unlike other processes using a die with micro-sized nozzles, this setup is die-free, with an orifice being macro-sized (2 mm). Thirdly, the size of the setup is even much smaller than a miniature of melt spinning - micro melt spinning. In fact, it

is a benchtop device and is easily portable. Fourthly, melt drawing usually refers to a process of stretching melted materials, and a typical scenario is that a piece of fiber being held at both ends is stretched at constant speed while the material is being heated. However, melt drawing also refers to a process of creating fibers. A typical scenario is that a needle is dipped into melt, breaking the surface, and lifted into air generating a fiber. As both melt drawing and melt spinning have the common meaning of creating fibers from melt, to emphasize drawing-initiation and small size, it is decided that microfiber melt drawing is the name of this new technique. This is in line with the naming of other drawing methods, such as plastic drawing and optical fiber drawing. Plastic drawing or cold drawing is a post-spinning process that draws solid fibers into high strength fibers. Optical fiber drawing is a process to fabricate optical fibers by feeding a rod pre-form into a furnace and drawing a fiber from the molten part of the pre-form. Finally, melt spinning implies the use of a spinning die, but melt drawing does not have this implication. Since the name of micro melt spinning has been used by a minimized conventional melt spinning system, to avoid unnecessary ambiguity, microfiber melt drawing is selected as the name of this device.

3.5 Validation of Fabrication of Microfibers

Polycaprolactone microfiber bundles were fabricated at orifice diameters of 2 and 3 mm, at melt temperatures of 100, 110, 120, 130, 140 and 150 °C, and at take-up velocities of 250, 500, 750, 1000, 1250 and 1500 rpm. The melt height was kept around 10 mm. The mandrel used had a diameter of 20 mm. For each melt temperature and rpm setting, at least six fiber bundles were fabricated, and each fiber bundle was continuously drawn for exactly one minute.

According to the model, it is possible to spin 1 denier microfibers if the orifice diameter is at 2 mm. However it is not possible to do so if the orifice diameter is beyond 2 mm. Therefore 3 mm was selected as a representative value to compare with 2 mm to show the effect of orifice diameter.

Polycaprolactone has a melt point of about 60 °C and a decomposition temperature of about 200 °C. To avoid thermal degradation, temperatures should be selected away from the decomposition temperature. A second consideration is viscosity. At higher temperatures, the melt is too viscous and breakage can happen due to capillary effect. At lower temperatures, the viscosity is too high and breakage can happen as a result of stress. The processing temperatures for polycaprolactone must be in a range that gives suitable viscosities. Therefore a series of representative temperatures ranging from 100 °C to 150 °C was selected to study the effect of temperature.

According to the model, 1 denier microfibers can be obtained only at certain take-up velocities. Take-up velocity equals to the product of rotational velocity and mandrel diameter. As the range of operation for the motor is up to 2000 rpm, the mandrel diameter has to be designed accordingly. Representative rotational velocities at an interval of 250 rpm were selected to study the effect of take-up velocity.

3.5.1 Mass flow rate

Figure 3.17 shows theoretical and experimental mass flow rates. Theoretical flow rates were determined from the mathematical model described in Section 3.3. Experimental flow rates were determined by the weight of the microfiber samples. As the fabrication time of each sample is one minute, the weight of the sample is measured as mg/min, which is the experimental flow rate.

The model predicts that for a larger orifice diameter (3 mm), the flow rate is much higher, and the effect of melt height is larger as indicated by a wider region between the two theoretical lines ($h = 9$ mm and $h = 11$ mm). This suggests that a smaller orifice diameter should be used in fabrication as for which the flow rate remains stable and varies less during fabrication.

Figure 3.17 shows that at 3 mm orifice diameter, the overall experimental flow rates trend with the theoretical lines. At 2 mm orifice diameter, the experimental flow rates agree well with the predicted range. In experiment, the production of a large number of samples resulted in a rapid loss of the melt in the holder, especially when a large orifice diameter was used. The larger the orifice, the faster the melt is consumed. In order to maintain a constant melt height, adding new polymer into the melt holder is required during the fabrication process and this is frequent in the case of using a larger orifice diameter. In fact, both fabrication of microfibers and fueling the melt holder causes a change of melt height, and more frequent changes occur when a larger orifice diameter is used. Therefore the fueling operation itself may affect the experimental flow rate. Moreover, the added new polymer is solid and takes time to melt, which may also have an influence.

Figure 3.17: A comparison of theoretical and experimental mass flow rates. (d: orifice diameter; h: melt height)

3.5.2 Measurement of microfiber diameter

To measure the diameter of microfibers, two methods can be considered. One is directly reading fiber diameter from scanning electron microscope (SEM) images of microfibers, and the other is an indirect method by computing the linear density based on the weight of the microfibers. Shown in Figure 3.18 is a comparison of fiber diameters obtained by these two methods. It can be seen that both methods deviate little from each other, showing that they are of equal effectiveness and accuracy in measuring fiber diameter. The samples in Figure 3.18 were fabricated under different conditions. The samples were first measured and then sorted based on their fiber diameters.

Figure 3.18: A comparison of SEM method and weight method.

However, if the total number of samples is large, the SEM method can be tedious. Thus a better choice is to use the second method, which measures fiber diameter by weight. In this method, the linear density (an equivalent to fiber diameter) can be obtained by the weight of a sample over its length. If denier, i.e. 1 gram per 9000 meters, is used as the unit of linear density, the following expression is obtained:

$$\text{Linear Density} = 9000 \cdot \frac{W}{N \cdot L} = 9000 \cdot \frac{N \cdot \rho \cdot \frac{\pi}{4} \varphi^2 \cdot L}{N \cdot L} = 2250 \rho \pi \varphi^2 \quad (14)$$

where W is the weight of the fibers, N is the number of microfibers (or the rpm value), ρ is density, φ is fiber diameter and L is the length of fibers (or the circumference of the mandrel). Here what appeared to be a microfiber bundle is actually a single thread wound onto the mandrel many times. There are many rounds on the mandrel. Hence N represents the number of rounds, or the number of microfibers, which is equivalent to the rpm value. From Table 3.3, it can be observed that the equivalent linear density to a fiber diameter of 10 μm is 0.8 denier.

Table 3.3: Comparison between linear density and fiber diameter.

Linear density (denier)	Diameter (μm)	Linear density (denier)	Diameter (μm)
0.1	3.6	2	16.0
0.2	5.1	2.1	16.4
0.3	6.2	2.2	16.8
0.4	7.2	2.3	17.2
0.5	8.0	2.4	17.6
0.6	8.8	2.5	17.9
0.7	9.5	2.6	18.3
0.8	10.1	2.7	18.6
0.9	10.8	2.8	19.0
1	11.3	2.9	19.3
1.1	11.9	3	19.6
1.2	12.4	3.1	20.0
1.3	12.9	3.2	20.3
1.4	13.4	3.3	20.6
1.5	13.9	3.4	20.9
1.6	14.3	3.5	21.2
1.7	14.8	3.6	21.5
1.8	15.2	3.7	21.8
1.9	15.6	3.8	22.1

3.5.3 Linear density map

Figure 3.19 shows the theoretical linear density map. At each temperature, it is clear that linear density decreases with increasing take-up speed, in accordance with the conservation of mass. For the 3 mm orifice diameter, no fibers were found below one denier, while for the 2 mm orifice diameter, a fraction of the curves stays below the line of one denier. Therefore, based on the model, it is predicted that microfibers of fiber diameter of 10 μm can be achieved for 2 mm orifice diameter.

Figure 3.19: Theoretical linear density map. $h = 10$ mm.

Figure 3.20 shows the experimental linear density map. It agrees well with the model except for that under certain extreme conditions, especially at a lower temperature and a higher take-up speed, the data points are missing because microfibers cannot be continuously fabricated for one minute. However, for 2 mm orifice diameter, a fraction of the curves still fall below one denier, indicating the feasibility of fabricating microfibers of $10\text{ }\mu\text{m}$.

Figure 3.20: Experimental linear density map.

3.5.4 Temperature speed map

Temperature-speed map is a useful reference to identify the conditions to spin microfibers for a desired diameter. In Table 3.4, two regions are clearly shown: the unspinnable region (marked by x) and the spinnable region (marked by linear density). In the spinnable region, the enclosed area contains the conditions at which 10 μm microfibers can be fabricated. However, conditions at the upper left corner are not recommended, because the production rate is comparatively low. Nor recommended is the conditions at the lower right corner, in which temperature is too high to allow the cooling of the fiber in air, as a result, fusion of fibers may occur. Therefore, the

conditions of 120 °C – 140 °C and 750 rpm - 1500 rpm are recommended to spin microfibers, with a preference of 130 °C and 800 - 1000 rpm. Note that the values of the rotational speeds indicated are for a mandrel of 20 mm diameter. For a mandrel with a larger diameter, the correspondent rpm value will be proportionally lower.

Table 3.4: Temperature-speed map (unit: denier).

	100 °C	110 °C	120 °C	130 °C	140 °C	150 °C
250 rpm	1.7	1.7	2.1	2.3	2.7	3.0
500 rpm	0.9	1.0	1.1	1.2	1.4	1.4
750 rpm	x	0.7	0.8	0.8	0.9	1.0
1000 rpm	x	x	0.6	0.6	0.7	0.8
1250 rpm	x	x	x	0.5	0.6	0.6
1500 rpm	x	x	x	x	0.5	0.6

3.6 Comparison with Existing Methods

The microfiber melt drawing device easily and simply produces microfibers with controllable diameter. The process is fast and cheap. Table 3.5 shows a comparison of different fiber generation methods. The effectiveness, simplicity and economy of this method can be readily seen by comparing their size, cost, forms of fibers and production rate.

Table 3.5: Comparison with existing methods.

	Microfiber melt drawing	Electrospinning	Melt-spinning
Set-up		Source: http://www.mecc.co.jp	Source: http://www.fourne-polymertechnik.de
Size	30 cm x 30 cm x 30 cm	63 cm x 83 cm x 88 cm	300 cm x 300 cm x 300 cm (Estimated)
Cost	USD 1,000 (Estimated)	USD 44,000 (Quoted from MECC, Japan)	High
Forms of fibers	Unfused parallel microfiber bundles	Fused non-woven web	Monofilament
Range of diameter	8 - 50 μm	0.01-20 μm	10 -150 μm
Production rate	Moderate	Low	High

3.7 Conclusion

Among three concepts, microfiber melt drawing has been found to be suitable for generating microfibers required in this project, while the other two, wheel-stretch and needle array drawing, are not suitable. Based on this concept, a novel mathematical model has been developed to understand and describe the process and a device

prototype has been conceived, designed and built. The model predicts that a linear density below 0.8 denier is achievable when 2 mm orifice diameter is used. Experiments have shown that a bundle of microfibers of 10 μm has been achievable within a range of temperatures and take-up speeds, enabling further experiments to be carried out. Characterization and biological evaluation of these microfibers will be discussed in later chapters.

CHAPTER 4 SURFACE TENSION MELT

4.1 Introduction

After tendon repair, adhesion formation always remains to be a risk and physical barriers are usually applied along with the repair as a measure to prevent adhesion of the repaired tendon to the surrounding tissue. Traditional barriers such as PTFE and e-PTFE are non-degradable and after use, they remain in the body permanently. Barriers made of natural materials such as hyaluronic acids can be used for adhesion prevention, but the major drawback is that they are degraded within a month and thus unable to perform the separating function over the entire period of tendon healing. In this project, a slow degrading biopolymer, polycaprolactone, is selected for long-term tendon adhesion prevention. Polycaprolactone is a proven biocompatible and biodegradable synthetic polymer, and its degradation time *in vivo* could last for one year, matching up with the time of tendon healing.

However, before use the material needs to be turned into a sheet form and the sheet should be porous. Generally, the first idea to fabricate such a porous membrane is by solution casting or solvent casting. In this project, it requires organic solvents or toxic materials to be avoided in the fabrication process. Besides, most of solution casted membranes are dense films without pores. Another requirement is that the pore size on the barrier must be suitable, that is, below 20 μm or the size of a tendon cell, as membranes with larger pores the barrier may not be able to act as a barrier to separate tissues.

To avoid the use of solvents, mechanical force may be used to stretch a material from a flat sheet to a single layer (Section 2.6). However, creation of pores on the layer cannot be simultaneously realized. An alternative approach is to take the advantage of some natural phenomena. It is known that accumulation of numerous particles on a liquid surface can create a single material layer with pores formed by the spaces among the particles. One example is duckweeds, the little flowering plants growing in a pond. These little particles cluster to cover the water surface, and a full coverage resembles a porous membrane when viewed from a distance. Another example is the accumulation of dust on the surface of still water. It takes some time for this to be noticeable though, the eventual outcome on the water surface is a thin film consisting of countless dust particles which resembles a porous membrane. In fact, the phenomenon of particles forming a layer is common in nature, and the natural existence of this type of membrane suggests an alternative perspective on the process development of membrane fabrication.

The examples present in nature usually involve particles and water surface, but what matters most is whether a sufficient number of particles would be able to cover the water surface and become a whole by fusing together. Making use of this idea, a new concept for fabricating a porous membrane is thus proposed. As illustrated in Figure 4.1, a layer of micro-particles can be considered as a set of two dimensional points and a porous membrane as a plane. The conversion between a layer of micro-particles and a porous membrane can be directly achieved by heating these micro-particles to fuse together. The spaces among micro-particles will naturally form pores on the membrane.

The PCL powder comprises fine, light and hydrophobic particles, which naturally float on water surface, forming a layer of micro-particles. When a layer of such micro-particles is melted and the particles joined together, a porous membrane is thus formed macroscopically.

