Regulation of smooth muscle cells using UV embossed microchannelled polymeric films

Shen, Jinye

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Regulation of Smooth Muscle Cells Using UV Embossed Microchannelled Polymeric Films

Shen Jinye

School of Mechanical & Aerospace Engineering

A thesis submitted to the Nanyang Technological University
in fulfilment of the requirement for the degree of
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Table of abbreviations

2-D	two-dimensional
3-D	three-dimensional
bFGF	basic fibroblast growth factor
EC	endothelial cell
ϵ -CL	ϵ -caprolactone
ECM	extracellular matrix
ePTFE	expanded polytetrafluoroethylene
FDA	Food and Drug Administration
GA	glycolide
LA	lactide
LBL	layer-by-layer
MMP	matrix metalloproteinase
Mn	number-average molecular weight
PBS	phosphate buffered saline
PCLLGA	poly(ϵ -caprolactone-co-lactide-co-glycolide)
PCL	poly(ϵ -caprolactone)
PDGF	platelet-derived growth factor
PDMS	polydimethylsiloxane
PGA	poly(glycolic acid)
PLLA	poly(L-lactide)
ROP	ring opening polymerization
SMC	smooth muscle cell
SIS	small intestine submucosa
TGF	transforming growth factor
TE	tissue-engineered
Tm	melting point
UV	ultra-violet
WHO	World Health Organization

SUMMARY

The reconstruction of a functional vascular tunica media, with circumferentially oriented contractile smooth muscle cells (SMCs) and extracellular matrix, appropriate mechanical properties and vasoactivity has yet to be demonstrated for tissue engineering of small diameter blood vessels. Synthetic SMC is desired for cell proliferation needed for vessel remodeling but the contractile phenotype is necessary for sufficient vasoactivity and inhibition of neointima formation. The switch of SMC phenotype from synthetic to contractile at the appropriate cell development stage has been hypothesized to be necessary for producing a functional vascular tunica media.

This study aims to regulate the phenotypic switch of SMCs using three-dimensional (3-D) microchannels in biodegradable photocrosslinked polyester films. 3-D microchannels (~65 μ m deep and 10-160 μ m wide) separated by high aspect ratio (8) microwalls were made by Ultra-violet embossing using a poly(ϵ -caprolactone-co-lactide-co-glycolide) (PCLLGA) diacrylate. We investigated the effects of the micropatterns and seeding densities on SMC responses.

Wide microchannels (80 to 160 μ m wide) were found to regulate the SMC morphology and orientation only nearing confluence. At low cell density, synthetic morphology and the desired cellular proliferation were observed. Nearing confluence, the physically constraining microwalls caused SMCs to show elongated morphology and orientation along the microwalls and up-regulated expression of contractile α -actin protein, indicating a switch to a more contractile phenotype. The seeding densities of SMCs on the microchannelled films (160 μ m wide) affect the incubation period necessary for SMC elongation, orientation and up-regulation of smooth muscle α -actin expression; higher seeding density resulted in more rapid cell elongation,

orientation and phenotype switch. The cellular proliferation as well as phenotype switch upon confluence suggests that these biodegradable 3-D microchannelled scaffolding films hold promise for guiding SMCs to grow into functional vascular media. A rapid rate of alignment and phenotype switch would be needed for fabricating vascular media comprising multiple layers of circumferentially oriented SMCs on a timescale short enough to be meaningful for clinical applications.

The biodegradable substrate should have tunable tensile properties and degradation rate. A new series of liquid photopolymerizable linear PCLLGA diacrylate with different molecular weights (M_n : 1800, 4800 and 9300 Da) and compositions (20%, 40% and 60% ϵ -caprolactone (ϵ -CL)) was synthesized. Upon UV irradiation, the linear PCLLGA reacted into highly crosslinked networks within minutes and the resulting polymer films are transparent and rubbery with high elongations at break (more than 50%) and moduli varying from 12.3 MPa to 1.7 MPa. *In vitro* degradation tests of these networks show a two-stage degradation behavior. In the first stage, the Young's modulus and ultimate stress were found to decrease linearly with time whereas their initial extendibility (maximum strain) was retained which was favorable for vascular tissue engineering. Lower molecular weight and lower ϵ -CL content led to a faster decrease in Young's modulus. In the second stage, which began when the Young's modulus dropped below 1 MPa, there was rapid weight loss and strain increase. The lower the ϵ -CL content, the earlier the second stage happened. By varying the molecular weight and the composition, the polymers can be adjusted to maintain their tensile properties for time periods ranging from several weeks to several months. This flexibility should make these networks attractive materials for vascular tissue engineering or a wide range of other biomedical applications. Films formed from these new polymers have excellent biocompatibility with SMC.

Chapter 1 Introduction

1.1 Background

Cardiovascular diseases are the leading cause of death and disability in the United States and most European countries ¹⁻³. For patients who require vascular bypass surgery, autologous veins are the preferred replacement but approximately 60% of them do not have a suitable vessel for grafting due to previous operations, size mismatch or venous disease ³. There is thus an urgent need for a blood vessel substitute ^{1, 4, 5}. Small diameter grafts needed for coronary artery bypass graft or peripheral vascular repair below the knee fail *in vivo* when synthetic materials such as Dacron or polytetrafluoroethylene are used; thrombogenicity, compliance mismatch, and neointima formation are the main failure causes ^{1,3,5}. Researchers have thus spent great efforts in developing biological small diameter substitutes and have achieved encouraging results ^{6,7}. Tissue engineering is one such approach. A functional small diameter vascular graft must exhibit all the functional characteristics of a normal blood vessel, including nonthrombogenicity, sufficient burst pressure and suture retention strength, viscoelasticity, appropriate remodeling responses and vasoactivity ^{8, 9}. It must also be cheaply manufacturable in a relatively short space of time, and in sufficient numbers with differing specifications (diameter, length, etc.). However, current tissue engineering approaches are unsatisfactory with many problems including thrombogenicity, inappropriate mechanical properties and vasoactivity, and extensively long culturing time ^{1, 5, 10, 11}.

There are three major types of blood vessels: muscular arteries, elastic arteries, and veins. This thesis focuses on a biosynthetic replacement for the muscular arteries, i.e., coronary arteries and femoral-popliteal (below the knee) arteries that are most

severely affected by atherosclerosis. A blood vessel is typically composed of three concentric layers: the tunica intima, the tunica media and the tunica adventitia. The tunica media significantly contributes to the tensile strength, compliance, and vasoactive response of the blood vessel and composed of circumferentially oriented smooth muscle cells (SMCs) and the secreted extracellular matrix (ECM), such as collagen (mainly type I) and elastin fibers. The plasticity of SMCs, i.e. SMCs cultured *in vitro* can assume synthetic or contractile phenotype^{5, 12}, needs to be exploited to achieve a functional graft. A synthetic phenotype of SMC allows the cells to rapidly proliferate and produce ECM whereas a shift to contractile phenotype with organized ECM at the appropriate developmental stage would provide a functional and quiescent vascular media. In addition, there are two other significant components of the muscular arteries. These are the internal elastic lamina (IEL), and the external elastic lamina (EEL), which separate the intima/media and media/adventitia, respectively. These are principally composed of elastic fibers and provide the compliance and elasticity to the muscular arteries *in vivo*.

Numerous efforts have been spent to regulate the phenotype, orientation, and ECM production of SMCs; these include mechanical and biochemical stimulation, and two-dimensional (2-D) adhesive protein micropatterning¹³⁻¹⁵. Cyclic mechanical strain has been shown to achieve the desirable histological organization and ECM production¹⁶⁻¹⁸ but usually long time of mechanical stimulation (usually several weeks) is needed. Mechanical stimulation was also found to increase the expression and activation of matrix metalloproteinase-2 (MMP-2) which might cause the undesirable disintegration of the matrix¹⁰. Growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor- β 1 (TGF- β 1), have been shown to affect

SMC phenotype but long-term patent grafts constructed with short culturing periods have not been achieved.

Others have used porous polymeric scaffold as scaffold for SMCs¹⁸. However, porous scaffold does not possess specific cues for guiding functional smooth muscle tissue development. 2-D protein micropattern whereby SMCs proliferate and align in the relatively narrow adhesive region as a monolayer has also been investigated^{14,19}; regulation of cell morphology and orientation was observed but cell growth and proliferation necessary for tissue regeneration were inhibited. However, these cells are growing as a monolayer and there is a need for a novel scaffold that can promote the cells to grow into 3-D tissues. A biomimetic scaffold can appropriately regulate the phenotype shift of SMC²⁰⁻²² and guide the three-dimensional (3-D) media re-creation. The scaffold for re-creation of a functional smooth muscle media must also be elastic, biocompatible with controllable degradation properties (i.e. mechanical strength and degradation products) and have suitable morphology. The degradation of the scaffold has been shown to affect SMC phenotype²³. The undesirable acidic environment due to rapid degradation of commonly used poly(glycolic acid) (PGA) scaffold in vascular tissue engineering causes SMCs to modulate to the synthetic phenotype²³. Further, the exposure to mechanical stretch or strain due to pulsatile blood flow needs a scaffold to sustain the systemic pressure and provide integrity during tissue development. Control of the biodegradation rate and products are thus important scaffold material design parameters. The scaffold should also be easily, reproducibly, timely and economically manufactured into a variety of shapes and sizes.

3-D microchannelled biodegradable films in which cells align in the microchannels are developed in this study (Fig 1.1). Ultra-violet (UV) embossing was applied for

fabricating the microchannelled film. The UV embossing process was adapted to produce fully biodegradable microchannelled film. The 3-D microchannel would potentially allow re-creation of the native-like 3-D vascular media eventually. Detailed studies of the effect of seeding densities and microchannel width on the SMC proliferation, alignment, elongation and phenotype switch were carried out. A series of novel biodegradable, biocompatible and photopatternable polymers are needed for the UV embossed scaffold.

Figure 1.1 Proposed 3-D microchannelled film. (a) Flat film, and (b) folded tubular structure.

1.2 Objective and Scope

The overall objective of this PhD project is to regulate the phenotypic switch of SMCs using our unique UV embossed microchannelled polymeric films made from biocompatible, biodegradable and photocrosslinked diacrylated polyester. The microchannelled polymeric films were made using poly(ϵ -caprolactone-co-lactide-co-glycolide) (PCLLGA) diacrylate (Scheme 1) by UV embossing (Scheme 2). The photocrosslinkable polymer is dispensed over a microstructured mold (usually a polydimethylsiloxane (PDMS) mold), covered with a temporary transparent carrier

and hardened in place. The hardened micropattern replica is then removed from the mold. The embossing is quick and can be easily adapted to mass production. After the removal of the temporary carrier, the microchannelled scaffold was composed of exclusively biodegradable materials when biodegradable PCL/LGA diacrylate was used in this study. The crosslinked film can have mechanical properties and degradation rate tunable to match the tissue regeneration rate so as to allow the proper reconstruction of a functional vascular smooth muscle media tissue.

Specifically, the objectives of this project are to:

- 1) Design and synthesize a liquid amorphous oligomer based on ϵ -caprolactone (ϵ -CL), L-lactide (L-LA) and glycolide (GA) monomers and endcapped with photocrosslinkable acrylate groups (Scheme 1).
- 2) Investigate the mechanical properties and degradation behaviors of the UV crosslinked polymers from the above oligomers.
- 3) Make microstructured polymeric films with microchannels separated by high aspect ratio microwalls using the oligomer synthesized in (1) above via UV embossing (Scheme 2).
- 4) Study the effect of different widths of the microchannels on SMC morphology, orientation and phenotype (Fig 1.1).
- 5) Examine the effect of different initial seeding densities on SMC behavior in the microchannels.

Scheme 1 Proposed chemical structure of PCLLGA diacrylate.

Scheme 2 UV embossing of a biodegradable microchannelled polymeric film.

1.3 Outline

This report is divided into 6 chapters. The first Chapter covers the background and objectives of this PhD project. The literature review is presented in Chapter two. Chapter three describes the synthesis of liquid photopolymerizable and biodegradable diacrylated PCLLGA oligomers and the degradation study of the corresponding UV crosslinked polymers. In the fourth Chapter, microchannelled polymeric films using the photopolymerizable PCLLGA diacrylates were fabricated and the response of

SMCs (i.e. cell proliferation, alignment, morphology and α -actin expression) to the microchannel width (varying from 10 to 160 μ m) was investigated. The effect of the initial seeding densities (10,000, 100,000, and 200,000 cells/cm²) on SMC response to the micropatterns was examined in Chapter five. In Chapter six, conclusions are given, salient observations are highlighted, and recommendations for future works are suggested.

Chapter 2 Literature review

2.1 Anatomy of muscular arteries

Blood vessels distribute blood to every part of the body; bring oxygen and nutrients to and carry away waste products from various organs; and form the body's circulating system together with the heart. The vascular network consists of a branched system of arteries, capillaries and veins that vary in size, mechanical properties, biochemical and cellular content, and ultrastructural organization, depending on their location and specific function. When the heart pumps, blood moves out of the heart passing through arteries of diminishing size to network of tiny capillaries and back to the heart via veins of increasing caliber. The blood vessels are elastic and dilate and contract as the heart pumps to move blood around the body. Blood vessels that carry blood away from the heart to the organs are called arteries. As the arteries get further from the heart, vessels are getting successively smaller in caliber size and greater in numbers. The arteries range in size from aorta which is about 25mm in diameter in man to smaller arteries (arterioles) (about 300 μ m to less than 50 μ m). The structure of their walls varies depending on their functions. Arteries are the thickest blood vessels as they are the higher-pressure portion of the circulatory system.