Figure 4.1: Conversion from a layer of micro-particles to a porous membrane.

4.2 Process Design and Validation

To implement the concept of a porous membrane can be created on water surface by heating a layer of particles, it is necessary to break it down into three practical steps (see Figure 4.2): first, formation of a two dimensional layer of micro-particles that are in close contact with each other; secondly, melting of these micro-particles into a layer of polymer melt; and thirdly, cooling the melt to allow the emergence of a porous membrane.

Figure 4.2: Schematic of process design.

4.2.1 Step 1: Preparation of a layer of particles on water surface

Figure 4.3 shows the process of preparing a layer of PCL particles. PCL powder was placed in a weighing boat and dispensing was done near the water surface by gently tapping the weighing boat. As the PCL powder particles are small, light and highly hydrophobic, after the dispersing process, about 0.1 grams of PCL powder floated on the water surface supported by surface tension and hydrophobic forces. The shape of the floated powder followed the geometry of the glass dish. Next, the glass dish was shaken in a circular manner and a wave sweeping anti-clockwise could be seen disaggregating the PCL particles and distributing them evenly across the water surface. Finally, the water surface was gently blown with a rubber bulb to remove any excess powder that remained on the top of the particle layer, or sometimes, certain large aggregates that had not been broken down by further shaking.

Figure 4.3: Preparation of a layer of particles on water surface.

0.1 gram of powder was used because any amount less than that cannot cover the glass dish and quantity more than that will lead to difficulty to achieve a single layer of micro-particles of even thickness. Figure 4.4 shows five glass dishes, each filled with 30 ml de-ionized water and loaded with PCL powder at, from right to left, 0.03 g, 0.06 g, 0.09 g, 0.12 g and 0.15 g, respectively. It is shown that for a glass dish with 10 cm diameter, 0.09 g of powder was just sufficient to cover the whole water surface. 0.03 g of powder resulted in fragmentation and incomplete coverage, while 0.15 g of powder resulted in a lot of waste. Glass dishes loaded with 0.09 and 0.12 g powder showed relatively good coverage without breakage in the surface or too much waste. Therefore, 0.1 g of PCL powder was recommended as a suitable quantity for fabrication.

Figure 4.4: Optimization of powder usage.

4.2.2 Step 2: Heating a layer of particles into a layer of melt

Following the success of creating a layer of micro-particles, the glass dish was put inside the bottom of a laboratory oven (BINDER, Inc.) and in full contact with a steel plate for heating. The temperature was set at 80 °C and the heating time was 20 minutes. Temperature and heating time have an effect on the resulted membrane, but this will be discussed later. In Figure 4.5a, it is shown that before heating, the layer of PCL particles appeared translucent, and during heating, micro-particles underwent phase change, from a semi-crystalline state to an amorphous state, and this phase change can be reflected by the transparency of the layer. A piece of paper with a written script was placed below the glass dishes to show the difference in transparency. Figure 4.5b shows a layer of PCL melt, which is transparent.

Figure 4.5: Heating a layer of particles into a layer of melt.

4.2.3 Step 3: Cooling a layer of melt into a membrane

The heated glass dish was cooled at room temperature, and it usually takes about 15 minutes for the melt to solidify. Figure 4.6 shows the appearance of the melt layer

after cooling for 5 minutes and 10 minutes. Once removed from the oven, the layer of melt gradually became white and translucent and eventually a firm membrane. A few wrinkles appeared on the membrane due to the non-uniform residual stresses generated in the crystallization process [189], that is, some parts of the membrane contracted more than the others, resulting in mild topographical distortion of the membrane.

Figure 4.6: Cooling a layer of melt into a membrane.

The concept that a layer of micro-particles can be directly turned into a layer of membrane has been demonstrated. Figure 4.7 shows a comparison between a layer of particles and a membrane it forms. Figure 4.7a shows a layer of dispensed particles resting on the water surface. Figure 4.7c shows a microscopic view these particles. They have irregular shapes and the spaces between them form a porous network. Figure 4.7b shows a fabricated PCL membrane, which appears light, soft, translucent, smooth and fine in texture. The size of the membrane appears roughly the same as the glass dish most of the time, but it can be smaller if some part of the membrane happens to detach from the glass wall and shrink. Moreover, when placed on water,

this membrane does not sink, and if pressed downwards, water can be seen seeping through the pressed area, suggesting that there are pores on the membrane. Figure 4.7d shows a microscopic observation of a portion of the membrane. Micro-sized pores are found peppered over the entire surface of the membrane. It is obvious that the pores are a direct result of the spaces among PCL particles not closing completely during heating, otherwise a dense film would have been resulted if all the spaces were filled by melt. It is believed that, in the heating process, most of small spaces closed up and disappeared by the coalescence of adjacent melt, whereas some large ones, driven by surface tension of the melt, became bigger and rounder. In addition, these pores are observed to be randomly distributed on the membrane at sizes ranging from a few micrometers up to nearly one hundred micrometers. The size of a pore may be influenced by the number of particles that form it (see Figure 4.7c). For example, six or seven particles enclose a larger space than three particles, and a space formed as a result tends to become a larger pore. However, the kind of space that tends to be closed or tends to be open is not yet clearly understood.

Figure 4.7: Comparison of a layer of micro-particles and a membrane.

4.3 *Fabrication Controls*

4.3.1 **Effects of temperature and heating time**

In order to investigate the effects of temperature and heating time on the resulting membrane, temperature was set at 60, 80 and 100 °C, and the glass dishes were heated for 10, 20 and 30 minutes before visual examination. The values of these parameters are based on the fact that the melting point of PCL is around 60 °C and the boiling point of water is 100 °C at atmospheric conditions. Figure 4.8 shows the effects of temperature and heating time on the membrane. At 60 °C, there was no melting or fusion of particles over the observed 30 minutes period. At 80 °C and 10 minutes, melting of particles near the wall of the glass dish was observed, and the

layer of particles remained white and opaque. At 20 minutes, the layer of micro-particles turned translucent near the glass wall then going radially inward, and eventually a layer of melt. At 30 minutes, the membrane was found to have shrunk in size and attached to one side of the glass dish. At 100 °C, a membrane could be formed within 10 minutes, but the quality of this membrane was poor as there were many visible holes. Perhaps, this is because the viscosity of melt at a higher temperature is low, making melt flow easily to enlarge the hole. At 20 minutes, small bubbles could be noticed at the bottom of the glass dish, and the membrane had more visible holes and distorted in shape. At 30 minutes, the membrane was found attached to one side of the glass dish and has shrunk in size. From this experiment, it can be concluded that given the current setting, the conditions of 80 °C and 20 minutes are more preferable than others for fabricating membranes.

Figure 4.8: Effect of temperature and heating time on the formation of a membrane.

It is important to note that the amount of water largely determines the heating time. In the fabrication process, the total amount of heat supplied by the oven is transferred to work in several ways: raising the temperature of the glass dish, raising the temperature of water, vaporizing water, and melting PCL micro-particles. The specific heat capacity of water is $75.3 \text{ J} \cdot \text{mol}^{-1} \text{ K}^{-1}$ at $25 \text{ }^{\circ}\text{C}$ or $4.18 \text{ J} \cdot \text{g}^{-1} \text{ K}^{-1}$. The specific heat of glass is $0.84 \text{ J} \cdot \text{g}^{-1} \text{ K}^{-1}$ at $25 \text{ }^{\circ}\text{C}$. The specific heat of PCL is $161.6 \text{ J} \cdot \text{mol}^{-1} \text{ K}^{-1}$ at $25 \text{ }^{\circ}\text{C}$ [188], or $0.003 \text{ J} \cdot \text{g}^{-1} \text{ K}^{-1}$ based on an average molecular weight of $50,000 \text{ g/mol}$. This means that water and glass can absorb more heat than PCL. Moreover, the mass of PCL powder in the experiment is merely a small fraction (0.1%) of the total mass of water and the glass dish. Therefore, heat is mainly absorbed by the glass dish and water, and it is the heated water that melts the micro-

particles at its surface. The total heat can thus be estimated based on specific heat capacities of glass and water by Equation (15)

$$Q = cm\Delta T \quad (15)$$

where Q is heat, c is specific heat capacity, m is mass and ΔT is temperature difference

$$Q_{\text{total}} = Q_{\text{glass}} + Q_{\text{water}} \quad (16)$$

$$= 4.18 \frac{\text{J}}{\text{g}}^{\circ}\text{C} \times 30\text{g} \times (80^{\circ}\text{C} - 23^{\circ}\text{C}) + 0.84 \frac{\text{J}}{\text{g}}^{\circ}\text{C} \times 58\text{g} \times (80^{\circ}\text{C} - 23^{\circ}\text{C}) = 9924.84 \text{ J}$$

Melting of micro-particles requires water temperature to reach to the melting point of PCL at first. The heating time, which starts from placing the glass dish in the oven to forming a layer of melt, depends on not only the oven temperature but also the amount of water in the glass dish. A higher volume of water and a lower temperature will require a longer time to melt micro-particles. The heating time required is 20 minutes in the experiments. Using equation (17), the power consumed by the glass dish and water was estimated to be about 8.3 watts.

$$P = Q/t \quad (17)$$

where P is power, Q is total heat and t is time.

4.3.2 Edge effect

When the particles melt in the oven, melt surface tension and gravity begin to drive the melt layer to contract and shrink on the water surface so that its surface energy can be minimized. PCL is an adhesive material, which when melted is able to adhere to glass. If the edge of the melt layer firmly attaches to the glass wall, the melt layer does not shrink in size, as the attachment resists the contraction. However, if some part of the edge happens to detach from the glass wall due to insufficient coverage of

powder and hence poor attachment, a hole near the glass wall can be resulted (see Figure 4.9a). If the detached part is large, the adjacent parts can be weakened and become partially detached as well (Figure 4.9b). As a result of the detachment, a smaller but thicker membrane can be formed after cooling (Figure 4.9c). To prevent these, the blowing step in the preparation of a layer of particles is critical. As the blown particles are usually stopped by the glass wall and accumulate there, forming a continuity of powder on the water surface and the glass wall. This facilitates the circumferential attachment of a layer of melt. But more importantly, temperature and heating time need to be controlled in order to reduce the risk of detachment.

Figure 4.9: Edge effect.

4.3.3 Effect of particle size

As membranes are fabricated directly from micro-particles that can be prepared in different sizes, it is important to investigate the effect of particle size. To study the impact of different particle sizes, 0.1 g of 100 μm PCL powder and 0.5 g of 600 μm PCL powder were used to fabricate membranes at the temperature of 80 $^{\circ}\text{C}$ and the heating time of 20 min. For particles of 600 μm , 0.5 g is needed, because of a lower surface to volume ratio, which requires more materials when the same area is covered.

Figure 4.10 shows membranes fabricated using two sizes of particles, (a) 100 μm and (b) 600 μm . Membranes formed from finer particles are smoother, thinner, softer and more flexible than those formed from coarse particles, indicating that particle size is an important factor in affecting the texture and rigidity of a membrane.

Figure 4.10. Effect of particle size on the texture of the fabricated membranes.

4.3.4 Effect of blowing

During the fabrication process, not all the powder used has gone on to form the membrane, some powder is blown out of the glass dish, and some stuck onto the glass wall. Thus it is necessary to investigate the efficiency of powder usage. Table 4.1 shows the average mass of a membrane after fabrication. The efficiency is determined by the average weight of the membrane over the weight of powder used. The efficiency of powder usage is about 91.5%, in other words, about 10 mg of powder is lost during the blowing process.

Table 4.1: Efficiency of powder usage.

Sample	Weight of powder (mg)	Weight of membrane (mg)
1	100	91
2	100	93
3	100	86
4	100	94
5	100	94
6	100	91
Average	100	91.5
Efficiency of powder usage		91.5 %

4.4 Name of the Method

This method, using water, a biodegradable polymer and an oven to fabricate membranes, has several features. First, the process does not involve any solvent or toxic additives, which is particularly important for biomedical applications, as toxic chemical residues are likely to alter the behaviors of cells or incur cell death. Secondly, it is simple and fast. The method does not require special equipment, merely a common laboratory oven is sufficient, and the membrane can be fabricated as quickly as possible. Using other methods such as solution casting, the membrane can be obtained only after the solvent has been fully evaporated. Thirdly, this method takes advantage of the spaces among the micro-particles, which eventually gives rise to the pores on the membrane, enabling a direct fabrication of a porous membrane. Finally, the substrate in this method is water. The fluidity of water allows convenient detachment of a flat membrane, avoiding bent or ruffled shapes.

However, there are some limitations in this method. The materials have to be in the form of fine powder, as coarse particles affect the texture of the membrane. Also, the powder has to be insoluble in water; otherwise, if the particles dissolve, the membrane

cannot be formed. Lastly, the melting point of the powder has to be much lower than the boiling point of water.

As to naming this method, the term “surface tension melt” is coined. “Surface tension” implies the presence of water, a critical element in this process, and it emphasizes the role of surface tension in supporting all the events occurred in the process (formation of a monolayer of particles, a layer of melt, and a membrane). On the other hand, the word “melt” stresses the molten state of polymer, or the transition state from a layer of particles to a porous membrane, which is a critical step to membranize a layer of particles. Therefore this method is named surface tension melt

4.5 Development of Double-Layer Bags

Besides being used as one piece for tendon adhesion prevention, the membrane can also be explored for alternative applications, especially when two pieces of membranes are heat-sealed to form a bag.

Figure 4.11 shows the method to make a PCL bag. Briefly, 30 ml distilled water was poured into a glass dish of 10 cm diameter, and then 0.1 g of PCL powder was gently dispensed on the water surface and blown into a monolayer. Next, the glass dish was transferred to an oven set at 80 °C for about 20 minutes. Once removed from the oven, the glass dish was allowed to cool to room temperature until a firm membrane was formed. Two stacked membranes were then cut and sealed by a heated mold with a circular edge.

Figure 4.11: Schematic drawing for fabrication of PCL bag.

The method to make a PCL monolayer bag consists of two steps: stacking two single layers of porous membranes and sealing them by a mould. The melting point of PCL is as low as about 60 °C, which makes it easy to be treated by heat. When a heated blade is slid through two membranes, the cut edges are melted, fused, cooled, solidified and hence sealed. Figure 4.12 shows a double-layer carrier, cut and sealed from two stacked membranes. The round shape is because the mould is round. Since solvent is not involved in the fabrication process, harmful residues are unlikely to be left in the membranes. Moreover, bi-layer materials are of particular importance in various aspects of tissue engineering, for example, engineered cartilage [190] , epidermal and dermal layers of skin [191], and double-layer nerve conduit [192]. Therefore, these bags may serve different roles and fulfill multiple purposes.

Furthermore, it is possible to fabricate multiple layers to make a “sandwich” bag, which may be used to culture multi-layers of cells as the bags have pores on each layer and sufficient spacing among the layers.

Figure 4.12: A polycaprolactone double-layer bag.

4.6 Conclusion

A polycaprolactone membrane is required in this project as a separation barrier for tendon adhesion prevention. Inspired by the natural presence of membranes formed from a layer of particles, an original and novel membrane fabrication process, named surface tension melt, has been developed and proved effective in fabricating a membrane with micro-sized pores. The process involves three steps: monolayer preparation, heating to melt the powder so that they formed together and cooling afterwards. Since these membranes are made of polycaprolactone, a biodegradable material, they are promising to be used for tendon adhesion prevention. Moreover, it can also be easily modified to produce a double-layer bag by simply stacking and sealing two membranes.

CHAPTER 5 EXPERIMENTS

Experiments were organized into two groups as shown in Figure 5.1. Polycaprolactone microfibers were fabricated, characterized, *in vitro* and *in vivo* evaluated for tendon repair. Polycaprolactone membranes were fabricated, characterized, *in vitro* and *in vivo* evaluated for tendon adhesion prevention. Procedures of the experiments will be detailed in this chapter.

Figure 5.1: Organization of experiments.

5.1 Materials

Poly(ϵ -caprolactone) powder (CAPA® 6501, Molecular weight: 50kDa) was purchased from Solvay Interlox, UK. The material density of poly(ϵ -caprolactone) is 1.1 g/cm³. The melting point is around 60 °C. The particle size is about 100 μ m. Powder of 600 μ m particle size was a product sample requested from Perstorp. Table 5.1 presents the material properties of the PCL powder used in this project. The information is obtained from material safety data sheet (MSDS) provided by Solvay, the supplier of PCL.

Table 5.1: Material properties of polycaprolactone.

Materials	Polycaprolactone (CAPA 6501, Solvay)
Molecular weight	50000 Da
Melting point	58°C-60 °C
Decomposition temperature	200 °C
Density	1.10 g/cm ³
Shear viscosity	1.5 million mPa·s @ 100 °C

5.2 Fabrication and Characterization of Microfiber Bundles

5.2.1 Fabrication of microfiber bundles

Microfiber bundles were fabricated using microfiber melt drawing technique describe in Chapter 3 at about 130 ± 5 °C and 500 - 600 rpm (for 3 cm mandrel diameter) or 800 - 900 rpm (for 2 cm mandrel diameter).

5.2.2 Scanning electron microscope

Fabricated microfiber bundles, cut into 0.5 cm segments with a blade, were attached to a metal stub by tape, and then these segments were sputter-coated using auto fine coater (Jeol JFC-1600) with gold for 30 seconds at 10 mA before SEM (Jeol JSM-5600LV) examination.

5.2.3 Microfibers in a wet environment

Microfiber bundles were made into a linear form, inserted into a short plastic tube, and placed in water over one month. Another microfiber bundle was put into a plastic test tube filled with about 30 ml water and picked up for visual inspection right after a vigorous shaking of the tube. Microfibers eventually function as a biodegradable scaffold *in vivo* and the *in vivo* environment is wet due to the presence of water, it is necessary to examine how these microfibers behave in a wet environment. This is important in terms of tissue infiltration. If the microfiber bundles are dense and solid,

tissue infiltration into the interior of microfiber scaffolds can become a problem. It is believed that these microfibers are unfused and loose in a wet environment and thus are able to create spaces between the fibers for tissue infiltration.

5.2.4 Mechanical test

Instron 5569 mechanical tester with a load cell of 50 kN was used to carry out tensile testing of PCL microfiber bundles at room temperature and a humidity of 50%. Two aluminum strips were fabricated to facilitate the test (see Figure 5.2). Each of seven circular specimens had about 500 single microfibers, as the take-up speed is 500 rpm/min and the time of fabrication is one minute. Each specimen was first looped over two metal strips and then was finely positioned and tested at a 60 mm/min.

Figure 5.2: PCL microfiber bundle specimen.

The tensile strength of a single microfiber could be tested by an atomic force microscope tip-based approach [193], but this test method is time consuming and requires familiarity with micromanipulators, which can be a challenge when several

samples have to be tested. Instead, single microfibers were tested on a micro tensile tester similar to [194]. Figure 5.3 shows the method to test a single microfiber. Briefly, a single microfiber was visually recognized and unwound from a microfiber bundle. The single thread was then placed on a rectangular cardboard and glued at the edges of the window. The size of the window was considered as gauge length, which is about 3 mm. Before experiments, the two ends of the cardboard were adhered to grips, and the cardboard was cut beside the window to allow a single microfiber to be stretched. The strain rate was set at 0.05 mm/s.

Figure 5.3: Testing of a single microfiber.

5.2.5 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (Q200, TA Instruments) was used to examine if there was a change of melting point in PCL microfibers. PCL powder (10.4 mg) and PCL microfibers (6.8 mg) were squeezed into aluminum pans, covered with lids, and manually crushed into single pieces. An empty pan with lid was used as reference. Because the melting point of the purchased PCL is about 60 °C, the heating

temperature was set from 30 °C to 70 °C. Heating rate was set at 5 °C / min. Experiments for both powder and microfibers were run twice.

5.3 In Vitro Evaluation of Polycaprolactone Microfibers

5.3.1 Cells on microfibers

5.3.1.1 Preparation of microfibers

Nine samples were prepared and each had an area of 10 mm x 5 mm. Before cell seeding, samples were sterilized with 70% ethanol overnight, washed three times with phosphate buffered saline (PBS) and immersed in culture medium over night

5.3.1.2 Cell culture

Dermal fibroblasts and tenocytes are similar as both have a spindle shape and are able to secrete collagens, and they appear distinct only in the anatomical site in the body and the expression of certain anatomical site-specific genes [195]. As a cell source for tendon tissue engineering, dermal fibroblasts have been demonstrated to be able to generate tendons either in a porcine model [83] or recently *in vitro* [196], which indicates that they are promising to be used as a replacement for tenocytes since they are more readily available. Adult human dermal fibroblasts (Lonza, USA) were thus used in this experiment (see Figure 5.4). First, they were thawed in water bath for 30 minutes at 37 °C degree. Then cells were centrifuged (4 minutes, 1500 rpm), transferred to 75 cm² flasks, and cultured with 15 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1 % penicillin and streptomycin. Culture medium was replaced every 2 – 3 days.

Figure 5.4: Adult human dermal fibroblasts.

5.3.1.3 Cell seeding

Cells were detached using cell scrapers, then centrifuged and counted using hemocytometer. 2×10^4 cells/ml were seeded on each sample as well as on 3 tissue culture plate (TCP) wells as control. The samples were placed in the incubator (5% CO₂, 37°C) for 3 – 4 hours to allow for cell attachment. Next, the samples were transferred to new wells and another 6 TCP wells were seeded with the same amount of cells as new controls. The experimental wells containing unattached cells and the previous 3 TCP control wells were sent for methylthiazol tetrazolium (MTT) test. The difference between the control wells and the experimental wells represents cell attachment on microfibers or the number of cells at Day 1. Following that, culture medium was replaced every 2 – 3 days.

5.3.1.4 Methylthiazol tetrazolium (MTT) test

At day 1, each well was added with 20 μl of MTT solution and incubated overnight. Next, 200 μl of lysing buffer was added to break up cell membrane. Then 100 μl of cell solution from each well was transferred to a 96-well cell culture plate.

Absorbance was measured at 570 nm wavelength by a micro-plate spectrophotometer (Tecan™ Ultra Evolution). At day 3, 3 samples and 3 control wells were taken for MTT test. The same procedure was repeated at day 7.

5.3.1.5 Scanning electron microscope

At day 7, 3 samples were fixed with 5% glutaraldehyde solution, washed with PBS and dehydrated with a series of 50%, 70%, 90%, 100% and 100% ethanol solutions. The samples were then dried and sputter coated with gold for 30 seconds at 10 mA before SEM examination.

5.3.2 Cells in-between microfibers

5.3.2.1 Preparation of fiber membrane

To seed cells in-between microfibers on a two dimensional film, a fiber membrane was prepared. Briefly, a scotch tape was first wrapped around the mandrel with the sticky side facing to the air and then the microfibers were fabricated on the tape. Four rectangular samples (20 mm x 10 mm) were prepared by cutting with a scissor and the fiber side. After sterilization in 70% ethanol overnight, the samples were rinsed three times with PBS and immersed in culture medium overnight before use.

5.3.2.2 Cell culture

NIH 3T3 fibroblasts were used. After thawing in 37 °C water bath, cells were centrifuged and transferred to 75 cm² flasks, and then cultured in incubator for 3 days. During first passage, cells were detached by adding 5 ml trypsin and incubated for 5 minutes. After that, 1 ml of 2×10^5 of the cells was seeded onto each microfiber and cultured for another 3 days.

5.3.2.3 Inverted microscope

Nikon TS-100 Inverted Microscope was used to observe the morphology of cells in-between microfibers, because the tape was transparent and the shape of cells could be observed as they were on cultured flasks.

5.4 In Vivo Evaluation of Polycaprolactone Microfibers

To investigate whether the microfiber bundles could be infiltrated by host tissues such as dermal fibroblasts, microfiber bundles were initially subcutaneously implanted in three nude mice. Figure 5.5 shows the microfiber bundle specimens. Nude mice were anesthetized before surgery. A wound was created on the back and closed by suture after implantation of microfiber bundles (each cut with a width of 2-3 mm and a length of 2 cm). Figure 5.6 shows the implantation of microfibers which caused a bump on the skin (white arrow). The animal experiment was conducted by Doctor Chen Chih-Hao and his research assistant at the Chang Gung Memorial Hospital, Taiwan. For histological staining, the microfibers and associated skin were removed *en bloc* and fixed in 10% (v/v) formaldehyde at 4°C for 24 hours. Then they were dehydrated in an ascending alcohol series, embedded in paraffin and thin-sectioned (5 µm). Sections were stained with hematoxylin and eosin (H&E) and evaluated under an optical microscope (NIKON E5000, Japan).

Figure 5.5: Microfiber bundle specimens.

Figure 5.6: Subcutaneous implantation.

To ascertain that the host issue of the intended implantation site (i.e. tendon) can infiltrate into the interior region of a microfiber bundle, a second study involving two white New Zealand rabbits was performed. After anesthetizing, the rabbit was positioned prone on the operating table. Both hind limbs were shaved, scrubbed and disinfected with iodine and 70% alcohol, after which a tourniquet was applied for bleeding control. A posterior longitudinal skin incision was made in both legs sequentially. After dissection of skin and subcutaneous tissues, the Achilles tendon was exposed. After opening the tendon sheath, a defect window (about 15 mm x 2 mm) was created in the middle of the rabbit Achilles tendon by removing original tendon tissues (see Figure 5.7). The defect was not a through thickness defect but was about a half of the full thickness of the tendon. Bare microfiber bundles (about 5000 - 6000 strands) were then filled into the defect as a tissue repair materials. The two ends

of the microfiber bundle were sutured to the defect extremities. Tendon sheath was closed after the microfibers implantation. The rabbits were allowed to move freely after surgery. The rabbits were sacrificed at 1 month and 2 months, and the microfibers and associated tendon tissue was removed *en bloc* and treated the same as above for histological examination.

Figure 5.7: Achilles tendon repair in rabbits.

5.5 Fabrication and Characterization of Membranes

5.5.1 Membrane morphology

In Section 4.3.1, it has been shown that 80 °C temperature and 20 min heating time were the preferred conditions for the successful fabrication of porous PCL membranes. Without stating otherwise, all the membranes undergoing characterization were fabricated under the conditions of 80 °C temperature and 20 min heating time. Membrane morphology was examined by scanning electron microscopy (SEM) (Jeol JSM-5600LV). The PCL membrane was cut with a scissor into small pieces and coated with gold at 10 mA for 20 seconds before SEM examination

5.5.2 Thickness measurement

A micrometer (Mitutoyo Coolant Proof Micrometer Series 293, accuracy: $\pm 0.00127\text{mm}$) was used to measure the thickness of six PCL membranes. For each membrane, ten measurements were taken at randomly selected points.

5.5.3 Roughness measurement

The surface roughness, R_a , of the PCL membranes was measured using the Confocal Imaging Profiler (PL μ , SENSOFAR). For each of six membranes, 10 readings were taken on 10 different positions.