There are three major types of blood vessels: muscular arteries, elastic arteries, and veins. Elastic arteries are large arteries such as aorta with very high blood pressure and flow to transport blood from heart whereas muscular arteries are smaller arteries deliver blood from large arteries to tissues and organs. This thesis focuses on a biosynthetic

replacement for the muscular arteries, i.e., coronary arteries and femoral-popliteal (below the knee) arteries that are most severely affected by atherosclerosis.

The muscular arteries are typically composed of three concentric layers^{11, 24} (Fig 2.1). The innermost layer, the tunica intima, consists of a monolayer of longitudinally oriented endothelial cells (ECs) which has direct contact with blood constituents. The intermediate layer, the tunica media (made up of circumferentially oriented SMCs and ECM such as collagen (mainly type I), elastin and proteoglycans) provides the structural integrity and controls blood flow. The outer layer, the tunica adventitia, is composed of fibroblasts and fibrous elements. The boundary between the tunica intima and tunica media is marked by the internal elastic lamina (elastic interna), which is especially well developed in arteries of medium caliber. Between the tunica media and the tunica adventitia, a thinner external elastic lamina (elastic externa) is also present in many arteries. These are principally composed of elastic fibers and provide the compliance and elasticity to these muscular arteries *in vivo*.

Figure 2.1 Graphic representation of the cross section of a small muscular artery, showing the concentric arrangement of tunica intima, media, adventitia, internal and external elastic lamina ²⁵.

2.2 Vascular diseases

Cardiovascular diseases, including coronary artery disease, peripheral vascular disease and cerebrovascular disease (stroke) (Fig 2.2), are the leading cause of death and disability in the United States and most European countries ^{1, 3}. Over 71.3 million Americans suffer from cardiovascular problems ²⁶. The most widespread form of cardiovascular disease worldwide starts with damage to the blood vessels, a few of which are listed below.

Figure 2.2 The cardiovascular disease triad²⁷.

Atherosclerosis is the hardening of blood vessels due to formation of plaques within the arteries and it is the principle pathology that leads to **ischemia** (inadequate or restriction of blood supply to a part of the body caused by constriction or blockage of the blood vessels) and possibly **infarction** (necrosis or tissue cell death of an organ due to prolonged ischemia). The earliest phase in atherosclerosis is the formation of fatty streaks accumulated by lipid-filled foam cells, such as macrophages and T cells (Fig 2.3). The fatty streaks will progress into atheroma with foam cells and extracellular lipids in the core region and surrounded by SMCs and collagen-rich matrix. Hardening of the plaque and artery occurs and a complicated lesion forms. Progression continues by the increase

of the size of the lipid pool in the plaque resulted from high serum cholesterol, hypertension, diabetes, etc. Increase of plaque size progressively decreases the luminal cross-sectional area, making it harder for the blood to flow through. Atherosclerotic plaques can rupture, continue to grow and then clot resulting in complete occlusion of the artery. Coronary artery disease due to atherosclerosis is the predominant cause of cardiovascular morbidity by a narrowing or clogging of the coronary arteries which supply the heart with oxygen and nutrients for the normal function. In America, 20% of patients with coronary artery disease died and approximately 500,000 coronary artery bypass graft surgeries were required in 2000 ³. The inadequate blood flow to the heart muscle causes a shortage of oxygen to heart muscle (**cardiac ischemia**), which initially leads to **angina** (chest pain) and can progress to **myocardial infarction** (heart attack) when the coronary arteries are completely blocked. In the case of brain and cerebral circulation, atherosclerosis may cause **stroke**. Atherosclerosis of lower limb arteries retards blood flow to the legs or arms, causes pain or even **gangrene** (death of tissue due to a loss of blood supply) of a limb, and may lead to eventual amputation.

Hypertension, a condition that blood pressure is persistently elevated, is another main process damaging the blood vessel. Normal systemic pressure is around 120/80 mmHg (systolic/diastolic) and pressure above 160/90 mmHg is generally considered as systemic hypertension ²⁸. Hypertension damages the smaller vessels, making them scarred, narrowed and less elastic over time. Patients with hypertension are more susceptible to the development of atherosclerosis and atherosclerosis can be accelerated by hypertension.

Figure 2.3 Progression of atherosclerosis^{29, 30}.

Thrombosis is the formation of thrombus (blood clot) in uninjured vessels or after relatively minor injury, obstructing the blood flow. It is a pathologic condition where normal hemostatic processes are inappropriately activated. It is mainly caused by the physical loss of endothelium or endothelial dysfunction resulted from stasis or turbulence of blood flow. The injury or activation of ECs leads to the production of von Willebrand factor that promotes platelet adhesion, the synthesis of tissue factor that augments the

coagulation and the secretion of inhibitors that suppress fibrinolysis; and these result in or augment the blood clot formation.

2.3 Historical replacements

There is a great need for vascular grafts to replace both peripheral and coronary vessels affected by arterial diseases or complications from other diseases such as diabetes. In many cases, the autologous saphenous vein or the patient's internal mammary artery is the ideal replacement vessel for bypass grafting^{1, 31, 32}. The anti-thrombogenic luminal endothelium, biological construction, compliant natural tissue characteristics and immune acceptance make the autologous arteries or veins ideal substitutes for diseased arteries. The internal mammary artery was found to be superior to saphenous vein and has become the preferred replacement vessel, although the anastomosis of the internal mammary artery was found to be more difficult and need longer operating time. Generally, 80% of these autologous internal mammary artery substitutes versus 40 to 50% of saphenous vein substitutes remain patent 10 years after grafting^{3, 5, 33}. The possible reasons of the superior performance of the internal mammary artery are supposed to be due to its superior endothelial function, better comparability in size to the coronary artery, and better blood supply via vasa vasorum^{33, 34}. Although being successfully used as coronary artery bypass grafts, healthy autologous arteries or veins are not available for grafting for many patients who require vascular bypass surgery^{3, 5}. Furthermore, it is not desirable to remove them from their normal positions.

Artificial materials, such as Dacron and expanded polytetrafluoroethylene (ePTFE) have been used as vascular grafts^{6, 35, 36}. Synthetic grafts have been applied successfully

to large diameter (6-10mm) or high flow blood vessels ^{6, 7}. Unfortunately, when applied to low-flow or smaller diameter sites (3-5mm), these synthetic grafts have shown poor patency rate mainly due to the thrombogenic problem or anastomotic hyperplasia. Various efforts have been spent to improve the antithrombogenicity of the graft surface through heparin coating, fibrin coating, or EC seeding ^{6, 36}. The results are promising yet mixed. Aside from this, these synthetic grafts are generally rigid; their compliance mismatch with the native vessels induces anastomotic hyperplasia at the anastomosed site. Anastomotic hyperplasia leads to both low flow and turbulent flow with the increased ability of thrombotic stenosis or occlusion of the synthetic graft ⁵. Another major disadvantage of these artificial grafts lies in the fact that they cannot grow, remodel, or repair *in vivo* ^{6, 37}.

2.4 Tissue engineering of small diameter vascular grafts

To obtain a biological substitute, tissue engineering has been applied to fabricate biological small diameter blood vessel replacements and great achievements have been made during the last two decades ^{1, 4, 5, 16}. By using autologous living cells (with or without a well-defined 3-D matrix ³⁸), tissue engineering introduces the possibility to recreate a small diameter blood vessel substitute with structural and functional properties similar to native vessels. A few approaches have been developed in constructing a TE small diameter vascular graft and these can be classified into three main representative methodologies (Fig 2.4) based on collagen, cell, and cell and biocompatible scaffold ^{1, 7, 10}.

Figure 2.4 Schematic representation of (a) collagen-based, (b) cell-based, and (c) cell and biocompatible scaffold-based vascular grafts ¹.

2.4.1 Collagen-based TE vascular grafts

In 1986, Weinberg and Bell reported the first completely TE blood vessel, which was developed by coculturing ECs and SMCs in type I collagen gel (Fig 2.4 (a)) ³⁹. Collagen is one of the major ECM components of the arterial wall, contains cell recognized RGD (arginine-glycine-aspartate) adhesive peptide sequence, and facilitates cell attachment and tissue growth. A vascular media was constructed with bovine vascular SMC cultured in a denatured collagen (obtained from porcine skin or rat tail tendon) tube. A layer of fibroblasts was then added around the outside of the media as the adventitia and ECs

were seeded onto the luminal surface. This study demonstrated the feasibility of producing a TE vascular graft with structures similar to the native vessel. The use of biological collagen scaffolding, which may elicit desirable biological responses such as cell-mediated remodeling and functional vasoactivity, is the main advantage of this approach. Unfortunately, the resulting vascular graft ruptured at very low pressures (<10mmHg). By alternating 2 Dacron meshes and 3 collagen layers to maximize the burst strength, the burst strength achieved was only 323 ± 31 mmHg which is significantly lower than that of the native arteries (~5000 mmHg) or saphenous vein (1680 mmHg) and not acceptable for clinical applications^{1,3}. The poor mechanical strength is attributed to poor chemical integrity of the denatured collagen gel due to the loss of its native triple helix, the random or longitudinal orientation of SMCs and collagen fiber, and the significantly lower SMC density in collagen gel (one-eighth to one-fourth of those in native blood vessels)^{35,40}. The SMCs proliferate poorly when encapsulated in collagen and do not align in the radial direction.

In order to improve the burst strength of the engineered vessels, various methods have been explored to induce the circumferential orientation of SMCs and collagen fibrils. L'Heureux *et al.* reported the circumferential orientation by SMC-induced compaction over a nonadhesive central rod (Fig 2.5), which significantly improved the circumferential stiffness compared to that of the freely compacted constructs⁴¹. Tranquillo *et al.*⁴² induced circumferential orientation of collagen fibrils and SMCs via a strong magnetic field during collagen fibrillogenesis and the stiffness of the media equivalent was increased. However, the effect was compromised with time in culture¹⁰. Mechanical stimulation has been applied to improve the mechanical properties of the

collagen-based engineered vessel: there was a significant improvement in circumferential orientation of cells and ultimate stress (58 kPa vs 16 kPa) and modulus (142 kPa vs 68kPa) of the engineered media equivalent after 8 days application of cyclic strain (10% distention at 1 Hz frequency) ⁴³. Despite the numerous efforts to improve the burst strength of the collagen-based TE blood vessel, the best reported construct can withstand a pressure of about 225mmHg, which is much lower than the burst strength of native saphenous vein (1680 ± 307 mm Hg) ¹. The inherent physical weakness of the reconstituted collagen gel continues to restrict the breakthrough of this model. The suppressed ECM secretion and lower SMC density when SMCs were embedded in collagen gel are other factors leading to the low burst strength of the collagen-based construct model.

Figure 2.5 Illustration of free collagen gel compaction over a nonadhesive central rod to induce circumferential alignment of SMCs and fibrils by L'Heureux *et al.* ¹².

Expanded on this model, decellularized small intestine submucosa (SIS), which was chemically treated to remove the cellular component leaving an intact matrix consisting

mainly of type I collagen, has been investigated as a scaffold in the tissue engineering of small diameter blood vessel^{32, 44}. The decellularized SIS possesses better mechanical properties than collagen gel scaffold in that the native collagen structure was retained. Huynh *et al.* implanted a decellularized porcine SIS with deposition of a thick layer of bovine type I collagen in a rabbit artery and obtained a construct with a burst pressure of 931 ± 284 mmHg. Infiltration of SMCs and ECs into the construct was found and after 90 days' implantation, vasoactive function was observed when subjected to chemical stimuli *in vitro*. Despite the promising results, immune response to exogenic collagen and thrombogenicity due to lack of EC lining are main problems of this approach^{3, 32, 40, 44}.

Another major disadvantage of the collagen-based approach is that synthesis of elastin, a critical structural and functional component of the arterial wall for dilation/contraction with heart pumping, was greatly suppressed when SMCs were cultured on or embedded in collagen gel even under mechanical stimulation^{11, 43}. Recently, fibrin gel has been used as a substitute due to its ability to act as a temporary matrix for tissue ingrowth during wound healing. A significantly higher (by a factor of 8) elastin synthesis was found when SMCs were cultured on fibrin gel than that on collagen gel⁴⁵. The results are encouraging though the elastin production of engineered vessels with fibrin gel after 15 weeks *in vivo* implantation into the low pressure external jugular vein of sheep⁴⁶ was still not as extensive as that of the native vessels.

2.4.2 Cell-based TE vascular grafts

In view of the poor mechanical properties and possible immune response of the collagen-based TE vascular grafts, L'Heureux *et al.* developed an autologous cell-based

TE vascular graft without the use of a scaffold support⁴⁷. Monolayer cell sheets were formed by culturing human vascular SMCs with ascorbic acid and dermal fibroblasts separately till confluence for extended periods of time. These cell sheets were then rolled around an inert tubular support to form a tubular tissue with the fibroblast sheets on the outside. After 8 weeks' culture for tubular tissue maturation, the tubular support was removed and the inner surface was seeded with ECs so that a three-layered native-like vascular graft was constructed with the basic structure representative of a native artery. These grafts were able to withstand over 2000 mmHg pressure, similar to native saphenous vein. The mechanical strength of this model was attributed to the well-organized collagenous matrix of the adventitia. Additionally, SMC secreted abundant ECM (with the presence of ascorbic acid), which improves the mechanical properties of the graft. The grafts demonstrated a modest patency rate of 50% after 1 week implantation in a canine model, with thrombosis happened in 3 out of 6 animals. Compared to the collagen-based method, constructs made with this method exhibit adequate mechanical strength (>2000 psi burst strength) for arterial implantation and eliminate immunological mismatch by the use of autologous cells. However, production of such a blood vessel substitute composed of autologous cells would require extensive culturing time (3 months) for formation of cellular sheets from the patient's cells and maturation of the vessel in advance of the required graft procedure. Moreover, the engineered vessel was much less compliant than native vessels⁴⁶. The compliance mismatch was attributed to the insufficient elastin production and may lead to intimal hyperplasia. Additionally, many other critical issues, such as the vasoactive function, and

most importantly, the long-term *in vivo* efficacy of the vessel, are yet to be clarified before such grafts are clinically useful.