5.5.4 Pore size measurement

Five rectangular samples (25 mm x 15 mm) were cut with a surgical blade from five locations (top, bottom, left, right and center) of the same membrane and sputter coated with gold at 10 mA for 20 seconds before SEM (JEOL JSM-5600LV) examination. Pore size was measured by SEM images using software SmileView (Version 2.05).

5.5.5 Mechanical test

The tensile strength of the PCL membranes was tested at room temperature using the Instron Microtester (Model 5547). A total of 10 samples (10 mm x 15 mm) were cut from the PCL membranes and prepared by sandwiching their two extreme ends between two pieces of adhesive cardboard (see Figure 5.8). The cardboard ends were then mounted onto the clamps of the Instron Microtester, and a load of 50 N and a strain rate of 10 mm/min were used.

Figure 5.8: Schematic of clamping membranes.

5.6 In Vitro Evaluation of Membranes

5.6.1 Preparation of membranes

Six samples were prepared and each had an area of 10 mm x 10 mm. Before cell seeding, samples were sterilized with 70% ethanol overnight, washed three times with PBS, and immersed in culture medium over night

5.6.2 Cell culture

Similar to the cell culture experiments on the microfibers, adult human dermal fibroblasts (Lonza, USA) were used to evaluate potential of the membranes for cell growth. Cells were thawed in water bath for 30 minutes at 37 °C degree. They were then centrifuged (4 minutes, 1500 rpm), transferred to 75 cm² flasks and cultured with 15 ml of DMEM supplemented with 10% fetal bovine serum and 1 % penicillin and streptomycin. The culture medium was replaced every 2 – 3 days.

5.6.3 Cell seeding

Cells were detached using cell scrapers, then centrifuged and counted using hemocytometer. 2×10^4 cells/ml was seeded on each of the samples as well as on 3 TCP wells as control. Then all the samples were placed in the incubator (5% CO₂, 37°C) for 3 – 4 hours to allow for cell attachment. Next, the samples were transferred to new wells and another 6 TCP wells were seeded with the same amount of cells as new controls. The experimental wells containing unattached cells and the prior 3 TCP control wells were sent for MTT test. The difference between the control wells and the experimental wells represents cell attachment on microfibers or the number of cells at day 1. Following that, culture medium was replaced every 2 – 3 days.

5.6.4 Methylthiazol tetrazolium (MTT) test

At Day 1, each well was added with 20 µl of MTT solution and incubated overnight. Next, 200 µl of lysing buffer was added to break up cell membrane. Then 100 µl of cell solution from each well was transferred to a 96-well cell culture plate. Absorbance was measured at 570 nm wavelength by a micro-plate spectrophotometer (Tecan™ Ultra Evolution). At day 3, 3 samples and 3 control wells were taken for MTT test. The same procedure was repeated at day 7.

5.6.5 Evaluation of double-layer PCL bags

Adult human dermal fibroblasts (Lonza, USA) were thawed and expanded in 75 cm² culture flasks using Gibco Dulbecco's Modified Eagle Medium added with 0.01% penicillin and streptomycin and 10% fetal bovine serum. After two passages, fibroblasts were detached using 5 ml trypsin and centrifuged at 1500 rpm for 4 minutes. The cell pellet was collected and re-suspended with fresh culture medium. Fibroblasts were then transferred at a density of 2.76×10^5 cells / ml into four PCL

bags through a small opening cut on the edge of each bag, large enough for a micropipette tip to be inserted into it. Then the four petri dishes containing four bags were put in a CO₂ incubator at 37 °C for three days. After that, the bags were transferred to four 15 cm² flasks, each filled with 30 ml fresh culture medium. Culture medium was changed every three days over one month. At day 11 and day 32, PCL bags were sampled and fixed by 5% glutaraldehyde solution. After washing with PBS, the bags were subject to dehydration in a series of ethanol solution at 50%, 70%, 90%, 100% and 100%. The samples were then dried and opened with a blade to expose the interior. Samples were cut from the center of each PCL bag and the inner side of the membrane was sputter-coated with gold at 10 mA for 20 seconds before scanning electron microscope examination.

5.7 In Vivo Evaluation of Membranes

Two white New Zealand rabbits was used for this study. After anesthetizing, the rabbit was positioned prone on the operating table. Both hind limbs were shaved, scrubbed and disinfected with iodine and 70% alcohol, after which a tourniquet was applied for bleeding control. A posterior longitudinal skin incision was made in both legs sequentially. On the left side, after dissection of skin and subcutaneous tissues, the Achilles tendon was exposed, cut in the middle, and then repaired with suture (Figure 5.9b). PCL membranes (Figure 5.9a) were wrapped around the repaired tendon. On the right Achilles tendons, the same procedures were applied without the membranes. Tendon sheaths were not repaired. The skin was closed in interrupted fashion with silk suture. Following surgical intervention, no wound dressing was applied. After surgery, the rabbits were housed individually and the mobilization was not limited. The rabbits were sacrificed after two months. The Achilles tendons in both hind limbs were excised *en bloc*. The histological sections (stained with

hematoxylin and eosin) were evaluated under an optical microscope (NIKON E5000, Japan).

Figure 5.9: Animal model for tendon adhesion prevention.

5.8 Statistical Method

All data obtained in experiments were expressed in average $\pm$ standard deviation and analyzed by two-tailed and unpaired student T test.

CHAPTER 6 RESULTS AND DISCUSSION

Two new methods have been introduced in Chapter 3 and Chapter 4. One is microfiber melt drawing, a process that is able to fabricate a bundle of PCL microfibers of 10 μm fiber diameter, and the other is surface tension melt, a technique to fabricate a porous PCL membrane. A series of experiments on these two innovations, including characterization, *in vitro* studies and *in vivo* studies, have been carried out and described in Chapter 5. The results from these experiments will be discussed in this chapter.

6.1 Fabrication and Characterization of Microfiber Bundles

6.1.1 Morphology of dry microfibers

Unlike electrospun microfiber web, microfibers fabricated by the microfiber melt drawing technique are tightly packed circular bundles with a silvery and shiny appearance similar to native tendons (see Figure 6.1, white arrows). Each dry microfiber bundle can be easily removed by hand. The amount of microfiber in a bundle can be controlled by time of fabrication, or, microfiber bundles with consistent weight can be fabricated by spinning each over a fixed period of time. During fabrication, the melt holder was shifted to keep microfiber bundles separate from each other, and a number of fiber bundles can be fabricated sequentially in this manner. The gap between microfiber bundles is recommended to be at least 2 mm for ease of distinguishing between adjacent bundles. In addition to fabricating a stacked microfiber bundle, a microfiber sheet (oval arrow) can be fabricated for a fixed time over a fixed spread.

Figure 6.1: As-fabricated polycaprolactone microfibers (white arrows: bundles, oval arrow: a microfiber sheet).

Figure 6.2 shows an SEM image of microfibers at the surface of a bundle. The surface is smooth and the shape is uniform. There seem to be many individual microfibers, but the shown microfibers are in fact a single thread which is wound about 500 times. Compared to the aligned microfibers fabricated by electrospinning (see Figure 6.3), which are a two dimensional sheet and consist of numerous discontinuous fibers, the microfiber bundles shown in Figure 6.2 are three dimensional and highly aligned. Moreover, the electrospun microfibers require a solvent for fabrication, and their morphology can vary from the form of a fiber to a string of spherical beads depending on the concentration of the solvent [197]. In contrast, the microfiber melt drawing technique is solvent free. Therefore, the microfiber melt drawing technique has significant advantages over electrospinning in producing aligned microfibers, especially in terms of fiber morphology, fiber continuity, fiber alignment as well as whether a solve is used or not.

Figure 6.2: Morphology of aligned microfibers by microfiber melt drawing.

Figure 6.3: Morphology of aligned polycaprolactone microfibers fabricated by electrospinning [197]. Scale bar: 10 μ m.

6.1.2 Morphology of wet microfibers

Dry microfiber bundles appear dense and compact, but when placed in water, they absorb water and become bigger. Because individual microfibers can separate from each other and spread in water (see Figure 6.4). The looseness of microfibers in a wet environment indicates that these microfibers are unfused in their dry form, and that spaces can be spontaneously created between individual microfibers as they spread and separate. As seen in Figure 6.5, the process of dispersing microfibers consists of the three stages suggested by Parfitt [198]. The first is the wetting of the external

surfaces of microfibers and displacing the internal air between the microfibers. The second is the breaking of the microfiber bundle into either individual microfibers or smaller bundles. The final stage involves the dispersing of the individual microfibers throughout the water. On the left of Figure 6.4, the microfibers are in the final stage of the dispersing process. On the right of Figure 6.4, the right end of this microfiber bundle is fused by heat, thus the final stage is constrained. The individual microfiber dissociates from the bundle due to the natural buoyancy of PCL and the hydrophobic property of the microfiber. Besides, the water between the individual microfibers can act as a hydrophilic barrier between these individual hydrophobic domains to avert them from re-bundling. There is no force that is strong enough to bring the microfibers together. Even if the attractive forces (e.g. Wan der Waals force and Coulombic force) between the microfibers were strong enough to bring them together, they would have to overcome the hydrophilic barrier before re-bundling happens.

Figure 6.4: A bundle of polycaprolactone microfibers in water.

Figure 6.5: Dissociation of microfibers in water.

Besides the spontaneity of dissociation in a wet environment, microfiber bundles can also be loosened in water by mechanical agitation. Figure 6.6 shows a dry microfiber bundle becoming tangled after being rigorously shaken in a tap water-filled plastic test tube. This mechanical energy broke the microfiber bundle into individual microfibers, but the pattern of alignment was broken as well due to the two ends of the bundle being left unconstrained. This simple demonstration suggests that mechanical force may play mixed roles in animal study. Firstly, the movement of the body part where microfibers are implanted may contribute to the spread-out of microfibers in favor of tissue infiltration. Secondly, the pattern of alignment may be disrupted by the movement of the animal if the two ends of a microfiber bundle are not constrained during implantation.

Figure 6.6: Microfibers can be agitated to form a loose structure in water.

6.1.3 Mechanical test

Figure 6.7 shows a typical load- extension curve of microfiber bundles fabricated over one minute. The curve shows an elastic behavior at the initial stage, followed by a zigzag portion for the plastic deformation. There are about 500 strands in each bundle, and the shown mechanical behavior is a collective property of these individual strands. The zigzags on the curve are due to the chronological fluctuation in the collective deformation behavior of microfiber strands during stretch. The reason for testing this collective behavior of microfibers is that the strength of a bundle cannot be simply calculated based on the strength of a single microfiber, although there were some attempts to work out a solution [199, 200]. In fact, the average strength of a bundle is always less than the strength of their individual components due to unequal extension of the individual strands (zigzags on the curve). Therefore, testing a microfiber bundle can help understand the strength of the bundle as a whole. Moreover, collagen fibers in a native tendon always respond to the external load in a collective manner, and testing the strength of a microfiber bundle better resembles the way the natural tissue responds.

The microfiber bundle has a stiffness of 1.11 ± 0.20 N/mm, a breaking force of 16.8 ± 1.84 N and an elongation at break of 115 ± 4 %. These properties can be significantly increased by increasing the fabrication time or the number of microfibers. Figure 6.8 shows the comparison of microfiber bundles fabricated over 1 minute and 2 minutes. The stiffness and breaking force of the latter is significantly higher than that of the former ($p < 0.001$ for both). However, there is no statistical difference in the elongation. The data suggest that with an increasing number of microfibers in a bundle, the curve can be gradually shift up, similar to what happens in a developing tendon (see Section 2.1.2 in Chapter 2). Also, this implies that the number of collagen fibers is a strong factor in determining the strength of native tendons and that our tissue can use the number of collagen fibers as a control to adjust the mechanical performance of tendon during development, healing or age-dependent degeneration. These data suggest that the mechanical property of a microfiber bundle could be tailored to suit to the strength of a variety of tendons.

Figure 6.7: A typical load-extension curve of microfiber bundles fabricated over one minute (number of strands in a circular bundle: about 500).

Figure 6.8: Comparison of microfiber bundles fabricated at about 500 rpm over 1 minute and 2 minutes.

Table 6.1 shows the result of tensile testing of a single microfiber. The stiffness for the tested six samples is 0.008 ± 0.003 N/mm, the breaking force 0.006 ± 0.001 N, and the elongation at break $61.78 \pm 8.64\%$. Compared to the stiffness of a microfiber bundle, the stiffness of a single microfiber is much lower. This is because the number of microfibers can significantly affect the stiffness of a bundle (see Figure 6.8) and a single microfiber can be considered as the smallest bundle.

The average breaking force of a single microfiber in a 1-min microfiber bundle can be estimated by its breaking force over 1000 strands (each microfiber bundle was tested in a loop and hence two sets of 500 strands), which is 0.0168 N. Similarly, the average breaking force of a single microfiber in a 2-min microfiber bundle is 0.0158

N. It was expected that the result from testing a single microfiber would be higher than 0.0168 N because the more microfibers in a bundle the lower average breaking force. However, Table 6.1 shows a result otherwise that the breaking force of a single microfiber (0.006 N) is almost three times lower than that estimated from a microfiber bundle. This unexpected result could be due to the following reasons. First, a single microfiber and a microfiber bundle were tested in two different testing systems. The former is about 3 mm long in gauge length and the latter 30 mm. Although fiber length impacts the breaking strength only slightly [194], the two different systems, measuring forces in a large and a small scale, could possibly give rise to some discrepancy in the data. When comparing the results tested in the same system, as expected, the average breaking force of a single microfiber estimated from a 2-min bundle is lower than that of 1-min bundle. Secondly, when preparing and handling a single microfiber, a manual unwinding process and direct contact with the microfiber are often required. Damage and compression on a microfiber is likely to happen in these handling processes, and the breaking force a single microfiber can be significantly lowered as a result of damage and transverse compression [194]. Although the two values are slightly different, they are still within the same magnitude.

The elongation of a single microfiber is lower than that of a microfiber bundle. This result is expected because stretching a single microfiber in the handling process is difficult to avoid, especially when mounting it to the cardboard and making it taut. It is very likely that a single microfiber had been already stretched before the tensile test was carried.

Table 6.1: Results of single microfiber tensile tests.

Sample	Stiffness (N/mm)	Breaking force (N)	Elongation at break (%)
1	0.012152	0.005498	54.83
2	0.010094	0.007379	77.73
3	0.008428	0.007134	56.53
4	0.006958	0.004694	55.83
5	0.00735	0.005704	61.23
6	0.004998	0.004155	64.53
Average	0.008 ± 0.003	0.006 ± 0.001	61.78 ± 8.64

Figure 6.9 shows the failure morphology of a broken microfiber. The breakage seemed to occur suddenly and a blunt end as shown (white arrow). This may be explained based on the observation reported by Zussman *et al* [201]. They observed two failure modes of electrospun microfibers, primarily multiple neck formation on a single microfiber and sometimes the formation of a fibrillar structure in the neck region. Figure 6.10 shows how a blunt end could be resulted. If multiple necks are formed on a single microfiber, there must be one which is the weakest. After breakage, the broken part is larger than the neck formed adjacent to the weakest neck, a blunt end thus appears. However, the fibrillar structure was observed in this study.

Figure 6.9: Failure morphology of a broken microfiber.

Figure 6.10: Formation of a blunt end.

6.1.4 Thermal property

Figure 6.11 shows DSC curves of PCL powder and PCL microfiber. The melting points for both lie within the narrow range of 59 to 61 °C, which suggests that the microfiber melt drawing process has changed the form of the material but not the melting point of PCL. If the drawing process has significantly altered the organization of polymer chains, the melting point would have shifted to a higher value, because a more crystalline phase is usually associated with a higher melting point. The reason for this unchanged melt point could be due to the fact that the take up speed in the microfiber melt drawing process is relatively low, and thus the microfibers was not stretched to a high crystallinity.

Figure 6.11: Melting point of microfibers.

6.2 *In Vitro* Evaluation of Microfibers

6.2.1 Cells on the surface of 3D microfibers

Figure 6.12 shows the MTT result of human dermal fibroblasts cultured on microfibers over seven days. Both microfibers and TCPs were able to support cell growth over time, as indicated by higher absorbance values at day 7. The experimental group displayed a significantly lower absorbance at day 1 because the number of cells being successfully seeded on the microfibers was less than that of TCP group due to the fact that the area of each TCP well was much larger than the microfiber samples. Moreover, the highly hydrophobic surface of polycaprolactone microfibers which does not favor the initial attachment of cells could be another

factor [184]. At day 3, it is observed that the absorbance remained flat or even dropped slightly, which deviates from the postulated proliferation curve of 2^n , where n is the number of cell replications. One possible explanation for this is that the seeded cells comprised of a mixed population of stronger and weaker cells. The stronger cells were able to survive and proliferate while weaker cells died off, the concurrence of these two events accounted for the flatness in absorbance. However, at day 7, both groups demonstrated a significant increase in absorbance, indicating that the cells were proliferating afterwards. Overall, the *in vitro* data show that the polycaprolactone microfibers are suitable for tissue engineering scaffolds. The TCP group is used as a control to assess how well the cells grow on the microfibers. The comparison can show whether the growth curve for cells on microfibers is normal or abnormal. As shown in the figure, at day 1 and day 3, the growth curve for cells on microfibers is almost flat. Without the TCP group, it would be difficult to know this phenomenon is normal or not. Since both groups remain flat from day 1 to day 3, it can be concluded that the cells grow well on the microfibers even though the growth curve remains flat.

Figure 6.12: Cell proliferation on TCPs and polycaprolactone microfibers.

Figure 6.13 shows the SEM observations of cells on microfibers at day 7. It does not agree with the literature [202], which reported that microfibers (about 10 μm fiber diameter) were able to align the cells seeded on fiber surfaces. The alignment of cells was not obvious in our experiment. Rather, the cells crossed adjacent microfibers or wound around a microfiber seemingly to engulf it. Cells on a single microfiber, having no place to migrate, may have to either orient along the fiber axis or wind around it. However, when the environment surrounding cells comprises of multiple microfibers, organization of cells other than alignment, such as whirling on a microfiber or bridging across microfibers, becomes possible. Therefore the alignment of cells on the surface of multiple microfibers is inconclusive, that is, some cells can align along microfibers while some can migrate around randomly, especially when adjacent microfibers are within their reach. The current observation agrees with the previous literature that cells migrating along fibers may attach laterally to other cells present on the fibers to form sail-like structure in a fiber mesh [203].

Figure 6.13: SEM images of microfibers without (a) and with (b) cells.

6.2.2 Cells between microfibers on a 2D surface

When seeded onto microfibers, cells may stay on the surface of microfibers or between the channels formed by microfibers. When cells stay in the channels between microfibers, the alignment of cells is obvious. Figure 6.14 shows an SEM image of a microfiber film for seeding cells into the channels between microfibers. Figure 6.15 shows a comparison of 3T3 fibroblasts cultured in tissue culture plate with those cultured between microfibers. The cells were observed to have a spindle shape orienting along the channels in head-to-head manner. Similar observations have been reported for cells seeded into micro-channels [204] or micro-grooves [144]. However, this type of alignment may be different from the alignment response of cells to ultrafine surface features such as nano-grooves [205], in which the cell is much larger than the surface features and the mechanism of alignment or contact guidance has not been fully understood. The observed alignment of cells in micro-channels, perhaps, is largely due to the topographical confinement of the migration of a cell, that is, the cell is constrained to protrude its process along the channels rather than in other directions. If the migration of a cell is constrained within a square, the cell has a square shape [75]. Therefore, constrained cell migration may account for the observed cell alignment between microfibers.

Figure 6.14: Microfibers fabricated on to the sticky side of scotch tape.

Figure 6.15: 3T3 fibroblasts on (a) tissue culture plate; (b) & (c) between microfibers (white arrows).

6.3 *In Vivo* Evaluation of Microfibers

Figure 6.16 shows a microfiber bundle (black arrow) subcutaneously implanted for two months in nude mice. The bundle is enveloped by a layer of fibrous capsule due to foreign body response. Tiny blood vessels can be seen running across the surface of the microfiber bundle.

Figure 6.16: Gross appearance of the implanted microfiber bundle.

Figure 6.17 shows histological images of the microfiber bundle under 40x and 100x magnifications. Nuclei of cells are colored blue by hematoxylin. Cytoplasm and collagen are colored pink by eosin. The microfibers are white in color. It can be seen that the microfiber bundle, including the interior region, is thoroughly infiltrated. The aligned pattern of the microfibers is well preserved, and the infiltrated tissue appears to follow the alignment of microfibers.

Figure 6.17: Histology (H&E). Above: Cross-section. Below: Longitudinal section.

Figure 6.18 shows a higher resolution of the cross-section. The microfibers are found almost evenly dispersed in the pink background, and nuclei of cells are found throughout the spaces between microfibers. It is not easy to distinguish fibroblasts from other cell types such as mast cells and macrophages, but fibroblast-like cells can still be seen in the inner region of the microfiber bundle by their long and narrow nuclei (thin black arrows). Multinucleated foreign body giant cells are found between the microfibers (thick black arrow). Three cross-sections of blood vessels (white arrows) are seen at the top and the bottom left of the figure, which are in or near the fibrous capsule and run in parallel with microfibers. Blood vessels can hardly be identified in the inner region of the bundle. In general, both tissue infiltration and vascularization are critical to tissue engineering application, as regeneration of new tissues largely relies on existing tissues growing into void spaces and surviving a larger tissue thickness under the support of blood vessels. However, in the case of

tendon, angiogenesis or in-growth of blood vessels is less desirable because tendon is naturally less vascularized. Aggressive invasion of blood vessels may cause the regenerated tissue to become very dissimilar to tendon tissue. Instead, tissue infiltration is more important. Without good tissue infiltration, a repaired tendon is unlikely to regenerate.

Figure 6.18: Histology (H&E).

A suggested mechanism of tissue infiltrating into a microfiber bundle is illustrated in Figure 6.19. In this cross sectional view, white spots represent the microfibers and the blue spots nuclei of cells. Knowing that microfiber bundles loosen and spread or dissociate in a wet environment, it is reasonable to observe that microfiber bundles, once implanted, will also loosen and spread *in vivo* and allow the cells to penetrate through the spaces between microfibers. As individual microfibers spread and separate from each other, cells can enter the interior region as shown in Figure 6.19b. On the other hand, if the microfiber bundle does not spread, Figure 6.19a is the likely

result, in which a cluster of tightly packed microfibers is surrounded by cellular materials.

Figure 6.19: Microfibers (white spots) and cells (dark spots)

The results of subcutaneous implantation in mice have shown a good tissue infiltration and aligned tissue structure, which suggests that a similar outcome should be expected if the microfibers are implanted in tendon. Figures 6.20 shows the results of Achilles tendon repair in a rabbit model. A-B and C-D show the result of one month and two month implantation, respectively. At one month (A-B), the microfiber bundle is thoroughly infiltrated, and individual microfibers (5000 – 6000 strands) are almost evenly distributed in the infiltrated tissue. No blood vessels are present apparently in the microfiber repaired region and the region of native tendon tissue. Between the normal region and the microfiber repaired region, as well as between individual microfibers, comprise a mixture of tendon-like tissues and foreign body reactions. The tendon-like tissue may come from the infiltration of the surrounding tendon tissues or from the newly regenerated tendon, as both can give rise to the structure that is histologically similar to the normal region. Foreign body reaction is usually considered as an undesirable event for a biocompatible implant. However, one of the end-products of foreign body reaction is the formation of a fibrous capsule, in

which the primary component is collagen, the critical component in tendon as well. In this particular instance, it is perhaps favorable to have foreign body reactions because more collagens could be produced in the end. Microfibers might be able to play a role in promoting the integration of regenerated tissue and the normal structure by inducing the production of collagen-rich materials such as fibrous capsules. Moreover, the region repaired by microfibers not only forms a tendon-like substance but also displays a well aligned structure, which suggests that the implanted microfiber bundle may have promoted and guided the healing and regeneration of tendon inside the bundle. At two months (C-D), the microfibers were still present in an aligned and evenly distributed manner, indicating that the microfiber bundle is able to provide a long-term support and guidance. Between the microfibers, a mixture of tendon-like tissues and foreign body responses were still dominant. The color of the microfiber repaired region appears much denser than that of the native tissue. This is because the cell density is naturally low in the native tendon tissue, and the foreign body responses have become more evident after two months, as multinucleated giant cells were formed extensively around individual microfibers. The alignment of tissues continued to follow the alignment of microfibers, but whether the cells in the tissue could orient between and along the microfibers and form a three dimensionally aligned cellular network remains inconclusive. In one recent study, a two dimensional sheet of aligned electrospun microfibers were used for tendon repair and they could induce the formation of spindle-shaped cells and tendon like tissue from tendon stem cells [75], but there is no mention on the formation of a three dimensionally aligned cellular network.

Figure 6.20: Histology (H&E). A-B: 1 month. C-D: 2 months.

The inflammatory response in this study may result from the degraded fragments of polycaprolactone. These degradation products cannot be avoided once polycaprolactone is implanted. Over time, they will degrade into smaller and smaller fragments and eventually will be cleansed by the body. At the moment, it is difficult to know if the inflammatory response will persist over a long-term implantation period (i.e. 6 – 12 months), but certain measure can be taken to reduce this response in future, for example, coating of anti-inflammatory drugs on the surface of microfibers.

The microfiber bundle used in this study is different from an artificial tendon made from braided non-degradable fiber bundles such as poly polyethylene terephthalate

fibers. In the latter the filaments are tightly packed in a braid and with no or little freedom to spread out. As a result, the host tissue treats the bundle of filaments as a single solid material and reacts to the whole peripherally, resulting in scarce tissue infiltration [206]. A knitted structure of poly(lactic-co -glycolic acid) fibers can give rise to pores in the stitches and improve tissue infiltration [99, 101, 102], but the fibers in the knitted structure have no overall alignment due to the interlocking and hence lack directional guidance on tissue regeneration. The current *in vivo* result shows that a loose bundle of three dimensionally aligned microfibers can simultaneously achieve good tissue infiltration and the preservation of aligned pattern of microfibers. Moreover, compared to the electrospun microfibers, in which the densely packed microfibers form pores smaller than the size of a cell and hence block cell infiltration, the current microfiber bundle is three dimensional and more favorable for tissue infiltration. In fact, the pores in electrospun microfiber web result from random stacking of numerous discontinuous microfibers, and these microfibers are fused together at their contact points during fabrication, leaving the pore size almost fixed and unchangeable, unless the fabrication process is purposely modified to increase the pore size [205, 207]. This is why electrospun microfibers seldom loosen and spread in a wet environment and why they have the problem of cell infiltration. Unlike electrospun microfiber web, the microfibers fabricated by the microfiber melt drawing technique are unfused. The “pores” or spaces between microfibers are open and their size is subject to the environment the microfibers are placed in, that is, the open spaces are naturally enlargeable in an *in vivo* environment. This is a significant difference between these two types of microfibers.