2.4.3 Biocompatible scaffold and cell-based TE vascular grafts

Scaffolds are used in tissue engineering to provide a 3-D growth environment for cell adhesion, migration, proliferation and differentiation. They guide tissue development with predefined structures and offer mechanical integrity. The use of porous biodegradable scaffold has provided encouraging results in vascular tissue engineering. The most promising result obtained to date have been reported by Niklason *et al.* who developed a functional small diameter TE autologous artery by culturing bovine SMCs and ECs on PGA mesh that was hydroxide-modified to obtain improved cell attachment under pulsatile conditions¹⁸. Porcine aortic SMCs were seeded onto a tubular porous scaffold and cultured for 8 weeks at 165 beats per minute and 5% radial distension (strain). Migration of SMCs into the interiors of the scaffold was observed. During this development process, the cell secreted large amounts of matrix proteins and simultaneously, the polymer scaffold degraded gradually. After 8 weeks culture, bovine aortic ECs were seeded on the smooth luminal surface of the vessels and cultured for another 3 days before implantation. Supplements (ascorbic acid, copper ion, and amino acids) were added to the culture medium and mechanical stimulation was applied to encourage ECM production and therefore improve mechanical properties. Pulsed vessels cultured with medium supplementation were demonstrated to display burst pressure of 570 mmHg after 5 weeks and exceed 2000 mmHg with a desirable histological organization after 8 weeks⁴⁸. Poorer mechanical properties (burst pressure of 270 mmHg after 7 weeks) and less organized histological structure were observed when the graft was

cultured without mechanical stimulation or ascorbic acid. Two pulsed grafts remained patent up to 24-28 days after implanted into swine saphenous arteries and failure occurred by thrombosis and vessel wall dilation. One possible cause was the inflammation response elicited by the highly acidic environment due to the rapid degradation of PGA scaffold¹⁰. Compared to the cell-based model, the cell and scaffold-based approach significantly shortened culturing time and thus holds much promise for a clinically viable graft. The mechanical properties of this graft would be due to SMCs and the ECM proteins produced after degradation of the PGA scaffold. However, several critical issues still need to be addressed. Full vasoactivity was not achieved in the engineered vessels. 8 weeks of *in vitro* incubation was necessary to obtain the desired mechanical strength, SMC differentiated function and histological organization and this raises the manufacturing cost and poses difficulty for providing off-the-shelf availability. The compliance mismatch due to lack of elastin fiber leads to irreversible creep and intimal hyperplasia. PGA breakdown products, mostly glycolic acid, leads to SMC dedifferentiation²³. It is also not clear whether a stable EC layer can be sustained under physiological flow conditions¹. Further optimization of culture conditions or scaffold design may be necessary for fabricating a functional blood vessel^{1, 6, 36, 49, 50}.

2.5 Requirements for growing TE vascular grafts

The *in vivo* patency rate of current TE vascular grafts in animals are not satisfactory. A successful TE vascular graft must meet various requirements^{2, 8} which include nonthrombogenicity, appropriate mechanical properties (sufficient burst pressure and viscoelasticity) and vasoactivity. It must also be cheaply manufacturable in a relatively short space of time, and in sufficient numbers with differing specifications (diameter,

length, etc.). Current TE vascular graft failed in achieving at least one of these characteristics.

2.5.1 Nonthrombogenicity and ECs

A leak-proof EC layer, i.e. endothelium, is a prerequisite for a successful graft to resist thrombosis *in vivo*.

EC has a dynamic nature and modulates the normal hemostasis and pathologic conditions by balancing its antithrombotic and prothrombotic properties⁵¹. Under normal circumstances, an intact endothelium acts as an antithrombotic and anticoagulant barrier between blood and the blood vessel wall by preventing platelets and plasma coagulation factors to contact with the highly thrombogenic subendothelial ECM. EC also synthesizes anticoagulant substances, such as heparin-like molecules, thrombomodulin and tissue factor pathway inhibitor, to inhibit coagulant formation. It has fibrinolytic effects by synthesizing tissue-type plasminogen activator (t-PA) which can promote fibrinolysis. In addition to that, the vascular endothelium have many other synthetic and metabolic properties^{38, 52, 53}, including the regulation of platelet activation and aggregation, SMC migration and proliferation, and immune and inflammatory responses, and modulation of vascular tone. However, injury to EC may lead to the activation of its prothrombotic properties that augment blood clot formation; injured ECs synthesize von Willebrand factor that promote platelet adhesion and tissue factor that induce clotting, and secrete inhibitors which suppress fibrinolysis. EC injury or dysfunction may result in vascular diseases such as atherosclerosis, restenosis (an abnormal narrowing of blood vessel or other tubular organ usually caused by the inflammatory response which induces cell

proliferation), and altered immune response⁵³. It is thus critical to maintain the functional integrity of the endothelium for the prevention of thrombogenicity and the preservation of blood flow.

EC seeding with the synthetic grafts prior to implantation has been attempted in various animal models and human clinical trials to reduce thrombosis and extend synthetic graft patency⁵⁴⁻⁵⁶. The single-stage seeding (seeding undertaken at the time of implantation) has shown high patency rate and complete endothelialization in dog models^{57, 58}. However, the low density seeding of immediately used ECs after harvest appeared to be disappointing in humans, with no difference found between 13 seeded grafts and 15 unseeded grafts in femoropopliteal bypass in a study carried out by Herring et al.⁵⁹. The high density seeding of *in vitro* expanded cells (two-stage seeding) has provided some encouraging results. In a study carried out by Zilla et al., patency rate of 85% was obtained with seeded grafts versus 55% of unseeded grafts after 32 months implantation⁶⁰ and similar results were achieved with another 9-year patency rate study⁶¹. However, the prolonged cell culture time (2-4 weeks) and the inability of the cells to proliferate effectively make this strategy not suitable for emergency cases. Despite of the extensive efforts spent in these areas, complete endothelialization of synthetic vascular grafts in human has never been achieved⁶; although successful trials have been performed in animal models and have provided a reconstitution of the endothelial layer with synthetic grafts^{62, 63}. The discrepancy between species is one of the weaknesses of the animal models for clinical trials. Other factors, such as age and healthy conditions, may also contribute to this discrepancy, i.e., usually clinical trial in human was carried out in elderly or unhealthy patients whereas young and healthy animals were used in animal

models³⁴. Thrombotic stenosis or occlusion is also a leading cause of failure of current small diameter TE vascular grafts. This is mainly due to the difficulty in obtaining an intact EC layer due to EC loss upon exposure to blood flow. The source for significant ECs is another issue since matured ECs do not have much proliferation capability. EC progenitor cells from circulating blood, or stem cells may provide alternatives and necessitate much more investigation¹⁰.

2.5.2 Mechanical properties and ECM

A functional graft must also possess mechanical properties such as compliance and burst pressure comparable to the native vessel in order to sustain anastomotic pressure and avoid rupture. The mechanical properties of a native vessel are mainly derived from the composition and orientation of ECM and cells. The bulk of the ECM of the media layer of blood vessels consists primarily of intermixed collagen (mainly Type I and III), elastin, and proteoglycans, which are produced by vascular SMCs. Each of these components contributes to the mechanical properties critical for blood vessel function. The collagen offers the tensile stiffness (mainly the linear region of the nonlinear stress-strain curve in Fig 2.6⁶⁴). The elastin provides the elastic properties at low strains (the toe part of the stress-strain curve in Fig 2.6⁶⁴); it stretches the vessel at each pulse and pulls the vessel back to its original diameter thus contributing to the compliance of a vessel. The proteoglycans contribute to compressibility. The combination of collagen and elastin is responsible for the vessel viscoelastic properties¹⁶. According to the ANSI/AAMI VP20-1994 standards for vascular graft prosthesis, an arterial construct is required to at least possess mechanical properties comparable to the saphenous vein, i.e., to withstand a normal physiological pressure in the 80-120mm Hg range, and have burst

strength of 1680 mm Hg and suture retention strength of 273g³⁷. Burst pressure up to 2000 mmHg has been achieved through tissue engineering using cell-based or cell and scaffold-based approaches^{18, 47}. Another very important requirement is a suitable compliance (0.24-0.46 MPa-1 for males aged 40-80 at 150 mmHg), which has been more difficult to achieve. Compliance mismatch has been correlated with graft failure by inducing intimal hyperplasia^{65, 66}, which is caused by limited elastin production for all the current tissue engineering approaches. Compliance mismatch was usually found in grafts with high burst strength^{18, 47} whereas burst pressure was insufficient when suitable compliance was achieved⁶⁶. SMCs are responsible for secreting large amounts of collagen and elastin; however, the plasticity of SMCs creates a hurdle in engineering of small diameter blood vessel. The delicate control of SMC phenotype might be the key to solve the conflicting event.

Figure 2.6 Stress-strain curve from a human vena cava⁶⁴.

2.5.3 Vasoactivity and SMC phenotype

Finally, a graft must contain circumferentially oriented contractile SMC layers to exhibit vasoactivity. Vasoactivity, i.e. constriction and dilation of blood vessels, is a biomechanical function that is mediated mainly by vascular SMCs and ECs. ECs receive and send neuronal or biochemical signals that control vascular tone. Vascular SMCs, the main cellular component of the blood vessel wall, generate the contractile force necessary to change the vessel volume and blood pressure by responding to nervous and hormonal signals. Vascular SMCs in normal blood vessels are known as contractile or differentiated SMCs. They have a spindle-shaped morphology with a prominent centrally located nucleus (Fig 2.7), proliferate at an extremely low rate and are cells almost completely geared for contraction. They are aligned in the circumferential direction so that when the cell contracts or relaxes, macroscopic vessel constriction or dilation will happen ⁸. However, under certain pathological conditions such as atherosclerosis and restenosis, vascular SMCs are known to undergo phenotype transition to a synthetic phenotype, resulting in morphological change to fibroblast-like (Fig 2.6), cell migration and proliferation, ECM secretion, and loss of contractile function ^{48, 67, 68}. A similar phenotype shift is found when vascular SMCs are isolated from native blood vessel and cultured *in vitro* ^{48, 67}, indicated by the gradual loss of morphologically and immunocytologically identifiable myofilaments as well as contractility.

Figure 2.7 Morphology of contractile and synthetic SMCs ⁶⁹.

2.6 SMC phenotype control in vascular tissue engineering

In vascular tissue engineering, the phenotype change of SMC is of paramount importance for constructing a functional vascular media. SMCs must modulate from a nonproliferative contractile phenotype to a proliferative synthetic phenotype as a prerequisite for cellular proliferation and ECM secretion for tissue regeneration to be possible ⁴⁸. The synthetic phenotype allows the rapid cell expansion in culture to acquire the large number of cells needed. However, synthetic SMCs do not possess vasoactivity and cause intimal thickening and narrowing of the vessel lumen leading to one of the prominent failure modes of vascular grafts ^{70, 71}. They must switch to a quiescent and contractile phenotype in order to mimic the functional properties of the native blood vessel. The switch of SMCs from synthetic to contractile phenotype at the appropriate cell development stage may possibly produce functional TE blood vessels ^{13, 72}. Furthermore, the cells need to be circumferentially oriented so that the normal function of vasoactivity can be realized and the mechanical properties of the vessel wall can be improved. An ideal vascular media is composed of circumferentially oriented contractile SMCs and the secreted ECM, and have appropriate mechanical properties (burst pressure

and compliance) and vasoactive response, all of which are relevant to SMC phenotype control. However, due to the plasticity of SMCs, none of current available tissue engineering approaches have resulted in all of the above key features of the vascular media^{5, 12}. Therefore, it is of great importance to control SMC phenotype and function, which could be used to tailor the properties of vascular graft developed *in vitro*.

In vivo cell phenotype and function are influenced by the entire cellular environment, such as ECM components, cell-cell interactions, growth factors, and local chemical and mechanical conditions. A number of studies have been carried out *in vitro* to identify the effects of various factors on SMC phenotype and the knowledge is beneficial for tissue engineering of a functional blood vessel.

2.6.1 Effects of ECM and growth factors

Native vascular SMCs are embedded in a 3-D ECM containing mainly collagen, elastin, fibronectin, laminin and proteoglycans, each of which has profound effects on SMC phenotype. The ECM affects cell adhesion, migration, proliferation or gene expression via cellular transmembrane receptors, usually known as integrins⁷³, which provides a signal transduction between ECM and the interior of the cell. Many studies have been carried out to explore the effects of various ECM components on SMC phenotype usually in 2-D monolayer culture and under serum free conditions. For example, fibronectin and collagen type I were usually found to induce a shift toward a synthetic phenotype, whereas laminin, elastin, heparin or collagen type IV, can revert this phenotype transition and retain cells in a quiescent and contractile state *in vitro*^{13, 70, 74}. The ECM also serves as a reservoir for growth factors^{75, 76}. Heparin sulfate proteoglycan was found to be able

to bind basic fibroblast growth factor (bFGF) that induces SMC proliferation⁷⁶. Other proteins have also been shown to play an important role in SMC phenotype control. For example, fibrin was found to stimulate elastin synthesis as compared to collagen¹¹.

Vascular SMC functions are also influenced by various localized produced growth factors, including the two main growth factors involved in blood vessel development: PDGF and TGF- β 1. *In vitro* studies show that PDGF-BB generally stimulates SMC migration, proliferation and ECM production, which indicates a shift to a synthetic phenotype in vascular SMCs⁷⁷. TGF- β 1 usually inhibits proliferation but promotes migration of vascular SMCs^{78, 79}, and increases ECM production⁸⁰.

However, these biochemical signals usually interact with each other exerting a complicated effect on SMCs, i.e., effect of growth factors or ECM components often depends on what other substances are present^{70, 77}. Furthermore, the delivery of growth factors requires a precise control of the timing and the concentration⁸¹. Re-creation of this complex system *in vitro* will continue to be a challenge for tissue engineering.

2.6.2 EC-SMC interaction

As the neighboring cells to SMCs, ECs have profound effects on SMC phenotype. ECs have been shown to produce or localize various growth factors for SMCs, including PDGF and bFGF, as well as growth modulators such as heparin and TGF- β , which can either stimulate or inhibit SMC growth⁴⁸. During *in vivo* blood vessel formation, mural cells (vascular SMCs or pericytes) are recruited and differentiated into SMCs by growth factors (PDGF or TGF- β 1) secreted by ECs on the newly formed endothelial tube⁸². It has also been reported that EC conditioned medium or coculture with ECs affects SMC

phenotype. A quiescent confluent endothelial layer produces heparin-like substances which inhibit SMC proliferation and holds SMC back to a more contractile phenotype, whereas the proliferating ECs cannot^{83, 84}. An injury to the endothelium or endothelial dysfunction can cause SMC proliferation, reduced contractile protein expression and increased matrix synthesis, which usually leads to intimal thickening. Similar results were also found when SMCs were cultured with EC conditioned medium. Chamley-Campbell reported that conditioned medium from confluent ECs can alter SMC phenotype toward a more contractile phenotype, while conditioned medium from proliferating ECs cannot inhibit SMC proliferation⁸³. However, other investigators also found conditioned medium from quiescent ECs can stimulate SMC proliferation. For example, Fillinger *et al.*⁸⁵ observed a stimulated proliferation of SMCs cultured with quiescent EC conditioned media compared to SMCs cultured alone. The results are mixed and relatively little is known about how ECs regulate the balance between stimulators or inhibitors for SMC differentiation and this awaits further study.

2.6.3 Effects of mechanical stimulation

Blood vessels are exposed to mechanical forces in the form of shear stress and cyclic strain as a result of the pulsatile nature of blood flow and luminal pressure. Vascular cells are equipped with numerous receptors that allow them to detect and respond to the mechanical forces generated by pressure and shear stress⁸⁶. Cyclic stretching is the dominant mechanical stimulus that exerts direct effects on the SMC phenotype. It has been reported that cyclic mechanical strain induces increased production of growth factors, cell proliferation, ECM production and contractile protein expression of cultured SMCs. Wilson *et al.* observed that cyclic strain (60 cycle/min) induced SMC proliferation

and production of an autocrine growth factor⁸⁷. Mooney *et al.* found more elastin and a more contractile SMC in smooth muscle tissues engineered with type I collagen sponges subjected to cyclic strain compared to control tissue (no strain) in which SMCs exhibited a structure more consistent with the synthetic phenotype⁸⁸. Stegemann *et al.* showed that 4 days' exposure to 10% cyclic mechanical strain at a frequency of 1 Hz could increase rat aortic SMC proliferation and a slightly higher smooth muscle α -actin expression¹⁷.

The effect of the mechanical stimuli was also found to depend on other factors, such as the SMC scaffolds, the magnitude and frequency of the applied strain, and the presence of serum or various growth factors. Kim *et al.* demonstrated an increase in SMC proliferation and ECM (collagen and elastin) production when SMC was adherent to culture surfaces coated with fibronectin or vitronectin rather than collagen or laminin in 2-D culture⁸⁹. Mills *et al.*⁹⁰ reported that SMCs exposed to a high strain (7-24%) demonstrated alignment perpendicular to the strain gradient, while SMCs subjected to lower strain (0-7%) remained randomly aligned.

Dynamic mechanical conditioning of vascular tissue culture leads to an improvement in mechanical properties and histological organization of the engineered smooth muscle tissue. Niklason *et al.* showed that the pulsatile flow (5% radial distension at a rate of 165 pulses per minute) can induce large amounts of ECM production and desirable histological arrangement of SMCs, which contributed to the high burst strength of the engineered vessel after 8 weeks exposure¹⁸. In another study by Seliktar *et al.*, rat aortic SMC seeded collagen constructs subjected to 8 days of 10% cyclic strain at 1 Hz displayed circumferentially oriented and homogeneously distributed SMCs throughout the thickness of the vessel wall compared to the randomly orientated SMCs observed in

the control samples (static culture)⁴³. Mooney *et al.* also found that cyclic strain increased elastin and collagen production and tissue organization, resulting in increase in the mechanical properties of the engineered smooth muscle⁹¹.

Although the results are encouraging, the lack of elastin fibers still causes compliance mismatch and irreversible creep. In addition, usually a long time of mechanical stimulation (usually several weeks) is needed to achieve desirable histological organization, ECM production and mechanical strength^{16, 18}. Mechanical stimulation was also found to increase the expression and activation of matrix metalloproteinase (MMP)-2; MMP-2 is an enzyme known to degrade short collagens, which may lead to the disintegration of the matrix^{10, 92}. The EC detachment upon exposure to mechanical stimulation is one issue that requires the delicate control of mechanical stimulation.

2.6.4 Effects of the scaffold

The scaffolds provide the mechanical strength and maintain cell spatial arrangement under aggressive *in vivo* conditions during tissue regeneration; and guide tissue development by specific morphology or biological cues of the scaffold. The composition, degradation and morphology/architecture of the scaffold thus significantly affect cell behavior or tissue regeneration.

2.6.4.1 Design of scaffold morphology

2.6.4.1.1 Micropatterning of SMCs

Traditional tissue engineering scaffolds have randomly porous morphology; however, more specific or complex architectures are necessary to provide specific topological cues

for tissue formation. These porous scaffolds lead to random spatial arrangement of the cells. A number of critical cell functions, including adhesion to matrix and other cells, migration, proliferation, differentiation, and apoptosis, have been shown to be affected by the architecture/morphology of the scaffold⁹³. In the medial layer of blood vessels, the SMCs are aligned unidirectionally. Microfabrication technologies have been applied to pattern biological or chemical molecules and micron-scale topographic features on the scaffold. Micropatterned surfaces offer phenotypic and genotypic control of living cells⁹⁴⁻⁹⁷; provide well-defined *in vitro* cellular environment replicating the cells' *in vivo* dimensional and compositional environment, thus facilitating native-like tissue reconstruction with living cells and scaffolds. Micropatterning of cell culture substrates has been shown to control the cell shape causing cells to switch between proliferation, apoptosis or differentiation^{98,99}.

Goessl *et al.*¹⁰⁰ produced micropattern of areas between 25 and 3600 μm^2 to control SMCs by plasma lithography and found that cells grown on the 10×100 μm^2 areas developed into a spindle-like form. Ra *et al.*¹⁰¹ showed that SMCs aligned as spindles along the collagen strips while cells appeared unoriented on uncoated areas. Another study by Thakar *et al.*¹⁴ showed that on 30- and 20- μm collagen strips, the proliferation, cell shape index and the smooth muscle α -actin expression of SMCs were decreased. They also showed vascular SMCs could be aligned in grooves that were 25 μm deep and 30 μm wide and separated by low aspect ratio (defined as height/width ratio) microwalls. However, these narrow micropatterns or microchannels restrict, and in some cases, inhibit cell proliferation necessary for tissue formation. Further, some topologies render a large proportion of the substrate area unavailable for cell growth. Sarkar *et al.*¹⁵ showed

that grooves (20 to 80 μ m wide and 5 μ m deep) in non-biodegradable polydimethylsiloxane (PDMS) can control the vascular SMC aspect ratio, alignment and oriented remodeling of the ECM. However, non-degradable scaffolds affect the vasoactivity of the remodeled vessel. Further, previous micropatterning techniques have mainly focused on cell and actin alignment and none has reported the desirable SMC phenotype shift from synthetic to contractile at the appropriate developmental stage necessary for a vasoactive vessel. 3-D micropatterned scaffolds can potentially regenerate the 3-D tissue with the desired morphology rather than a monolayer of cells. It is generally acknowledged that currently-employed micropatterning techniques are not satisfactory for the requirements of vascular tissue engineering²⁰⁻²².

It has been suggested that cells on elastic substrates can actively sense the presence of a close-by surface, such as a surface of a tissue or organ, to align and elongate themselves by the boundary-induced strain which has a similar effect to that of external strain, e.g. cyclic strain^{102, 103}. Cells generate their own internal tension against the adhering substrate to maintain a state of isometric tension¹⁰⁴⁻¹⁰⁶. The self-generated forces or the associated changes in cell shape, as suggested by Chicurel *et al*, can modulate cellular growth, differentiation and ECM remodeling^{104, 105}. The aligned cells are also expected to produce strain fields which can be detected as external strain by other cells, and induce more cells to adopt the same direction^{103, 107}. By this analogy, we hypothesize that wide microchannels (relative to cell widths of 5 – 10 μ m) separated by microwalls might be a potential scaffold for regulating SMC growth, orientation and phenotype. The wide microchannels allow the cell proliferation needed for vessel remodeling; the physically constraining microwalls are expected to trigger cell elongation and alignment and affect

cell function, especially with confluence when the signals can be transmitted through not only cell-matrix interaction but also cell-cell contacts.

2.6.4.1.2 UV embossing applied in tissue engineering

UV embossing, a quick and convenient process involving low temperature and low pressure, could be used for producing microscale scaffolds for tissue engineering application^{108, 109}. In UV embossing, a liquid polymer is dispensed onto a microstructured mold and subsequently polymerized. Typically, the microstructured mold used in UV embossing is a child mold which can repeatedly replicated from a master mold prepared by conventional photolithography (Fig 2.8). Conventional photolithography generates patterns by selectively exposing the photoresist-coated surface with UV light through a photomask, followed by etching to yield the structures. The fabrication must be done in a cleanroom which inevitably increases the fabrication cost and decreases the accessibility. Comparatively, in UV embossing, large numbers of highly precise and complex structures from a single master mold can be produced easily and economically¹¹⁰. The substrate can consist of a glass plate as well as flexible plastic films and the materials used can be biodegradable. With a single master, the identical structures can be produced over large areas. High aspect ratio microstructures can be fabricated to emulate the thickness of 3-D tissues. In our lab, high aspect ratio (up to 15) UV embossed microstructures have been produced using non-degradable polymers¹¹¹.

Figure 2.8 Schematic illustration of generations of microstructured molds or films.

2.6.4.2 Design of scaffold material

The scaffold material can be natural or synthetic. Collagen and fibrin are the main naturally derived materials used in scaffolds for SMCs. They elicit the desired biological responses through their functional groups. However, like other natural materials, they suffer from batch-to-batch variations or scale-up difficulties. They also lack proper mechanical strength for transplantation¹¹². Synthetic polymers, on the other hand, allow precise control over the mechanical strength, degradation rate, microstructure and other attributes through tailoring their composition and molecular weight. They can be processed with various techniques and supplied in large quantities. Synthetic scaffold has been extensively used in tissue engineering application since the various processing techniques offer the ability to arrange cells/tissue in myriad 3-D morphologies and guide tissue formation relatively rapidly with the well-defined structures reproducibly and economically¹⁶. The biodegradable polymers are attractive for tissue engineering application in that the delayed degradation allows the development of completely natural

tissue after fulfilling their functions such as structural support and guidance for initial tissue formation. Aliphatic polyesters, synthesized from three common monomers - LA, GA, and ϵ -CL - usually by ring opening polymerization (ROP) (Table 2.1), are approved by Food and Drug Administration (FDA) to be used in therapeutic devices and widely used in tissue engineering scaffold. The degradation of these polyester scaffolds has been reported to affect the phenotypic changes of SMCs.

Table 2.1 Chemical structure of GA, LA, ϵ -CL and the resulting repeating units ¹¹³

Monomer and Resulting repeating units	
GA
LA	
a) L-LA
b) D,D-LA
c) <i>meso</i> -LA
ϵ -CL

Chiral center marked with *

Aliphatic polyesters degrade mainly by hydrolytic attack of the ester bond (Fig 2.9) and yield hydroxy carboxylic acids, which in most cases are ultimately metabolized by the body ^{113, 114}. During hydrolytic degradation, water penetrates the bulk of the scaffold,

attacks the ester bonds in the amorphous phase initially and converts the long polymer chain to shorter water-soluble fragments, so that the polymer molecular weight is reduced. A rapid loss of polymer mass will follow this phase due to the enzymatic attack and metabolization of the fragment. Degradation of aliphatic polyesters is usually controlled by the rate at which water penetrates the bulk, since it typically undergoes bulk degradation^{115, 116}. The degradation properties of such polymer are affected by the combined effects of hydrophilicity, crystallinity, and glass transition temperature of the polymer, which can be manipulated by molecular weight of the polymer and copolymerization with various monomers¹¹⁷.

Figure 2.9 Pathway of hydrolysis of the ester bond¹¹⁸.

PGA is highly crystalline with crystallinity ranging from 35% to 75%, a high melting point (T_m) (225~230 °C) and very low solubility in most organic solvents. It degrades rapidly due to its hydrophilic nature and tends to lose 50% and 100% of its mechanical strength after 2 weeks and 4 weeks respectively of water immersion^{112, 119}. In small diameter blood vessel tissue engineering, PGA accounts for the major scaffold material and has provided the most promising result to date¹⁸. However, Higgins *et al.* demonstrated that PGA breakdown products, mostly glycolic acid, could lead to changes in SMC phenotype²³. SMCs grown in high concentrations of PGA breakdown products showed decreased expression of calponin, a marker for SMC differentiation. A lower elastin production was also found using PGA as a scaffold compared to PGA/PLLA

(poly(L-lactide)) composite scaffold; PLLA imparts elasticity and decreases the degradation rate of PGA¹²⁰. Degradation of PGA produces acidic products and creates a low pH environment, which causes the undesired loss of SMC contractility and may also stimulate chronic inflammation and fibrosis. In addition, the loss of mechanical stability is also noteworthy during degradation, which can be undesirable if it is too fast. Blood vessels are subjected to mechanical stretch or strain due to the pulsatile nature of the blood flow. Therefore, scaffolds for vascular engineering generally need to have not only sufficient initial but also “partially degraded” elasticity¹²¹⁻¹²³. Control of the mechanical properties during degradation through material design is thus critical. The rate of degradation must be tuned to match the regeneration of the new tissue. The scaffold should maintain its mechanical properties and structural integrity until the seeded cells proliferate sufficiently and produce enough ECM, and only then completely degrade and be absorbed by the body^{124, 125}. PGA is unable to sustain systemic pressures during the tissue remodeling due to its rapid degradation (loss of 50% of its mechanical strength after two weeks). Therefore, commercially available PGA might not represent an optimal scaffold for vascular tissue engineering and an optimization of the scaffold is necessary to minimize the acidity of the degradation products and improve the mechanical stability during degradation.