It been shown that the microfiber bundle, regardless of the implantation sites (subcutaneous tissue or Achilles tendon), is able to loosen and spread in an *in vivo* environment and allows host tissue to penetrate into the interior of the bundle. This study has shown a strong clinical relevance to tendon repair, in particular, to the cases when partially degenerated or torn tendon tissues have to be removed and replaced. The observed rapid and thorough infiltration, the persistent directional guidance and the formation of tendon-like tissues, all together have unveiled the real host response to a three dimensionally aligned biodegradable PCL microfiber bundle. The current study has completed the first step and generated necessary knowledge towards a fully functional regeneration of injured tendons. The real situation of a microfiber bundle implanted *in vivo* is complex as multiple events happen concurrently. However, tissue infiltration, a major event required in tendon repair, has been demonstrated successfully. Based on the current results, a larger scale animal study can be designed and carried out in future to characterize the regenerated tendon and to evaluate the regeneration of tendon in a broader and wider spectrum including healing time, mechanical strength, ultra-microstructure and biochemical compositions around microfibers. Additionally, investigation on how the three dimensionally aligned microfibers effect on the infiltrated or regenerated tendon tissue will possibly open up a new research area and bring our understanding of cell alignment from two dimensions to three dimensions.

6.4 *Fabrication and Characterization of Membranes*

6.4.1 Membrane thickness and roughness

Table 6.2 shows that the thickness of membranes ranged from $25.5 \pm 2.9 \mu\text{m}$ to $33.4 \pm 2.6 \mu\text{m}$. This is much smaller than the size of the PCL particles used, which is

around 100 μm . Initially, particles should form a layer of a thickness of about 100 μm as well as a network of spaces among these particles due to their irregular shapes. When melted on the water surface, the particles become round and spread and fill their adjacent spaces. The overall thickness of the layer is thus reduced as a result of material re-distribution. Figure 6.21 shows SEM observation of the cross section of a membrane, which indicates the measured results.

Table 6.2: Thickness of fabricated membranes.

Sample	Thickness (μm)	Sample	Thickness (μm)
1	26.2 ± 2.9	4	33.4 ± 2.6
2	25.5 ± 2.9	5	26.3 ± 2.6
3	26.1 ± 2.0	6	26.3 ± 3.5

Figure 6.21: The cross-section of a membrane.

The roughness of these membranes, measured by confocal imaging profiler, is $3.4 \pm 2.9 \mu\text{m}$ (see Table 6.3). This may have explained the perception of a fine texture on the membrane. The membrane being even is largely due to the small size of particles that form it, but consistency in particle sizes and natural flatness of water surface may also contribute to the overall evenness.

Table 6.3: Roughness of membranes.

Sample	Ra (μm)			
1	2.939	3.453	4.355	2.666
2	4.545	3.800	2.892	3.371
3	2.724	3.563	4.324	2.611
4	2.877	4.599	3.779	3.548
5	2.196	2.108	3.336	1.994
6	2.018	3.875	5.579	3.693
Average	3.4 ± 2.9			

6.4.2 Distribution of pore size and two dimensional porosity

Figure 6.22 shows an SEM image of the membrane. Pores (black spots) are randomly distributed and have variable sizes. Table 6.4 shows the distribution of pore size of three membranes. Pore size ranged from a few micrometers to nearly one hundred micrometers and the average pore sizes for the three membranes were 16.2 ± 9.2 , 16.7 ± 10.9 and $16.2 \pm 6.3 \mu\text{m}$. The median pore size was about 14 - 15 μm . There is no statistical difference among the pore sizes measured, indicating that the fabricated membranes have a relatively consistent distribution of pore size. Figure 6.23 shows a typical graph of the distribution of pore size. Certain size of pore appeared to have zero frequency, this is because of the limitation of the software used during measurement. For example, in an SEM image of 100x magnification, instead of displaying 7 μm , a neighbouring value 6 μm or 8 μm were given by the software. However, this limitation has little impact on the overall distribution profile of pore size and the conclusion drawn.

Figure 6.22: SEM image of the membrane.

Table 6.4: Distribution of pore size.

Sample	1	2	3
Number of pores measured	949	885	1000
Average (μm)	16.2 ± 9.2	16.7 ± 10.9	16.2 ± 6.3
Median (μm)	14	14	15
Max size (μm)	95	80	46.1
Min size (μm)	2	3	6
Student t-test	$p(1,2) = 0.26$, $p(1,3) = 0.27$, $p(2,3) = 0.92$

Figure 6.23: Distribution of pore size.

This porous membrane will be used as a barrier to isolate repaired tendon tissue to its surroundings (see Figure 6.24). Due to the presence of pores, there is a risk that the membrane may be infiltrated by fibroblasts from the surrounding tissues. This risk is imposed not only by the sizes of pores but also by the number of pores on the membrane that are in direct contact with the surrounding tissues. Hence a ratio of the total area of pores to the area of the membrane is needed to indicate the percentage of the area that is at risk of cell infiltration. Alternatively, this area ratio can be called a two dimensional porosity. If this ratio is high, a large portion of the wrapped tendon can be exposed to the surrounding tissues, and the risk of cell infiltration and adhesion formation will be higher.

Figure 6.24: Porous membrane as a barrier.

The area ratio was estimated based on SEM images. To make the calculation of the area of a pore simple, all the pores were assumed to have a round shape, and the following equation was used for each membrane.

$$Area\ Ratio = \frac{\sum_i^{total\ No.\ of\ pores\ on\ all\ SEM\ images} area\ of\ pore\ i}{No.\ of\ SEM\ images \times area\ of\ SEM\ image} \quad (18)$$

Table 6.5 shows the estimated ratios for three membranes were 0.62%, 0.72% and 1.2%, and the average ratio was 0.85%, or roughly 1%. This means that only about 1% of the area of the membrane will allow the possibility of cell infiltration, and hence

that the membrane is at a low risk of being infiltrated. This result, together with result of the average pore size of about 16~17 μm , suggest that this membrane may be favorable for anti-adhesion study. Because a separation membrane that is too porous in terms of a two dimensional porosity will be at a high risk of adhesion formation, unless the size of the pores is far below the size of a cell (about 20 μm). On the other hand, if the membrane is dense and without pores, it is slippery and can move away from the defect site, as some pores are needed for surrounding tissue matrices to slightly grow in to anchor the membrane [208]. A low porosity might allow the anchorage of the membrane by the presence of pores but block the cells passing through the membrane by the small size of the pores. Moreover, penetration of water and tissue fluid will be limited to the two ends of the wrapped membrane if without pores. Pores on the membrane could allow a broader area of passage of tissue fluid as well as extrinsic healing factors that facilitate tendon healing and could prevent tissue necrosis due to insufficient nutrient diffusion. Therefore, the membranes fabricated by surface tension melt are different from solvent-casted dense films or porous fiber meshes, and they are unique in preventing tendon adhesion formation.

Table 6.5: Two dimensional porosity.

Sample	Membrane 1	Membrane 2	Membrane 3
Number of images	34	31	100
Area of image (μm^2)	1280 x 960	1280 x 960	512 x 384
Total area of pores (μm^2)	258493.4	276895.3	236109.8
Ratio	0.62%	0.72%	1.2%
Average ratio	0.85% (~ 1%)		

6.4.3 Mechanical test

Figure 6.25 shows a typical load-extension curve of the membrane. The stiffness of the membranes was 2.4 ± 0.4 N/mm, and the ultimate tensile load was 1.6 ± 0.3 N. These values mean that these membranes can be handled without ease of fracture

when wrapped around a repair site, eliminating the concern that porous membranes formed from particles may be easily fractured. Moreover, these data are important when the membranes are to be evaluated for other applications, such as scaffolds for skin and blood vessels, in which initial strength is required to support the growing engineered tissues.

Figure 6.25: Mechanical test.

Figure 6.26 shows the microscopic observation of a stretched membrane. It can be seen that pores were the crack initiation sites and small cracks horizontally adjacent to each other converge into a long crack perpendicular to loading. Thinning usually started at the periphery of pores and regions with less or no pores decreased in size and became connected by multiple thinned strips. This morphological observation implies that when applying the membrane to a repair site, the wrapping process needs to be carefully handled with gentle stretch so that the enlargement of the pores on the membrane can be minimized.

Figure 6.26: Failure mode. (a) Near the cardboard. (b) In the middle of a sample.

6.5 *In Vitro* Evaluation of Membranes

Figure 6.27 shows the result of cell proliferation on tissue culture plates and polycaprolactone membranes. Similar to the previous result for microfibers described in Section 6.2.1, at day 3, the absorbance for the polycaprolactone membranes remained almost unchanged. At day 7, a significantly higher absorbance was observed, indicating that the cells were proliferating normally. This result demonstrates that the membrane is able to support cell growth and suitable for animal implantation. However, in the case of adhesion prevention, this lessened cell proliferation (compared to TCP control) on polycaprolactone membranes can be an advantage. The less the cells staying attached to the membranes, the lower the risk of cell infiltration and adhesion formation. This preventive application is different from those that require the promotion of tissue interface integration, in anti-adhesion study, promotion of separation is preferred instead.

Figure 6.27: Cell proliferation on TCPs and membranes.

Figure 6.28 shows the result of one month cell culture in a double-layered PCL bags. Each sample was cut from the middle of the bag and the exposed area is the inner side of the bag. Cells are observed spreading and stretching on the inner side of the membrane. It appears that pores on the membrane have played a role in supporting the growth of cells. However, this does not rule out the possibility that the small opening on the edge may have also contributed to the nutrient transport and growth of cells. At day 11, fewer cells were found attached to the membrane and pores on membrane could be clearly seen under SEM. At day 32, more cells and extracellular matrices were found covering the inside of the membrane and pores on membranes seemed to be covered by cells and proteins as well. In certain instances, cells do bridge open spaces, such as in a fibrous mesh [203] or on micro-patterned ridges with groove width of about 10 – 20 μm [209].

Figure 6.28: Cell culture in double layer bags. a.& b. After 11 days of cell culture
c.&d. After 32 days of cell culture.

6.6 *In Vivo Evaluation of Membranes*

The membranes fabricated from the surface tension melt technique have three unique features. First, the membrane is made of polycaprolactone, a highly hydrophobic synthetic material. The hydrophobicity, which is unfavorable for cell attachment, could reduce the adhesion rate by allowing less cells attaching to the membrane. Secondly, polycaprolactone has a slow degradation rate, which enables a longer period of separation and thus diminishes the possibility of re-adhesion after the membrane completely degrades. Thirdly, the membrane has about 1% of its area comprising of pores about 16 – 17 μm in size. Usually, healing factors such as basic fibroblast growth factor effect on both tendon tissue and the surrounding tissue by promoting cell proliferation and collagen production, therefore they can increase the

adhesion rate when facilitating tendon healing [204]. The low porosity structure can be a solution to this problem by blocking the entry of cells through the pores while allowing the delivery of extrinsic healing factors to the defect site. Besides, these pores could also facilitate the diffusion of nutrients to the barrier-coverer area, preventing possible tissue necrosis. It was expected that this low porosity membrane would have an effective prevention of tendon adhesion in the animal experiment.

Figure 6.29 shows the result of anti-adhesion study in a rabbit model. The experimental (with membrane) group and the control (without membrane) group shows completely different outcomes after two months implantation. Multiple peritendinous adhesion sites (black arrows in b) are obvious in the control group, while the experimental group shows no visually observable adhesion. Here adhesion means the integration of tendon tissue and the surrounding subcutaneous tissues. Inflammatory response is noticed in both groups, as indicated by the redness on the tendons. Histological examination on the cross-section of tendon shows that, the membrane barrier (white arrows) is still present after two months and has separated the tendon tissue from the surrounding subcutaneous tissues, which leads to the absence of adhesion. A dark purple crescent is found close to the barrier, confirming the presence of inflammation. In the control group, the tendon tissue (on the right of d) has completely integrated with the surrounding subcutaneous tissue (on the left of d), indicating that the gliding of tendon (direction of gliding: into the paper) could be restricted during the healing period. A normal tendon is protected by a tendon sheath and hence does not integrate with the subcutaneous tissues. In fact, the membrane barrier serves as a sheath to protect the tendon tissue from integrating with its surroundings. However, a systematic study on the effectiveness of the membrane in

tendon adhesion prevention needs to involve a significant number of rabbits, a year-long period of time and a large amount of technical work, which is not possible to be included in the current research project. This is the limitation of the current animal study.

Figure 6.29: Anti-adhesion study at 2 months: repair site implantation in a rabbit model. (a)&(b): Gross view. (c) &(d): Cross-sectional view. Scale bar: 200 μ m.

Animal study can be performed on mouse, rat, rabbit, dog or pigs. The choice of an animal model depends on a number of factors, such as scientific objective, size of the animal and cost. Since this study is on tendon adhesion prevention, the main consideration is the size of the tendon. Mice and rat were not selected because their limbs are short and small. It is difficult to create reparable injuries on their tendons. Animals with larger tendons such as rabbit, dog or pig are therefore suitable. However, animal model selection should follow the minimum principle, i.e., selecting the

smallest animal that is sufficient for scientific experiment. Hence rabbit was selected in this study.

Although the animal experiment is preliminary (two rabbits) and involves only visual inspection and histological observation, it is a necessary step toward a large scale animal study that compares the efficacy of this membrane with other adhesion barriers, such as non-degradable barriers, non-porous barriers and fast-degrading barriers. Without the knowledge obtained from this study, the real situation of the low porosity membrane *in vivo* would have not been known. The current results are real and substantial, and have provided a strong indication that the membranes fabricated from surface tension melt are promising devices that can effectively prevent the adhesion of rabbit Achilles tendons. Therefore studies at a larger scale and in greater details can proceed in future.