GA has been copolymerized with poly-4-hydroxybutyrate¹²⁶ or polyhydroxyalkanoate¹²⁷ to decrease the degradation rate and thus the acidity of the degradation products, improve the mechanical stability during degradation and increase the elasticity of the scaffold. However, these polymers are not approved by FDA to be used in therapeutic devices and more investigation regarding the toxicity of the degradation products is

necessary. GA can be copolymerized LA or ϵ -CL which has been approved by FDA to be used in a host of therapeutic devices. Poly(lactide) (PLA), with an extra methyl group, is more hydrophobic and thus degrades more slowly than PGA. Due to the presence of two chiral centers, there are three forms of the LA monomer: L-LA, D,D-LA, and D,L -LA (Table 2.1). Polymerization of D,D-LA, and D,L -LA results in amorphous material. L-PLA is semicrystalline with high mechanical strength and more frequently used as tissue engineering scaffolds as its degradation product is naturally occurring L-lactic acid.

Poly(ϵ -caprolactone) (PCL) is of interest for tissue engineering scaffold applications since its degradation products are non-toxic and it can be highly elastic. Linear PCL is a rubbery semi-crystalline ($T_m = 55\text{ }^{\circ}\text{C}$) polymer at physiological temperature with a low T_g ($-60\text{ }^{\circ}\text{C}$). It has high molecular (e.g. water, oxygen and drugs) permeability and is the most elastic polymer among the polyesters approved by FDA to be used in therapeutic devices because of its low T_g . However, ϵ -CL degrades very slowly and has been commonly copolymerized with other monomers for preparing a diversified family of copolymers with tunable degradation rates and mechanical and thermal properties; ϵ -CL copolymers can be rubbery or glassy, slowly or quickly degrading and rigid or highly elastic^{125, 128-134}. Linear block copolymers with intermediate to high amount of ϵ -CL (eg. 55/45mol-% ϵ -CL /D,L-lactide¹³⁵) are generally crystalline; amorphous ϵ -CL copolymer displays high mobility because of its low T_g and would not retain its shape at body temperature^{135, 136}. However, the crystalline copolymer degrades relatively slowly and inhomogeneously which may not be favorable in tissue engineering applications. Photocrosslinking is another route to produce amorphous copolymers of ϵ -CL capable of maintaining their shape at physiological temperature. Further, photocrosslinkable liquid

polymers can be micro- or nano-patterned by UV embossing for cell guidance^{137, 138}. The synthesis and photocrosslinking of liquid biodegradable copolymers composed of ϵ -CL and trimethylene carbonate monomers and endcapped with coumarin¹³⁹ or phenylazide¹⁴⁰ functional groups have been demonstrated. However, the crosslinking efficiency was generally low. Acrylate and methacrylate groups have been widely used for UV photopolymerization because of their high reactivity¹⁴¹ leading to rapid and near complete conversion. Therefore, in this thesis, we design and synthesize photopolymerizable diacrylated oligomers based on L-LA, GA and ϵ -CL which are subsequently rapidly photocrosslinked into amorphous polymers. ϵ -CL increases chain flexibility, lowers the bulk modulus and decreases the degradation rate together with L-LA. The liquid oligomers can be arbitrarily molded into any shape under mild environments and room temperature, which is an advantage over high molecular weight linear polymers where high temperature or aggressive solvents are usually needed for polymer processing.

Little has been reported on the degradation behaviors of photocrosslinked aliphatic polyesters^{135, 142}. Amsden *et al.*¹⁴² prepared an acrylated three-armed star-poly(ϵ -caprolactone-co-D,L-lactide) for delivery of thermosensitive drugs and showed that the physical properties of the elastomers can be designed through manipulation of the molecular weights. However, the degradation rate of the polymers was generally similar whereas the greater weight loss was found in polymers with highest crosslink density, which was in accordance with a previous report of Helminen *et al.*¹³⁵. They prepared four-armed methacrylated star-poly(ϵ -caprolactone-co-D,L-lactide) and found a greater mass loss with higher crosslink density, which they attributed to the autocatalytic effect

due to the inability of the degradation products to diffuse from the bulk as a result of the highly tight network. Therefore, the crosslinking density can affect both the inward and outward diffusion of water and acidic products respectively in these networks. There is no report on the degradation behavior of photocrosslinked diacrylated linear aliphatic polyesters. Degradation studies are typically tedious but the ability to predict the degradation rate is highly useful. If the crosslinking density of photocrosslinked biodegradable polyesters is not too high, it is envisaged that the degradation rate will depend on the composition and molecular weight, similar to the linear counterparts – and opposite to the reported trend observed in the highly crosslinked networks^{135, 142}.

2.7 Summary

Small diameter vascular grafts are urgently needed in patients needing coronary artery bypass or peripheral vascular surgery due to cardiovascular disease or complications of other diseases such as diabetes. Autologous veins are the ideal substitutes but approximately 60% of patients who require vascular bypass surgery do not have healthy vessels for grafting. Small diameter synthetic grafts made of Dacron or polytetrafluoroethylene fail *in vivo* due to thrombogenicity, compliance mismatch, and neointima formation. The relatively new field of tissue engineering attempts to re-create biological small diameter vascular grafts using living cells with or without a scaffold. A successful small diameter TE vascular graft must meet certain requirements which include nonthrombogenicity, sufficient burst pressure and suture retention strength, viscoelasticity, appropriate remodeling responses and vasoactivity. It must also be manufactured cheaply in a relatively short space of time, and in sufficient numbers with differing specifications (diameter, length, etc.). However, current approaches for making

small diameter TE vascular grafts are unsatisfactory and fail in achieving at least one of these requirements. The collagen-based small diameter TE vascular grafts have poor mechanical properties due to the poor chemical integrity of the reconstituted collagen, low SMC density and largely longitudinal orientation of ECM and SMC. The possible immune response is another issue of this approach. The PGA-scaffold based TE vascular grafts need an undesirably long culture time to obtain desirable histological organization and burst strength; the undesired loss of SMC contractility was also found due to the acidic environment created by rapid degradation of the scaffold. For the cell-sheet based approach, although modest patency rate and adequate burst strength were achieved, the extensive long culturing time was a significant disadvantage.

Native vessel comprises three concentric layers: tunica intima, tunica media and tunica adventitia. Tunica media, one of the key components of the blood vessel wall, is responsible for the mechanical strength, compliance and vasoactive response of the blood vessel. An ideal vascular media is composed of circumferentially oriented contractile SMCs and their secreted ECM, such as collagen and elastin fibers. However, none of the currently available tissue engineering approaches have resulted in all of the key features of the vascular media due to the plasticity of SMCs. SMCs have two phenotypes (contractile and synthetic) and are known to show reversible phenotype changes with appropriate environmental stimuli. The synthetic phenotype is desired for cell proliferation needed for vessel remodeling whereas circumferentially oriented contractile SMCs are necessary for sufficient vasoactivity and inhibition of neointima formation. The SMC phenotype shift at an appropriate developmental stage is believed to provide functional vascular media.

Mechanical and biochemical stimulations, and micropatterns have been applied to regulate the phenotype, orientation, and ECM production of SMCs. Usually a long time of mechanical stimulation (of the order of several weeks) is needed to achieve the desirable histological organization and ECM production. The increase of MMP-2 expression and activation induced by mechanical stimulation might also lead to undesired matrix disintegration. SMC phenotype control in tissue engineering through biochemical stimulation requires stringent control over the timing and concentration of the biochemical stimulus (such as growth factors) over a long time period.

The degradation and morphology of 3-D scaffolds has been found to affect SMC phenotype. PGA porous scaffold has provided the most promising result for tissue engineering of small diameter vascular graft whereas its degradation product causes the loss of SMC contractility. Further, porous scaffold does not contain specific cues needed for functional smooth muscle tissue development guidance. 2-D micropatterns whereby SMCs grow attached on a surface have demonstrated the regulation of cell morphology and orientation but inhibition of cell growth necessary for tissue regeneration as well. 3-D microchannels for SMC alignment, elongation and phenotype switch are relatively unexplored. The scaffold for engineering of smooth muscle tissue also needs to have controllable degradation rate so that the mechanical strength of the scaffold should be enough to provide structural support during tissue regeneration and the effects of degradation products (such as acidity) on cell differentiation should be minimized. The scaffold should also be manufactured into a variety of shape and sizes easily, reproducibly and economically.

UV embossing, involving the use of a micro-mold to imprint liquid polymer which shall be subsequently UV polymerized, is a quick and convenient technique that can be applied in tissue engineering to produce film scaffolds with micro-scale patterns. A liquid, amorphous, photopolymerizable and biodegradable acrylated polymer is required for producing a biodegradable UV embossed scaffold. The mechanical properties and degradation rates of the scaffold should be easily tunable to match tissue regeneration rates. Development of UV embossed micropatterns for the tissue engineering of smooth muscle medial layers is the focus of this thesis. Suitable liquid oligomers have been also developed and their mechanical properties and degradation behaviors have been studied.

One of the requirements for a TE product to be clinically useful is that it must be manufactured cheaply in a relatively short space of time. The long culturing time is a serious problem for all current approaches of TE small diameter vascular grafts. Therefore, it is important to investigate the effect of cell culturing conditions, e.g., cell seeding density, so as to shorten the culturing period and reduce the cost.

Chapter 3 Synthesis, characterization and *in vitro* degradation of biodegradable photocrosslinked film from liquid PCLLGA diacrylate

3.1 Introduction

We wish to develop amorphous biodegradable and photocrosslinkable materials for vascular tissue engineering scaffolds in which the mechanical properties and biodegradation rates can be easily manipulated to match the tissue regeneration rates. There has been little study on the effect of composition or molecular weight on the biodegradation rates of photocrosslinked biodegradable aliphatic polyesters though such information is important and of great significance for tissue engineering scaffolds.

This chapter reports the design, synthesis and characterization of a new series of liquid biodegradable random linear oligomers based on ϵ -CL, L-LA and GA, which are endcapped with photocrosslinkable acrylate groups. The diacrylated linear oligomers were designed with different molecular weights (1800, 4800, and 9300 Da) and compositions (by varying ϵ -CL molar ratio from 20%, 40%, to 60%) so that the crosslinked polymers have varying mechanical properties and degradation rates. The high (9300), medium (4800) and low (1800) molecular weight oligomers were denoted by H-x, M-x and L-x respectively where x represents the % ϵ -CL content. Specifically, five oligomers (i.e. L-60, M-60, H-60, H-40 and H-20) were synthesized where each have an equal molar ratio of GA to L-LA. The physical, chemical and photocrosslinking characteristics of the polymers were studied. The change of tensile properties (Young's

modulus, ultimate stress and maximum strain), weight loss and water absorption during degradation were systematically studied over a 10-week period. Biocompatibility of these polymers with rat SMCs was demonstrated by culturing on thin films of crosslinked polymers.

3.2 Experimental details

3.2.1 Materials

L-LA and GA were purchased from Purac Asian Pacific (Singapore) and recrystallized from ethyl acetate. ϵ -CL, acryloyl chloride, stannous octoate, and tetra (ethylene glycol) were obtained from Sigma-Aldrich and used as received. Triethylamine was purchased from Fluka and used as received. 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651) was obtained from CIBA chemicals. All other chemicals were purchased from Sigma-Aldrich. They were of reagent grade and used without further purification. All cell culture reagents were obtained from Invitrogen unless otherwise specified.

3.2.2 Synthesis of PCLLGA diacrylate

The PCLLGA diols were prepared by ring opening polymerization of ϵ -CL, L-LA and GA, which were initiated with tetra(ethylene glycol) in the presence of stannous octoate as a catalyst and PCLLGA diacrylates were prepared by acrylation of diols using acryloyl chloride (Scheme 3.1).

(1) PCLLGA diol synthesis by ring opening polymerization of GA, L-LA and CL with tetraethylene glycol as initiator and catalyzed by stannous octoate.

(2) Acrylation of PCLLGA diol.

Scheme 3.1. Reaction scheme of synthesis of PCLLGA diacrylate.

Predetermined amounts of tetra(ethylene glycol), L-LA, GA and ϵ -CL, and stannous octoate (controlled by the designed molecular weights and composition) were added into a 100 ml round-bottomed flask. For example, for H-60, 4.32g L-LA, 3.48g GA, 20.52g ϵ -CL, 0.59g (3mmol) tetra(ethylene glycol) and 0.03g stannous octoate were added respectively. The reaction flask was equipped with a stirring bar and high-vacuum stopcock which was connected to a dual bank manifold with one end connected to a vacuum pump and the other to argon gas. After repeated evacuation and purging with argon gas (3 times), the reaction mixture was put into an oil bath preheated to 145 °C and polymerization was carried out under stirring for 24 hours at 145 °C under argon atmosphere. The reaction mixture was cooled to room temperature, precipitated in hexane and dried at 45 °C under reduced pressure to give a clear viscous liquid.

The isolated polymer was re-dissolved in dichloromethane in a three-neck round-bottomed flask and cooled to 0 °C in an ice bath. Acryloyl chloride and triethylamine dissolved in dichloromethane were added dropwisely into the flask. For the H-60 example, 6mmol acryloyl chloride and 6mmol triethylamine were added. The mixture was reacted at 0 °C for 6 hours and then at room temperature (~25 °C) for 18 hours. The mixture was washed with dilute HCl and NaHCO₃ solution to remove triethylamine and HCl respectively. The mixture was then further washed with doubly distilled (dd) H₂O twice to remove traces of NaHCO₃. Following this, the mixture was dried with anhydrous MgSO₄ and microfiltered. Dichloromethane was then removed by rotary evaporation and the residual amount was removed under reduced pressure at 65 °C for 48 hours. The obtained diacrylated oligomers were colorless or clear light yellow viscous liquids.

The triethylamine should be added slowly dropwisely into the reaction flask; otherwise the reaction mixture will become yellowish or even brownish which will cause poor UV transmission and autofluorescence. The reason is not completely clear. However, the rapid addition of triethylamine was suspected to accelerate the diacrylation reaction by rapid consumption of HCl, which led to local overheat and caused some other side reactions such as oxidation of the PCLLGA diols or diacrylates. The autofluorescence will cause difficulties in cell characterization where fluorescence microscopy is frequently used. By adding triethylamine dropwisely, we can afford light yellow or even colorless oligomers even with very low molecular weight where large amount of triethylamine is added.

3.2.3 Chemical and thermal characterizations

^1H -NMR spectra were recorded on a Bruker DMX-300 spectrometer, working at 300.130 MHz. The solvents for ^1H -NMR measurement were CDCl_3 and acetone- d_6 . The solvent contained 0.5% tetramethylsilane as the internal standard.

Number- and weight-average molecular weights (M_n and M_w respectively) and molecular weight distribution (M_w/M_n) were determined by gel permeation chromatography (GPC) measured with an Agilent 1100 Series equipped with a GPC-SEC (Size Exclusion Chromatography) data analysis software. Samples were analyzed at 25 °C with tetrahydrofuran as eluent at a flow rate of 1.0 ml min $^{-1}$. Polystyrene standards were used and the results were used only qualitatively to check the peak shape and size distribution of the different oligomers.

Fourier transform infrared (FTIR) spectra of the oligomers were recorded on a Nicolet Magna-IR 560 spectrometer equipped with a Golden Gate single reflection diamond attenuated total reflection (ATR) unit over the wavenumber range of 4000-400cm $^{-1}$. The spectra were collected with resolution of 4cm $^{-1}$ and scan number of 32 using an MCT (Mercury Cadmium Telluride) detector.

Differential scanning calorimetric (DSC) measurements were carried out using a TA DSC 2920 Modulated DSC. The crosslinked sample (see Section 3.2.5 for preparation) was heated from room temperature (at 20°C/min) to 200°C which was maintained for 1 minute. It was cooled to at 10°C/min to -60°C which was maintained for 1 minute. It was then re-heated at 20°C/min to 200°C. The oligomers were heated to 80°C only. For the three oligomers made with 60% ϵ -CL, due to their low T_g reaching the lower temperature

limit of the instrument (-60°C), after the first heat-up scan, the samples were cooled to -100°C by manually adding of liquid nitrogen and re-heated at $20^{\circ}\text{C}/\text{min}$ to 80°C . All DSC data presented here are from the second heating scans.

Viscosity of the diacrylates was measured using a Controlled Strain Rheometer (ARES Rheometric Scientific) using a 25mm-diameter cone. The Rheometer was calibrated using a Fluka silicone oil (DC200) which has a reported viscosity of $100\text{ mPa}\cdot\text{s}$ at 25°C under the shear rate of 10 rad/s . The viscosities of the diacrylates reported were those measured at 10 rad/s and 25°C .

UV transmittance in the range of 190-500nm of the diacrylates was measured by scanning a $75\mu\text{m}$ -thick diacrylate resin squeezed between two pieces of quartz with a $75\mu\text{m}$ -thick spacer using a UV-VIS-NIR scanning spectrophotometer (EV-3101PC, SHIMADZU).

3.2.4 Curing analyzed by FTIR spectra

The photopolymerizing resins were prepared by stirring each of the diacrylated oligomers for 24h at 65°C with 0.5 wt% of Irgacure 651 photoinitiator which was pre-dissolved in butanone (10/90(w/w) Irgacure 651/Butanone); the excess butanone was removed under reduced pressure at 65°C for 2h.

The dynamic FTIR spectra of photopolymerizing resins were recorded with a Nicolet Magna-IR 560 spectrometer equipped with an MCT detector. A single reflection diamond ATR device was used for sampling. UV-irradiation was performed with a mercury lamp. The light was guided to the sample using a fiber-optic cable with an inner-diameter of

1cm. The 365nm intensity of UV light at the sample on the diamond crystal was adjusted to approximately 10mW/cm². Prior to use, the ATR diamond crystal was thoroughly cleaned using acetone. For the background spectrum collection, the scan number was set to 256 and resolution set at 4cm⁻¹ and nitrogen was used as purge gas throughout the spectrum recording (background and sample). For the actual sample scan, the scan number was set at 5 (i.e. 23 points/min) and resolution at 4cm⁻¹. A “picture frame” of 300-gage (75µm thick) Melinex® 454 film with a cutout of 2cm by 2cm was used as a spacer to control the thickness of the resin. The spacer was placed around the crystal and a bead of the sample was dropped gently onto the crystal. Then a piece of clean glass, previously cleaned with acetone and dried, was pressed onto the resin carefully to ensure that no air bubbles were created. The spectra were continuously collected during the dynamic UV curing process. The kinetic conversion was calculated from the decay of the absorption bands of the CH=CH₂ deformation mode at 810 cm⁻¹ by integration of the peak areas. The symmetrical stretch vibration of -CH₃ at 2862 cm⁻¹ was used as a reference signal to compensate for differences in sample thickness since this peak remained constant throughout the course of the experiments. The conversion was calculated from the following equation:

$$Conversion(\%) = 1 - \frac{(A_t / B_t)}{(A_0 / B_0)} \quad (3-1)$$

where A_t, B_t are the area under peak 810 cm⁻¹ and 2862 cm⁻¹ at time t respectively, and A₀, B₀ are the area under peak 810 cm⁻¹ and 2862 cm⁻¹ at time 0.

3.2.5 Fabrication of photocured films

The UV resin formulation was dispensed onto a piece of horizontal picture-frame glass mold which had a 125 μ m-thick polyester film (Melinex® 454 from Dupont) peripheral spacer. The resin was degassed in a vacuum oven at 0 atmosphere at 65 °C for 24h. Afterwards, another piece of cleaned glass was carefully placed over the resin which was then UV polymerized for 10min using a flood UV exposure system with an Hg-lamp, specifically a 350W Mercury lamp with 365nm-intensity of 10mW/cm² in a MA6 UV imprint system supplied by SUSS MicroTech, Germany.

3.2.6 Mechanical testing of crosslinked films

The cross-linked polymer samples were cut into dumbbell shape (according to ASTM D-1457, Fig 3.1) which was 14mm long and 5mm wide at the narrow and gripping sections respectively. An Instron 5569 equipped with Merlin Software and a static 10N-load cell was used. The samples were pulled at 2mm/min until breakage occurred. Only those samples breaking at narrow region were included in the analysis and results reported are averages of five to ten valid measurements.

Figure 3.1 Dimensions (in mm) of dumbbell shape used for tensile testing.

3.2.7 *In vitro* degradation study of photocured films

The initial weight of the photocured films (W_0) was recorded. The photocured films were then incubated in phosphate buffered saline (PBS, pH 7.4) solution at 37°C for up to 10 weeks in an orbital shaking machine with a shaking speed of 50 rpm. The pH of the solution was checked weekly and the solution was replaced when pH dropped below 7.0.

After 1, 2, 4, 6, 8 and 10 weeks, the swollen weight (W_s) and dried weight (W_d) were measured after wiping the surface water with paper and after vacuum-drying respectively. The weight loss (WL) and water absorptivity (WA) were calculated using equations (3-2) and (3-3) respectively. The results reported are averages of three measurements.

$$WL (\%) = 100 \frac{(W_0 - W_d)}{W_0} \quad (3-2)$$

$$WA \text{ (\%)} = 100 \frac{(W_s - W_d)}{W_d} \quad (3-3)$$

The tensile properties of control and degraded films at these intervals were also measured as described in section 3.2.6.

3.2.8 Cell culture on photocured films

SMCs used were from rat aorta (ATCC, CRL-1444). SMCs were maintained in 75cm² tissue culture polystyrene flasks in complete growth medium (Dulbecco's Modified Eagle's Medium with 4mM L-glutamine supplemented with 10% fetal bovine serum, 50IU/ml of penicillin and 50μl/ml of streptomycin). Cells were passaged by trypsinization with 0.25% (w/v) Trypsin-0.03% (w/v) EDTA solution before reaching confluence, usually every 3rd day. The different polymer films were cut into 1.5cm diameter discs and sterilized in 70% ethanol/30% ddH₂O solution for 24h. Before cell seeding, the films were moved to a 24-well culture plate and equilibrated with PBS for 30mins. Then SMCs together with culture medium were added to the plate (10,000 cells/cm²) and incubated for 3 days. Cell density after 1 days and 3 day culture was assessed by cell counting using a hemocytometer after detachment from the substrates and trypan blue staining. Results reported are averages from three measurements. Phase contrast images were taken using a ZEISS inverted microscope.

3.3 Results and discussion

3.3.1 Synthesis and characterization of uncrosslinked oligomers

The five diacrylated oligomers synthesized were characterized by ¹H-NMR (300 MHz, CDCl₃, δ, ppm). As an illustration, the spectrum of the diacrylated oligomer L-60 is

shown in Fig 3.2 (a). The total proton assignment of L-60 in the ^1H NMR spectrum is specified in Fig 3.2 (b): (I) $\text{CH}_2=\text{CH}$ vinyl groups at 5.84-6.54 ppm (peak i, j) (II) CH_2CH_2 groups attached to oxygen atoms at 3.65 ppm (peak a) (III) CH tertiary carbon groups attached to oxygen atoms and carbonyl groups at 5.11 ppm due to LA moiety as a broad peak (c) (IV) lateral methyl CH_3 groups and the middle CH_2 groups in $\text{CH}_2\text{CH}_2\text{CH}_2$ groups at 1.38-1.65 ppm (peak d, g) (V) CH_2 groups attached to oxygen atoms at 4.04-4.32 ppm (peak h) (VI) CH_2 secondary carbon groups attached to oxygen atoms and carbonyl groups at 4.60-4.73 ppm (peak b) due to GA moiety (VII) CH_2 groups attached to carbonyl groups at 2.31-2.46 ppm (peak e) (VIII) two side CH_2 groups in $\text{CH}_2\text{CH}_2\text{CH}_2$ groups at 1.35-1.68 ppm (peak f). Some peaks can be further split corresponding to different stereosequences¹⁴³. For example, peak e was split: e1 and e2 doublets corresponding to COCH_2 groups (e1) attached to GA or LA units which are more polar than $\epsilon\text{-CL}$ unit, and those (e2) attached to another $\epsilon\text{-CL}$ repeat unit respectively. However, clear elucidation of the stereosequences of the oligomers need high resolution scanning. Pack *et al.*¹⁴³ used 600-MHz ^1H -NMR scanning. The molar ratios of $\epsilon\text{-CL}$, LA and GA in each synthesized diacrylate were calculated according to the relative intensities of the corresponding protons and were similar to the designed values (Table 3.1).

(a)

(b)

Figure 3.2 (a) ^1H -NMR spectrum of PCLLGA diacrylate oligomer (L-60); (b) molecular structure of PCLLGA diacrylate.

Table 3.1 GPC, NMR and DSC analysis of PCLLGA diacrylates.

Diacrylate	$M_{n,th}^a$	$[CL]:[LA]:[GA]^b$		$M_{n,NMR}^{**}$ (Da)	M_n^+ (Da)	M_w^+ (Da)	D	Viscosity (Pa·s)
		Monomer feed	Polymer [*]					
L-60	1800	60:20:20	62:18:20	1869	2080	3060	1.47	1.8±0.1
M-60	4800	60:20:20	62:18:20	5529	7150	8810	1.23	21.6±1.6
H-60	9300	60:20:20	59:21:20	10193	12200	15700	1.28	95.5±0.9
H-40	9300	40:30:30	45:27:28	9300	9850	12400	1.26	361±9
H-20	9300	20:40:40	23:38:39	9023	12400	18700	1.51	1506±31

^aTheoretical number average molecular weight. ^b Molar ratio. *Calculated from ¹H-NMR. **Calculated from ¹H-NMR using the H in the central tetra(ethylene glycol) block (“a” as designated in Fig 3.2a) was used as a standard. ⁺Measured using GPC.

Fig 3.3 shows the typical FTIR spectra of the PCLLGA diol and diacrylate using L-60. The peak at 3540 cm⁻¹ due to the hydroxyl group in diol disappeared completely after the acrylation reaction suggesting complete or nearly complete conversion of diol to diacrylate. The CH=CH₂ group gave two absorption bands at 810cm⁻¹(C=C bond twisting) and 1635cm⁻¹(C=C bond stretching) confirming the acrylation was successful. The absorption bands at 1730cm⁻¹ are attributed to ester stretching in the oligomer backbone.

Figure 3.3 FTIR spectrum of PCLLGA diol and diacrylate (L-60).

GPC results (Table 3.1) gave a narrow and normal distribution (1.23~1.51) of the molecular weight. The measured number-average molecular weights (M_n) were similar to the designed M_n , indicating that the polymerization proceeded to near completion.