CHAPTER 7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

This research has generated two novel methodologies, named microfiber melt drawing and surface tension melt, for the fabrication of a bundle of unfused microfibers and a low porosity membrane using biodegradable polymer. This research has also explored the potential of these unique microfibers and membranes for tendon graft replacement and prevention of post-operative adhesion, which are two significant problems commonly found in tendon surgeries. The major contributions of this research are as follows:

7.1.1 Development of microfiber melt drawing

Microfiber melt drawing, a novel method to create a bundle of unfused biodegradable microfibers has been developed. A mathematical model has been developed to understand it and identify the important parametric controls. A device prototype consisting of five subsystems has been designed and built. The device has been investigated under different fabrication conditions to produce required microfibers.

7.1.2 Development of surface tension melt

Surface tension melt, a solvent-free method to create a piece of thin membrane with a low porosity has been designed with three simple steps: preparation of a layer of particles on water surface, heating and cooling. This novel process has been validated with experiments and proven reproducible. Control parameters such as temperature and heating time have been studied, and the fabrication condition optimized.

7.1.3 Characterization of microfibers and membranes

Another contribution of this research is the investigation of various properties of these new microfibers and membranes. Both microfibers and membranes have been characterized by several methods including visual inspection, scanning electron microscopy, thermal analysis, porosity analysis and mechanical analysis. It has been found that the fabricated microfibers were a bundle of linear, unfused and biodegradable microfibers, with fiber diameter being as low as 10 μm and able to loosen and spread in a wet environment, largely resembling the collagen fibers found in native tendon tissues. The fabricated membranes were a low porosity, solvent-free and biodegradable membrane, with a thickness of 20 – 30 μm , average pore size of 16 μm , and average two dimensional porosity of about 1%. The stiffness of the membranes was $2.4 \pm 0.4 \text{ N/mm}$ and the ultimate tensile load was $1.6 \pm 0.3 \text{ N}$.

7.1.4 *In vitro* and *in vivo* evaluation of microfibers and membranes

Both microfibers and membranes have been evaluated *in vitro* and *in vivo* for their potential for tendon repair and adhesion prevention. *In vitro* studies have shown that both microfibers and membranes were able to support the proliferation of fibroblasts, demonstrating they were potentially suitable for tissue engineering scaffolds as well as for animal implantation. Subcutaneous implantation in nude mice has showed that unfused microfiber bundles were highly infiltrated by host tissue, largely owing to their unique characteristic in a wet environment. The results of rabbit Achilles tendon implantation have shown the infiltration of host tissue into the interior region of microfiber bundles and suggested a well aligned structure of the healing tissue. The results of anti-adhesion study have shown that low porosity membranes were effective in preventing tendon post-operative adhesion as compared to the control.

7.2 Future Work

7.2.1 Development of microfiber melt drawing

Although the microfiber drawing device is able to fabricate a bundle of unfused polycaprolactone microfibers, fabrication of similar microfibers from other biomaterials remains to be explored. Moreover, as the same device is able to fabricate a flat sheet of microfibers, production of a microfiber sheet with controlled spacing is another visible new research direction. The controlled spacing may be achieved by controlling the speed of the melt holder during its movement. The microfiber sheet with controlled spacing will be highly valuable to studies trying to understand the interactions between microfibers and a variety of cells such as fibroblasts, endothelial cells and nerves. Furthermore, extending the controlled spacing into three dimensions will be a huge challenge ahead but it is of paramount importance. An ideal biodegradable microfiber bundle, besides having an adequate fiber diameter and fiber alignment, should possess adequate spacing among microfibers as well. Currently, how fiber diameter and fiber spacing affect tendon regeneration in three dimensions remains largely unknown. Therefore, further development of the microfiber melt drawing technique toward a more advanced and sophisticated scientific apparatus will be essential for studying tissue-polymer interactions in three dimensions. More years ahead, commercialization of the microfiber bundle for tendon repair or for an enhanced result of tendon repair may start coming into sight if all the development and results are successful.

7.2.2 Development of surface tension melt

The current practice of surface tension melt still relies on manual operations. An automated device needs to be designed to replace the manual practice, and this will

involve a significant amount of work on conceiving novel and feasible ideas as well as on building prototypes for proof of concept. Also, there is a need to understand how the fabrication controls of the automated device affect the final results of a membrane, such as pore size, pore distribution and thickness. Mathematical modeling of the automated fabrication process and validation experiments will be needed, too. In fact, the most challenging task in surface tension melt is to control the pore size and porosity, which is, as understood currently, a natural and random occurrence. Deciphering the code of the random portrait and establishing an order in this chaotic event will enormously enhance our ability to precisely control the pores. Therefore looking for order will be the most important future work in the development of surface tension melt. An alternative explanation to the pore formation is the effect of vapor. When the vapor finds a way to go out, it may create new pores or may maintain the opening status of pores. However, this will depend on the pressure of the vapor and the surface tension of the molten material. A new setup would be needed as well to carry out the experiment. This work will help improve the quality of the membrane, making it more consistent for clinical applications.

7.2.3 Further *in vitro* and *in vivo* studies

Cell culture and animal study are critical in evaluating implantable medical devices such as microfiber bundles and adhesion barriers. The initial tissue responses to these biodegradable materials and structures cannot be completely known until they are tested in reality. The preliminary experiments conducted in a small number of animals have revealed the potential of polycaprolactone microfibers and membranes for tendon repair and adhesion prevention, and have set a base on which future works can be progressively carried out, that is, the observation that an injured tendon can be repaired by a polycaprolactone microfiber bundle and a polycaprolactone membrane

in the way they are designed to function. Future work will include the characterization of the repaired tendon over the entire healing period to understand the healing and regeneration events in a broader, bigger and more detailed manner. This work will involve a larger scale animal study over a longer period of time as well as a combination of various characterization techniques, such as electron microscope, histochemical staining, histological grading system, mechanical test and micro computed tomography. Moreover, future work will have to include the study of the degradation of polycaprolactone microfibers and membranes *in vitro* and *in vivo* to gain a full understanding of their behaviors. Furthermore, a study combining microfibers and membranes may be included in the study design to know if these two medical devices can work together to achieve an optimal repair.

7.3 List of Publications

7.3.1 Journal papers

- Ming-Yih Lee, Wen-Wei Tsai, Zhi-Hong Tang, His-Jung Chen, Jyh-Ping Chen, Chih-Hao Chen and Jia An, Laser sintered porous polycaprolactone scaffolds loaded with hyaluronic acid and gelatin-grafted thermo-responsive hydrogel for cartilage tissue engineering, Biomedical Materials (Under review)
- Jia An, Chee Kai Chua, Kah Fai Leong, Chih-Hao Chen and Jyh-Ping Chen, Solvent-free fabrication of a bundle of three dimensionally aligned polycaprolactone microfibers for engineering of anisotropic tissues, Biomedical Microdevices (Under review)
- Jia An, Chee Kai Chua, and Kah Fai Leong, Chih-Hao Chen and Jyh-Ping Chen, Solvent-free fabrication of a low-porosity membrane from

polycaprolactone micro-particles for tendon adhesion prevention, Biomedical Microdevices (In preparation)

- Jia An, Chee Kai Chua, and Kah Fai Leong, Three dimensionally aligned microfiber scaffolds for tendon tissue engineering, Acta Biomaterialia (In preparation)

7.3.2 Conference papers

- Chua, C. K., J. An, K.F. Leong, C.M. Cheah and H. Chang, Spinning of Biomaterial Microfibers for Tendon Tissue Engineering. Proceedings of 4th International Conference on Advanced Research in Virtual and Rapid Prototyping, Leiria, Portugal, 6 to 10 Oct 2009
- J. An, C.K. Chua, and K.F. Leong, Automated Fabrication of Three Dimensional Porous Microfiber Scaffolds for Tissue Engineering, 4th IEEE International Conference on Robotics, Automation and Mechatronics (RAM 2010), Singapore, 28-30 June 2010
- A. Liu, J. An, C.K. Chua, and K.F. Leong, A Portable Device for Fabricating Biomaterial Microfiber Bundles, ICoPE2010 and 13th ICPE Int'l Conference on Precision Engineering, Singapore, 28-30 July 2010
- A. Liu, J. An, C.K. Chua, and K.F. Leong, A Novel Membrane Formation Method: Inspiration from Duckweeds, 4th International Conference PMI2010, Ghent, Belgium, 15-17 Sep 2010
- J. An, C.K. Chua, and K.F. Leong, 3D Microfiber Scaffolds by Micro Melt Drawing Technique, 2010 International Symposium of Materials on Regenerative Medicine, National Health Research Institutes, Taiwan, 3-5 Nov 2010

- J. An, C.K. Chua, and K.F. Leong, Biodegradable Double Layer Cell Carriers for Tissue Engineering, 1st International Symposium on Bioengineering, Nanyang Technological University, Singapore, 19 Jan 2011

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APPENDICES

Table A1 and table A2 show the raw data of sample fiber bundles spun for one minute at orifice diameter of 2 mm and 3 mm respectively .

Table A1: Mass (mg) of sample fiber bundles spun at the orifice diameter of 2 mm.

2mm	100°C	110 °C	120 °C	130 °C	140 °C	150 °C
250 rpm	3.0	3.0	3.8	4.2	4.8	5.1
	2.7	2.5	3.5	3.9	5.2	5.0
	3.1	3.0	3.7	3.8	4.1	5.1
	2.7	3.1	3.3	3.9	4.5	4.9
	2.8	2.8	3.5	3.8	4.6	5.6
	3.2	3.0	4.1	4.0	5.0	5.3
500 rpm	3.4	3.5	3.8	4.3	5.2	5.0
	3.3	3.6	2.9	4.3	5.0	4.9
	3.1	3.1	4.6	4.2	4.6	4.7
	2.8	3.5	3.9	4.0	5.2	5.1
	3.7	3.7	3.6	4.3	5.0	4.7
	3.6	3.3	3.7	3.9	4.6	4.9
750 rpm	---	3.3	4.3	4.0	5.3	5.2
	---	3.4	4.1	4.6	4.8	5.5
	---	3.5	4.1	4.1	4.7	5.2
	---	3.6	4.1	4.4	4.8	5.1
	---	3.4	4.8	4.4	4.9	5.6
	---	4.0	4.3	4.6	5.0	5.0
1000 rpm	---	---	4.5	4.3	4.7	5.7
	---	---	4.0	4.3	4.5	5.2
	---	---	4.3	4.5	5.1	4.8
	---	---	4.3	4.4	5.1	5.1
	---	---	4.6	4.4	5.7	5.0
	---	---	4.4	4.2	4.5	5.7
1250 rpm	---	---	---	4.3	5.0	5.8
	---	---	---	4.8	5.4	5.5
	---	---	---	4.5	4.9	5.6
	---	---	---	4.3	5.3	5.2
	---	---	---	4.4	4.7	5.5
	---	---	---	4.3	4.5	5.5
1500 rpm	---	---	---	---	4.5	5.8
	---	---	---	---	5.3	5.9
	---	---	---	---	5.0	5.9
	---	---	---	---	5.2	5.9
	---	---	---	---	5.0	5.7
	---	---	---	---	5.0	5.4

Table A2: Mass (mg) of sample fiber bundles spun at the orifice diameter of 3mm.

3mm	100 °C	110 °C	120 °C	130 °C	140 °C	150 °C
250 rpm	11.3	12.2	13.5	15.8	18.1	18.8
	10.9	11.8	13.5	15.0	17.5	20.3
	10.8	11.8	12.6	14.5	17.4	19.9
	10.8	11.8	13.1	15.8	17.2	19.5
	10.7	11.8	12.6	15.3	17.3	19.3
	10.9	11.7	13.1	15.1	18.1	19.9
500 rpm	11.6	11.8	13.1	15.1	17.8	20.6
	11.7	12.2	12.7	15.2	16.3	19.6
	11.8	12.1	14.3	15.3	16.8	19.9
	12.1	12.4	13.4	16.9	17.0	19.5
	13.3	12.5	12.7	16.7	17.1	18.7
	12.0	13.5	13.7	15.1	17.3	19.9
750 rpm	---	8.6	13.5	16.2	17.6	19.6
	---	12.4	13.8	15.0	16.3	20.7
	---	12.2	13.3	15.1	16.7	18.8
	---	13.1	13.2	15.2	16.7	18.5
	---	13.2	13.2	15.4	17.4	19.8
	---	12.4	13.4	15.3	18.3	20.0
1000	---	12.2	14.1	15.7	17.0	19.2
	---	13.7	14.3	15.3	18.1	19.4
	---	12.8	14.8	15.5	19.6	20.4
	---	13.8	13.1	15.3	18.6	18.8
	---	14.6	13.5	16.2	18.6	19.2
	---	13.1	14.6	15.0	18.9	19.3
1250 rpm	---	---	14.4	15.5	17.3	20.1
	---	---	13.8	15.1	17.8	18.2
	---	---	14.0	15.8	17.6	18.1
	---	---	14.0	15.7	17.1	20.0
	---	---	14.8	15.7	17.6	19.7
	---	---	13.9	16.3	17.3	19.5
1500 rpm	---	---	17.7	15.0	17.5	18.7
	---	---	14.3	15.4	16.6	19.4
	---	---	15.0	16.2	18.5	20.3
	---	---	14.8	16.2	17.7	21.2
	---	---	15.2	15.7	17.9	20.2
	---	---	14.9	15.3	18.5	19.0

Table A3: Fiber diameters measured by SEM images (Number of samples: 16).