The viscosity of the diacrylate was observed to decrease as the ϵ -CL content increases but increase with increasing molecular weight (Table 3.1). With the molecular weight kept almost constant, viscosity decreases significantly from 1506 Pa·s with H-20 (low ϵ -CL content) to 96 Pa·s with H-60 (high ϵ -CL content). When more ϵ -CL is present, the polymer chain is longer and the chain is more flexible so that the resistance to flow is lower. The viscosity increases from 1.8 Pa·s with L-60 to 95.5 Pa·s for H-60 as the molecular weight increases from 1800 to 9300 Da (ϵ -CL kept at 60mol%).

DSC scans of the (uncrosslinked) diacrylates show no melting endotherms indicating the absence of crystalline domains (Fig 3.4). T_g of the amorphous oligomers decreases from -12.8°C (H-20) to -42.3°C (H-60) with increasing ϵ -CL molar ratio (with molecular weight kept almost constant) (Table 3.2). But the T_g increases from -57.1°C to -42.3°C as the molecular weight increases from 1800 to 9300 Da (ϵ -CL kept at 60mol%, Table 3.2). Unlike other high molecular weight ($M_n > 150,000$ Da) ϵ -CL/LA copolymers¹⁴⁴, our oligomers displayed one clear single glass transition peak which may be due to the relatively low molecular weight preventing blocky structure formation.

Figure 3.4 DSC result of the PCLLGA diacrylates.

The UV transmittance of the diacrylates was investigated by measuring the 75 μ m-thick resin. The spectrum was shown in Fig 3.5. Each of these diacrylates was shown to have good UV transmission (>97% at 365nm).

Figure 3.5 UV transmittance of PCLLGA diacrylates.

Table 3.2 DSC, real-time FTIR, and mechanical measurement results.

	L-60	M-60	H-60	H-40	H-20
Tg/diacrylate(°C)	-57.1	-54.2	-42.3	-36.6	-12.8
Tg/crosslinked(°C)	-42.2	-47.2	-39.5	-26.9	-0.4
ΔT_g (°C)*	14.9	7.0	2.8	9.7	12.4
Final conversion (%)**	99	99	97	98	98
$t_{1/2}$ (s)	4.2	8.6	11.6	15.7	17.5
E (MPa)	12.29±0.47	6.40±0.49	3.36±0.10	2.73±0.05	1.66±0.11
σ (MPa)	2.46±0.26	3.76±0.47	2.83±0.11	2.69±0.20	2.39±0.21
ε (%)	21±2	79±12	129±3	137±12	176±11

* $\Delta T_g = T_g/\text{crosslinked (°C)} - T_g/\text{diacrylate (°C)}$.

** The final conversion was the conversion calculated after 5 mins UV irradiation.

The results were expressed as average±STDEV (standard deviation) (n=5 to 10).

3.3.2 Photocuring characteristics

Fig 3.6 shows the dynamic acrylate conversion versus UV exposure time for different PCLLGA diacrylates. The final conversion and time to reach 50% of final conversion ($t_{1/2}$) are summarized in Table 3.2. The reactions were nearly completed after 1 min UV irradiation for all oligomers except H-20 which had the highest viscosity (Table 3.1). Free radical polymerization kinetics is known to be controlled by diffusion¹⁴¹. The high viscosity of H-20 can accelerate the diffusion controlled propagation causing enhanced autodecceleration so that the reaction is slowed down^{141, 145, 146}. However, all formulations were highly converted with prolonged UV irradiation, with final conversions reaching more than 95% after 5 mins of UV irradiation so that leaching of unreacted oligomers prior to cell culture was not necessary. With decreasing molecular weight, the reaction proceeded faster due to the higher concentration of functional groups: $t_{1/2}$ is 4.2s for L-60 and increased to 11.6s for H-60 (Table 3.2). 10 minutes used in preparation of photocured films for testing was confirmed to be sufficient.

Figure 3.6 Photocuring yield of PCLLGA diacrylates.

3.3.3 Thermal and tensile properties of photocrosslinked films

The thermal behavior of the photocrosslinked polymers was also examined with DSC (Fig 3.7) and the results are summarized in Table 3.2. Crosslinking led to an increase of the glass transition temperature (ΔT_g) compared to the uncrosslinked oligomers. ΔT_g ranged from 2.8°C to 14.9°C for H-60 and L-60 respectively. This trend is accounted for by the higher crosslinking density and tighter network formed from oligomers with lower molecular weight. In addition, the size of the T_g increase was found to anti-correlate with the ϵ -CL content among the high molecular weight oligomers/polymers. When more GA and LA were present (keeping the same molecular weight), the chain-chain interaction is

increased due to increased chain polarity so that crosslinking which brings chains to closer proximity has a more pronounced effect on the ΔT_g of more polar oligomers. All the crosslinked polymers had glass transition temperatures well below body temperature.

Figure 3.7 DSC result of the photocrosslinked PCLLGA polymers.

Representative uniaxial tensile stress-strain curves of the crosslinked PCLLGA copolymers are shown in Fig 3.8. Average values for the tensile Young's modulus (E), ultimate stress (σ) and maximum strain (ϵ) are listed in Table 3.2. A rather similar ultimate tensile strength was measured for all formulations; it increases by only 50% from the lowest (2.39 MPa for H-20) to the highest (3.76 MPa for M-60). However, there is a substantial range of Young's modulus (6.4 fold increase from the lowest to the highest) and maximum strain in these samples (7.4 fold increase from the lowest to the highest). The crosslinked PCLLGA polymers were generally elastomeric with high maximum strain exceeding 50% except for L-60. The very stiff L-60 has a low strain at failure and it is less suitable for applications in which significant elasticity is desired,

such as arterial grafts. As the molecular weight of the oligomer decreases from 9300 Da for H-60 to 1800 Da for L-60, the corresponding crosslinking density increases so that the polymer becomes stiffer and less extensible: L-60 has modulus of 12.29 ± 0.47 MPa and maximum strain of 21 ± 2 % compared to 3.36 ± 0.10 MPa and 129 ± 3 % respectively for H-60. However, the ultimate stress did not exhibit a monotonic dependence on molecular weight. Crosslinked polymer made from intermediate molecular weight (M-60) had the highest ultimate stress among the three copolymers with the same ϵ -CL content (i.e. L-60, M-60 and H-60). The effect of ϵ -CL content on the tensile properties was not significant. Increased ϵ -CL content resulted in a slightly stiffer and less extensible polymer: H-20 has E of 1.66 ± 0.11 MPa and ϵ of $176 \pm 11\%$ compared with corresponding values of 3.36 ± 0.10 MPa and $129 \pm 3\%$ for H-60. The mechanical properties and regeneration rate vary for blood vessels at different sites of the body so that the matching with the surrounding tissues requires scaffold with different mechanical properties and degradation behaviors. The polymers developed in this study have mechanical properties in the same order as elastin ($E=1.1$ MPa, $\sigma=2$ MPa, and $\epsilon=150\%$)¹⁴⁷ and have a comparable strength (2.4-3.8MPa) to human native arteries which has an ultimate tensile strength of 1.72-3.64MPa

148, 149

Figure 3.8 Representative stress-strain curves of crosslinked PCLLGA polymers.

3.3.4 *In vitro* degradation study

Theoretically, we expect both the ϵ -CL content and network density to affect the water uptake and degradation rate. As the relatively hydrophobic ϵ -CL content increases, the CL/GA/LA copolymer is expected to degrade more slowly. Also, as the crosslink density of the polymer increases, diffusion of water into the crosslinked rubbery networks decreases so that degradation rate should decrease. Our experimental results confirm these two effects.

The effect of ϵ -CL content on the water uptake (%) and weight loss (%) on the three polymers with about the same molecular weight (H-20, H-40 and H-60) shall first be discussed. Fig 3.9 shows that crosslinked samples made from H-20 which had the lowest ϵ -CL content and crosslink density of the five samples had the fastest biodegradation and

water intake rates. After 2 weeks' immersion in PBS, the H-20 crosslinked samples had absorbed much water (34%) and had lost around 7% of their original weight (Fig 3.9). After 4 weeks immersion in PBS, they had become paste-like with no definable form so that accurate weighing of the samples became impractical; the degradation study for this polymer was then stopped. Samples made from crosslinked H-40 with intermediate molecular weight had the second highest biodegradation and water absorption rates; H-40 samples absorbed 27% water and lost 9% weight after 8 weeks immersion in PBS; by week 8, they became too weak to withstand the pressure of the clamps in tensile testing machine so mechanical testing was stopped and after 10 weeks immersion in PBS, the H-40 samples disintegrated. The H-60 polymers were the most durable of the H-series polymers, absorbing less water (7%) and losing less weight (2.5% of the initial weight) after a longer (10 weeks) immersion in PBS.

(a)

(b)

Figure 3.9 (a) Water intake and (b) weight loss of crosslinked PCLLGA polymers during *in vitro* degradation in PBS (pH 7.4).

Fig 3.9 also shows that for M-60 and L-60, there were little water intake and weight loss observed during the 10-week period as these polymers have both higher crosslinking density and higher ϵ -CL content.

Fig 3.10 shows the changes in the mechanical properties (E , σ and ϵ) of the crosslinked polymers with respect to time during *in vitro* degradation. A linear decrease in the Young's modulus (E) and ultimate stress (σ) was observed regardless of the polymer composition indicating a bulk degradation mechanism, resembling the behavior of high molecular weight linear biodegradable polyesters. With a surface-eroding polymer, erosion only occurs at the surface of the sample so that generally the mechanical properties will be maintained^{115, 116}.

Figure 3.10 Change in mechanical properties of crosslinked PCLLGA polymers during *in vitro* degradation in PBS (pH 7.4). (a) Young's Modulus, E ; (b) ultimate stress, σ ; and (c) maximum strain, ϵ . The results were expressed as average $\pm$ STDEV; $n=5$ to 10.

We represent the degradation of Young's modulus or ultimate stress with time by:

$$E(t) = -A_E * t + E_0 \quad (3-4),$$

and

$$\sigma(t) = -A_\sigma * t + \sigma_0 \quad (3-5),$$

where t (weeks) is the immersion time in PBS.

The values of A_E , A_σ , E_0 and σ_0 for each crosslinked polymers based on linear regression fit to the experimental data are summarized in Table 3.3 and also noted in Fig 3.10. The values of E_0 and σ_0 obtained from extrapolation to zero time (Table 3.3) are generally similar to E and σ summarized in Table 3.2. As the amount of ϵ -CL increased, the rate of decrease in Young's modulus (A_E) and ultimate stress (A_σ) were found to decrease: A_E is 0.61 MPa/week for H-20 and decreases to 0.26 MPa/week for H-60. This correlation of degradation rate of tensile properties with ϵ -CL content is similar with the trend noted for the water absorption/weight loss (Fig 3.9). The Young's modulus was also found to decrease faster as the molecular weight between crosslink decreases: A_E is 0.26 MPa/week for H-60 versus 0.43 MPa/week for L-60. The ultimate stress decreased faster with increased molecular weight: A_σ is 0.22 MPa/week for H-60 versus 0.07 MPa/week for L-60.

Table 3.3 Linear regression coefficients for Young's Modulus (E) and ultimate stress (σ), A_E , A_σ , E_0 and σ_0 , and "half-decay lifetime" ($A_E/2E_0$) of PCLLGA polymers during degradation *in vitro* in PBS (pH 7.4).

	L-60	M-60	H-60	H-40	H-20
A_E (MPa/week)	0.43	0.32	0.26	0.36	0.61
E_0 (MPa)	12.15	6.28	3.07	2.60	1.67
A_σ (MPa/week)	0.07	0.19	0.22	0.30	0.70
σ_0 (MPa)	2.27	3.85	2.72	2.45	2.48
$\tau_{1/2}$ (week)	14.1	9.8	5.9	3.6	1.4

$X(t) = -A_x * t + X_0$ ($X = A, \sigma$); t is the immersion time in PBS in weeks.

Fig 3.10 (c) shows the change in maximum strain with increased PBS immersion time. Strain is less sensitive to degradation for these crosslinked polymers. No significant changes in maximum strains were found except for H-20 and H-40. For H-20, the strain was increased from 176% to 210% (an increase of 19%) over 2 weeks; after the 2-week period, the samples absorbed significant water and disintegrated (as shown in Fig 3.9 (a)). Additionally for H-40, the maximum strain increased by 17% from week 4 to week 6 though little change was noted before the 4-week period. The maximum strain of an elastomer depends on the distribution of end-to-end distances between crosslink points within the matrix¹⁴⁷ so that the distance between the crosslink points did not change much when only some chains had been cleaved. However, as more chains were cleaved, the crosslinked polymer became somewhat linear so that the maximum strain increased.

The relatively stable strain for lightly degrading crosslinked biodegradable polyesters has also been observed with star-shaped acrylated aliphatic polyesters^{135, 142}. The retained extendibility of partially degraded crosslinked copolymers was a significant improvement over the corresponding linear copolymers. With the latter, it has generally been observed that, extendibility decreases when molecular weight decreases and this happens usually from the initial period of immersion^{131, 132, 150}. The retained initial extendibility during the first stage of degradation (from weeks to months) of our crosslinked polyesters is useful in the engineering of vascular tissue or other soft tissues in which the scaffold must be elastic during tissue regeneration.

The degradation rate of our crosslinked system can be tuned by varying the molecular weight or composition. Compared to the star-shaped networks, it appears that the acidic degradation products in our diacrylated network can diffuse out more easily from these networks so that lower network density (with higher molecular weight) or ϵ -CL content causes faster degradation; thus the degradation of our linear crosslinked polyesters can be easily tuned via manipulation of molecular weight or composition.