21.2	8.9	17	8.2	21.7	10.4	12.6	17.6	15.1	8.2	10.4	17.6	10.9	16.1	8.8	21.2	11.7	25.2	10.5
18.2	9	16.4	8.2	23.2	11.4	11	19.3	16.9	9	9.7	20.1	12.1	16.1	8.6	21.3	11.5	24.3	10
22.8	8.8	17.1	8.4	23.4	10.7	12.7	18.6	18.6	8.8	10	17.3	10.5	17	8.8	22.3	11.7	18.7	9.6
17	8.7	15.6	8.9	17.9	10.2		20.1	17.7	9.2	9.3	19.5	10.8	16.2	8.5	22.5	11.2	21.5	10.4
19.5	9.2	16	8.4	17.1	10.9		16.3	18.8	8	8.7	19.1	9.7	16.9	8.9	25.4	11.4	19.3	9.8
16.4	9.1	16.4	8.9	16.7	12.1		18		8.2	9.5	19.8	10.9	15.8	10.3	24.1	10.8	28	9.8
16.3	9.9	16.2	9.9	21.3	12.1		17.5		12.1	8.5	17.5	10.7	16.9	8.1	22.9	10.2	26.5	10.6
16.6	9.2	16.6	9.8	21.4	10.9		17.4		8.3	9.8	20.3	9.7	14.2	7.9	23.5	10.2	17.3	10
	9.2	15.7	9.07		11.6		16.4		8.1	9.4	19.9	10.9	13.7	8.1	23.2	11.9	16.9	10
	10.1	16.4	8.8		11		18		8.2	8.9	16.6	10.3	14.5	7.9	19.7	11.5	23.9	10.4
	8.9	15.7	8.4		12		16.8		9.7	11.3	16.3	10.9	15.9	8.3	22.2	12.2	22	10
	8.5	15.8	10.2		10.4		14.8		10.9	11.6	21.1	9.9	17.9	8.4	20.1	10.6	20.1	9.8
	8.5	16.8	8.8		11.9		15.3		11.9	9.6	20.8	9.7	16.2	8.7	20.1	10.3	20.7	11.6
	9.6	16.8	10		11.1		16.1		9.2	9.7	20	10.8	14.7	8.5	23.1	9	17	10.5
	8.7	16.8	9.5		10.7		16.4		9	8.6	19.9	9.7	15.3	8.5	19.7	11.7		10.3
	9.2		9.7		12.2		16.1		8.6	9	15.6	9.8	15.7	8.9	20	12		9.7
	9.5				11.2		18.2		11.2	10.6	15.6	10.1	16.7	8.2		12.3		9.5
	9.4				10.6		19.1		11.5	10.6	22.9	11.1	16.2	8		13.6		9.9
	9.1				11.9		17.2		10.8			10.9	16.7	8		11.6		9.5
	8.5				12.5		16.2		8.4			10.3	17	8.7		12.4		9.3
	8.6				11.3		17.2		11.5			10.1	16.7	8.4				
	9.2				11.3		16.6		12			10.6	16.7					
	9.5				11.3		16.6		8.4			9.5	16.2					
	9				12		17.2		9.1			10.1						
	8.5				11.1		15.4		9.4			10.3						
	8.6				11.1		16.4		9.3			10.2						
	8.7				11.5		21		8.5			11.1						

Table A4: Mechanical tests of microfiber bundles.

1 min	Linear density (denier)	Break Force(N)	Elongation (%)	Stiffness(N/mm)
1	0.9	14.6	135.20	0.94
2	0.9	18.5	145.14	1.05
3	0.8	14.4	139.17	1.09
4	1.0	18.4	145.14	1.14
5	1.0	18.3	151.10	0.99
6	0.9	17.6	143.15	1.53
7	0.8	15.5	139.17	1.04
2 min				
1	0.9	33.4	149.11	1.30
2	1.0	34.1	153.09	1.52
3	0.9	32.2	153.09	1.55
4	1.0	27.9	155.08	1.50
5	0.9	37.0	141.16	1.82
6	1.0	28.4	145.14	1.85
7	0.9	28.4	145.14	1.85

Table A5: Membrane thickness measurement (Number of samples: 6).

Reading	1	2	3	4	5	6
1	32.0	26.0	23.0	21.0	21.0	26.0
2	36.0	30.0	22.0	25.0	22.0	25.0
3	34.0	29.0	25.0	24.0	26.0	24.0
4	32.0	28.0	24.0	23.0	24.0	24.0
5	29.0	28.0	23.0	28.0	30.0	25.0
6	36.0	23.0	28.0	30.0	23.0	29.0
7	31.0	26.0	28.0	29.0	28.0	27.0
8	35.0	23.0	27.0	26.0	26.0	29.0
9	32.0	23.0	31.0	27.0	27.0	28.0
10	37.0	27.0	32.0	29.0	28.0	24.0

Table A6: Mechanical tests of membranes.

Sample	Stiffness (N/mm)	Break Force (N)
1	2.6	1.7
2	1.7	1.05
3	2.2	1.65
4	2.8	1.9
5	2.4	1.45
6	2.4	1.7
7	2.4	1.65
8	2.0	1.25
9	3.2	2.05
10	2.4	1.8

Table A7: Pore size measurement.

Sample 1

14	15	5	49	15	13	9	13	6	10	23	18	10	15	6	13	66	19
13	11	6	60	13	20	8	11	6	8	13	26	20	4	4	13	16	14
19	10	11	24	15	8	13	11	6	26	18	21	10	6	11	23	18	14
21	9	10	39	11	14	10	13	14	9	14	23	15	6	9	6	18	4
11	10	25	11	14	23	15	20	11	6	35	19	15	20	24	8	21	13
16	13	11	45	16	14	19	8	11	14	15	10	11	18	36	20	16	15
18	11	10	10	11	28	6	13	14	15	24	34	15	18	14	13	10	6
25	30	13	16	5	9	16	14	8	15	19	14	16	20	76	6	9	9
11	13	21	44	14	11	15	20	26	20	21	25	13	16	29	13	9	15
31	10	11	16	46	14	10	14	14	14	14	9	16	6	11	18	9	26
45	18	30	61	21	14	5	15	9	10	14	54	18	6	11	8	10	44
9	22	15	23	10	10	11	14	19	16	6	26	9	8	15	26	14	28
10	8	18	13	8	18	9	21	10	14	13	41	10	11	15	16	16	15
33	9	9	8	14	15	11	23	16	20	11	13	16	14	11	13	10	10
19	25	14	38	11	19	14	33	5	32	9	19	9	9	20	13	28	13
19	14	16	25	14	23	9	29	14	14	16	31	11	14	71	21	21	18
18	8	21	13	14	14	6	14	11	18	16	23	20	9	11	23	15	20
31	20	11	23	35	10	21	11	9	11	18	16	30	9	8	8	11	10
33	14	14	19	9	28	10	14	10	11	15	11	10	20	31	9	14	18
44	23	21	24	13	10	18	23	11	10	38	38	16	25	69	9	8	25
15	8	34	15	15	13	13	11	10	13	9	8	11	30	9	11	25	19
14	8	11	38	28	3	9	8	28	10	10	10	23	12	6	21	11	11
10	13	11	34	13	5	9	16	10	11	10	31	15	8	6	9	33	10
15	10	15	23	16	8	11	29	14	24	10	39	6	13	13	9	11	23
15	10	8	21	10	4	35	11	15	10	20	8	20	9	26	13	14	18
14	10	13	39	11	18	20	11	8	9	14	8	8	38	6	6	5	15
14	13	25	33	26	14	20	23	11	11	28	30	14	14	20	18	11	18
14	9	19	9	8	13	11	21	19	14	10	16	8	25	8	11	18	5
10	11	9	20	16	9	18	18	15	10	11	58	16	4	13	15	9	9
14	10	16	23	25	9	10	11	24	13	16	33	15	10	49	6	19	15
8	10	13	16	14	10	23	10	24	11	29	13	5	10	18	10	11	24
13	19	21	8	4	21	21	6	15	66	25	80	15	8	44	13	13	8
14	30	9	10	24	9	8	35	6	19	16	18	41	20	9	23	20	15
11	23	15	6	13	8	15	30	24	10	13	46	18	8	16	11	15	18
18	32	35	34	9	18	15	24	13	22	25	75	10	9	13	8	16	11
18	6	15	39	29	15	4	23	10	15	20	14	13	11	14	21	28	
9	27	11	55	14	19	9	14	23	10	23	33	15	10	14	8	13	
10	16	13	33	11	5	13	74	10	32	10	75	14	14	11	18	9	
19	9	10	30	10	13	9	19	59	30	10	50	10	18	28	14	11	
18	10	28	63	9	8	13	10	21	13	14	9	51	18	11	10	10	
43	10	8	25	9	10	8	19	11	11	30	20	9	6	19	8	11	
6	15	13	8	9	18	11	24	11	22	14	16	8	16	9	9	13	
16	16	15	6	13	10	14	19	38	18	10	21	13	14	9	13	15	
10	10	16	9	25	10	10	6	15	10	9	16	43	16	8	13	49	

13	11	39	11	10	24	16	9	15	14	15	55	6	11	18	11	24
14	18	30	8	11	6	9	11	16	14	14	11	9	5	8	15	19
10	14	16	23	10	9	10	10	10	13	16	30	9	9	5	9	39
13	9	11	16	6	9	6	19	11	32	11	45	19	14	34	16	19
18	9	6	11	14	11	10	25	8	23	15	16	19	15	14	11	8
23	9	14	13	14	13	23	29	23	32	15	59	3	14	14	13	23

Sample 2

13	33	16	20	10	28	18	11	14	16	10	18	6	25	23	13	9	33	10
10	14	10	19	11	19	23	9	10	6	23	14	13	14	28	13	13	24	8
16	11	13	14	8	10	16	19	9	20	19	2	31	20	20	15	11	25	10
48	18	11	28	31	20	15	34	20	20	33	23	45	8	14	13	13	23	13
11	24	13	18	19	16	6	11	9	18	10	30	16	4	30	44	26	11	11
9	15	11	20	23	16	6	21	13	11	18	9	11	28	13	8	19	10	29
24	13	10	14	18	19	18	28	21	8	25	6	18	5	13	8	11	30	21
13	11	15	8	30	9	55	13	10	41	18	8	25	23	24	18	33	11	21
14	11	23	8	29	8	15	33	8	16	15	14	11	8	19	29	14	14	10
10	14	18	16	25	20	19	63	8	26	11	14	14	11	13	15	29	23	8
9	8	18	20	9	10	8	23	14	11	14	6	20	16	10	16	10	18	13
8	13	33	5	14	11	14	41	21	30	19	6	21	23	19	9	18	14	13
16	10	18	11	13	23	4	9	25	19	11	10	14	8	16	6	11	18	4
11	23	18	11	11	28	10	6	14	10	10	16	11	15	18	9	8	13	24
9	19	14	10	16	13	9	19	18	16	20	40	11	13	25	13	14	10	15
18	15	15	18	11	18	9	11	71	9	11	10	14	9	11	19	19	18	10
9	16	10	24	8	8	8	10	95	11	21	9	15	5	30	43	20	10	8
30	11	13	16	25	20	23	21	23	16	16	6	5	6	10	11	5	18	9
16	13	16	18	18	6	18	25	5	10	11	45	26	11	23	18	6	15	16
9	29	21	16	40	11	8	25	8	13	10	21	21	14	19	9	16	19	19
9	19	11	13	16	13	10	24	19	11	15	13	6	10	31	10	9	16	13
10	44	35	21	11	10	5	8	13	11	15	14	8	18	18	19	9	29	20
18	11	28	10	8	25	6	24	15	10	9	13	24	3	6	10	19	15	4
13	13	12	15	21	9	28	16	14	20	15	43	8	20	6	10	39	13	8
13	29	14	9	14	9	28	15	6	14	16	16	9	8	18	10	16	8	18
21	18	28	20	23	13	4	20	15	14	19	21	6	5	28	24	10	19	11
10	14	18	21	19	14	49	30	11	14	10	15	6	9	13	10	21	14	11
8	28	16	15	18	14	20	13	13	13	8	21	34	20	16	8	8	10	13
11	15	10	19	16	11	29	45	13	26	14	14	11	11	13	31	11	18	9
10	21	14	93	10	13	9	21	38	9	28	18	16	30	23	16	9	9	26
21	16	16	20	21	35	11	21	10	8	11	10	8	18	14	6	6	13	9
33	16	15	14	20	8	9	15	23	10	16	13	14	8	11	9	16	11	15
11	10	19	38	16	6	29	15	13	3	18	11	15	5	26	18	15	9	6
18	13	15	16	34	10	41	9	10	16	18	14	20	13	8	13	16	31	24
16	20	10	6	9	11	6	18	9	14	24	25	5	20	10	9	13	14	16
11	20	16	9	29	6	6	21	16	14	10	24	24	19	13	19	16	13	25
13	11	14	5	39	11	38	24	16	14	14	19	25	10	35	34	33	11	5
15	16	16	19	14	15	4	13	8	11	15	24	19	30	5	13	11	8	41

15	15	14	10	9	14	11	14	8	9	13	26	11	8	11	9	18	19	11
14	16	15	11	49	4	6	9	13	13	8	15	16	8	6	10	13	19	10
16	29	11	6	19	14	8	24	10	18	11	13	11	11	19	8	9	14	9
19	11	24	8	10	11	23	14	16	13	29	34	18	28	8	24	23	51	31
8	18	14	11	15	19	9	25	13	8	19	11	10	10	29	14	19	18	20
23	18	21	10	24	11	5	21	15	13	13	14	16	13	8	9	10	39	29
20	21	25	18	15	16	3	14	8	10	11	16	9	15	15	5	9	16	6
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Sample 3

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