From Fig 3.10 (a) and 3.10 (b), the Young's modulus and ultimate stress of H-20, H-40 and H-60 dropped to below 1 MPa after 2, 6, and 10 weeks respectively; by then, these polymers also absorbed water quickly, lost weight rapidly (Fig 3.9) and became paste-like. This suggests that the degradation of these networks can be roughly divided into two stages. The first stage is characterized by mechanical weakening evidenced in a linear decrease in Young's modulus and ultimate stress with almost constant maximum strain and insignificant water absorption/weight loss. The second stage beginning after the Young's modulus and ultimate stress have dropped to below 1 MPa is accompanied by

rapid weight loss of the polymer which may be attributed to its loose network with rapid water penetration. By extrapolation, E and σ are estimated to drop below 1 MPa simultaneously after 17 weeks and 26 weeks for M-60 and L-60, respectively, upon which rapid weight loss are expected to happen. Also, for each of the networks, the ultimate stress and Young's modulus degrades to about 1 MPa almost simultaneously. Degradation of the scaffold should match the tissue regeneration rate. Currently, 1 to 3 months are needed to achieve a tissue engineered small diameter blood vessel for grafting^{1, 40}. The PCLLGA polymeric films developed in this study have been shown to maintain their mechanical properties ranging from several weeks to several months (3 weeks for H-20 to 4 months for M-60 and 6 months for L-60). This suggests the newly developed polymers have mechanical properties and degradation rate suitable for tissue engineering of blood vessels and these properties can be manipulated according to specific requirements through adjustment of the component and molecular weight of the polymer.

To further estimate the degradation process, the “modulus half-life” defined as “time required for Young's modulus to decline to half of its initial value” was also computed for the five copolymers according to:

$$\tau_{1/2} = \frac{E_0 - E_0/2}{A_E} = \frac{E_0}{2A_E} \text{ (week)} \quad (3-6).$$

The results for the five copolymers are calculated (Table 3.3).

Figure 3.11 (a) shows the correlation between $\tau_{1/2}$ and ϵ -CL content for the three high molecular oligomers (H-60, H-40, and H-20). The “modulus half-life” obeys a linear

relationship versus their ϵ -CL content (admittedly there is only three points) (Fig 3.11 (a)) which can be represented by

$$\tau_{1/2}^{9300} = 1.4 + 2.2 * \frac{([CL] - 20\%)}{20\%} \text{ (week)} \quad (3-7),$$

where [CL] is the molar ratio of ϵ -CL. Using equation (3-7), we can predict that H-80 would have a modulus half life of about 8 weeks. Equation (3-7) is not a strict relationship, though, since the actual M_n is not constant (Table 3.1).

Similarly, for the series of polymers with fixed ϵ -CL content, the “modulus half-life” also obeys a linear relationship versus their measured M_n (Fig 3.11 (b)) which can be represented by

$$\tau_{1/2}^{60CL} = 14.1 - 0.0008 * (M_n - 2080) \text{ (week)} \quad (3-8).$$

An increase of 5000 Da in the M_n would lead to a decrease of 4 weeks in the modulus half-life at 60% ϵ -CL content.

From the above, we can predict how long the scaffolds made from these copolymers can retain their mechanical/structure integrity and this is important for tissue engineering scaffolds.

Figure 3.11 Modulus half-life of PCLLGA diacrylates (a) with roughly fixed molecular weight (9300) versus ϵ -CL content; (b) with fixed ϵ -CL content (60%) versus measured M_n .

3.3.5 *In vitro* biocompatibility with SMCs

The crosslinked films were sterilized by soaking in 70% ethanol/30% ddH₂O solution for 24 hours. The *in vitro* biocompatibility of the sterilized films was tested using a SMC cell line. After 3 day culture, SMCs were found to grow to near confluence on these polymer films, with cell organization and morphology resembling that on the tissue culture plate (Fig 3.12). Cell density on these polymer films was also found to be comparable to that on tissue culture polystyrene plate (TCPS) (89%~99% of TCPS at day 1 and 82%~95% of TCPS at day 3 (Fig 3.13)). These crosslinked polymers are biocompatible and are expected to be useful as tissue engineering scaffold materials.

Figure 3.12 Phase contrast images of SMC grown on photocrosslinked films from different diacrylates (L-60, H-60 and H-20) and tissue culture polystyrene plate after 3 day culture. All pictures were taken at 10X magnification.

Figure 3.13 SMC density on photocrosslinked films from different diacrylates (L-60, M-60, H-60, H-40 and H-20) and tissue culture polystyrene plate (TCPS) at day 1 and day 3.

3.4 Conclusion

A new series of biodegradable photopolymerizable linear PCLLGA diacrylates with different molecular weights and different compositions were successfully synthesized by a two-step reaction: (I) ring opening polymerization of ϵ -CL, L-LA and GA initiated by tetra(ethylene glycol) in the presence of stannous octoate catalyst followed by (II) acrylation with acryloyl chloride. The obtained oligomers and its corresponding crosslinked networks were found to be amorphous with low Tgs.

The oligomers reacted via photopolymerization to form highly crosslinked networks within minutes upon UV irradiation and the obtained polymer films have higher than 95% conversion and are transparent and rubbery. The networks mostly have high elongations at break (more than 50%) and the modulus vary from 12.3 MPa to 1.7 MPa.

The degradation results suggest a two-stage degradation behavior. In the first stage, the Young's modulus and ultimate stress were found to decrease linearly with time for all five networks with almost no weight loss. Lower molecular weight and lower ϵ -CL content led to a faster decrease in Young's modulus. However, the crosslinked polymers maintained their initial extendibility (maximum strain) during this stage. This is an improvement over linear copolymers whose extendibility has been found to decline during degradation and is a desirable feature since partially degraded scaffold must often be elastic during tissue regeneration. The second stage of degradation began after the Young's modulus and ultimate stress had dropped to about 1 MPa and was characterized by rapid weight loss and water uptake. The lower the ϵ -CL content and the higher the molecular weight, the earlier the second stage occurred. Diacrylate with higher molecular weight leads to longer linear chain and less crosslinking point after crosslinking. The linear chain is more prone to degradation than the crosslinking point. By varying the molecular weight and the composition, the crosslinked polymers can be adjusted to maintain their tensile properties for time periods ranging from several weeks to several months. This flexibility should make these networks attractive materials for vascular tissue engineering or a wide range of other biomedical applications. The linear relationship between composition or M_n and the modulus half-life suggests that the lifetime should be easily predictable.

SMC culture showed that these crosslinked polymers are highly biocompatible, nearly as good as tissue culture polystyrene plate, and they can be expected to be useful as tissue engineering scaffolds.

Through adjustment of the composition and molecular weight of diacrylated aliphatic polyester, crosslinked networks with different mechanical properties but controllable biodegradation rate can be designed to meet various requirements in tissue engineering, especially soft tissue engineering applications. The results may also be helpful in predicting the degradation behavior of similar polymer networks.

Chapter 4 3-D microchannels in biodegradable polymeric films to control orientation and phenotype of vascular SMCs

4.1 Introduction

Given the plasticity of SMCs, one of the main limitations of current scaffolding techniques is the difficulty in controlling SMC phenotype shifts *in vitro*. A synthetic phenotype allows the cells to rapidly proliferate and produce ECM whereas a shift to contractile phenotype with organized ECM ultimately provides a functional blood vessel. Narrow micropatterns have been shown to control the orientation or phenotype of SMCs. However, cell growth was also inhibited by the narrow micropatterns. We hypothesize that SMC proliferation, phenotype and orientation can be regulated by physical micropatterning involving relatively wider 3-D microchannels (relative to the width of SMC of about 5-10 μ m) separated by high aspect microwalls. The wide microchannel allows for cell proliferation needed for tissue regeneration and the presence of microwalls provides a physical guidance to cells, especially with confluence. Norman *et al*¹⁵¹ showed that fibroblasts encapsulated in collagen gel inside 3-D microchannels elongated and aligned along the channels throughout the height of the scaffold but their micropatterned scaffold was made from non-biodegradable PDMS. Biodegradable scaffolds with 3-D microchannels separated by high aspect ratio microwalls are useful for emulating the microenvironment and thickness of *in vivo* tissue. However, there are no reports of the fabrication of wide microchannels separated by high aspect ratio microwalls with biodegradable polymer to date; high aspect ratio microwalls are difficult to demold because they tend to break in the micro-mold.

The objective of this chapter is to investigate the effect of 3-D microchannels with varying width and separated by high aspect ratio microwalls on the orientation and phenotype of vascular SMCs. The liquid UV polymerizable biodegradable macromer (PCLLGA diacrylate) synthesized as described in the previous chapter was used for the UV micro-embossing. 3-D biodegradable microchannelled films with microchannels (60-70 μ m deep, widths varying from 10 to 160 μ m, 2cm long) separated by high aspect ratio (8) and narrow (10 or 25 μ m) microwalls over a large area (5cm by 5cm) were made. The effect of the microstructures on cell growth, orientation and phenotype control of vascular SMCs was studied. This study demonstrates two new results: 1) the formation of deep and wide 3-D microchannels separated by high aspect ratio microwalls in flexible and biodegradable films through UV micro-embossing and subsequent lift off from a carrier substrate film and 2) the induction of SMC alignment and shift to a more contractile phenotype nearing confluence, demonstrated by morphological, orientational (both cellular and smooth muscle α -actin) and contractile protein (smooth muscle α -actin) expression changes.

4.2 Experimental details

All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise specified. The PCLLGA diacrylate (H-60) used in this chapter was synthesized as described in Chapter 3. The H-60 polymer was selected because it is most durable among the H-series polymers, absorbing less water (7%) and losing less weight (2.5% of the initial weight) after a longer (10 weeks) immersion in PBS.

4.2.1 Fabrication of microstructured polymeric films

The fabrication of a microstructured biodegradable PCLLGA film is depicted in Fig 4.1. A master Si mold was first prepared using a Surface Technology System

(STS) Deep Reactive Ion Etching (DRIE) according to a published procedure¹⁵². Additionally, the microstructured Si (4-inch <100> p-type silicon wafers) master mold was surface-treated with a passivation step to deposit a Teflon-like polymer on it critical for clean demolding. C₄F₈ was used for the passivation using the DRIE system. The plasma power, C₄F₈ flow rate and pressure in the chamber were 300 W, 100 sccm and 26 mTorr, respectively and the duration was 90 sec. The master mold had 10 groups of microstructures each of which were 60-70μm deep (Fig 4.2). Five groups of the microstructures had microwalls which were 10μm wide and the remaining 25μm wide; the microwalls were all 2cm long. The microchannels between them were either 10, 40, 80, 120 or 160μm wide. For ease of reference, the microstructure geometries are hereafter denoted as w/c where w is width of the microwall and c is width of microchannel. Silastic 184 silicone rubber (Dow Corning Corporation) was poured on the silicon master mold to form flexible and reusable child PDMS molds¹⁵³ from which UV embossing was performed.

Figure 4.1 Schematic representation of fabrication process of a microstructured biodegradable film using PCLLGA diacrylate.

Figure 4.2 Schematic illustration of (a) the micropattern on silicon wafer, and (b) cross-sectional profile of micropattern. All dimensions are in micrometers (μm).

Silastic 184 silicone rubber base and its additive were mixed with the ratio 10:1. The silicone rubber mixture was stirred using a mechanical rod mixer at 200rpm for about 10 minutes. After uniform mixing, the mixture was degassed in a vacuum oven at 0 atmosphere for 15 minutes. After degassing, the mixture was carefully poured onto the master mold. Then the assembly was put into a vacuum oven at 0 atmosphere for further degassing until no bubble was visibly observed. A polyester film (Melinex ® 454 from Dupont) was used to cover the liquid rubber before another glass sheet was pressed onto it. The polyester film prevents the cured silicone rubber from adhering to the glass sheet so that it could be peeled from the master easily. Then this sandwich with the two glass sheets was clamped tightly to get a flat surface and heated at 65°C for 10 hours to harden the rubber. This curing step was enough to make the rubber attain sufficient mechanical strength to cohere during demolding.

After peeling out, the silicone rubber mold was heated at 80°C for another 2 hours. The cross-sectional profile of the microchannel was determined using scanning electron microscopy (SEM). SEM was done using the JEOL JSM-5600LV. Before SEM measurement, all samples were coated with a thin layer of gold using the Palaron SC7640 Sputter Coater. The gold coating time was 200s.

Diacrylated macromer (PCLLGA diacrylate H-60) was stirred for 2h at 65 °C with 0.5 wt% of 2,2-dimethoxy-2-phenylacetophenone (Irgacure 651 photoinitiator) (CIBA Chemicals) predissolved in butanone (10% Irgacure 651 in Butanone); the excess butanone was removed under reduced pressure at 65 °C for 2h. The UV resin formulation was dispensed onto the PDMS mold and allowed to spread in an oven set at 65 °C. Following this, degassing in a vacuum oven at 0 atmosphere at 65 °C was carried out to remove air bubbles and promote resin formulation filling of the mold micro-channel. A polyester film (Melinex® 454 DuPont Teijin Films, 125µm thick) was then carefully overlaid onto the resin to avoid the creation of any air bubbles. The polyester substrate was chosen to have marginal adhesion to PCLLGA so that the PCLLGA would adhere to the substrate more strongly than to the PDMS or Si mold but could be easily peeled from it after demolding. Finally, the resin was polymerized under 365nm UV for 10min. The patterned films were then carefully peeled from the PDMS mold and then the microchannelled film was lifted up from the polyester film. The UV source was a flood UV exposure system with an Hg-lamp, specifically a 350W Mercury lamp with intensity of 10mW/cm² of a mask aligner system supplied by SUSS MicroTech, Germany.

The one exception to this embossing protocol was in the case of 10/10 pattern. This pattern was directly UV embossed from the silicon master mold without the PDMS

child mold intermediate due to the pairing of neighboring microwalls of this dense PDMS soft mold (refer to Fig 4.5 (a3) in section 4.3.1). Aside from the use of the silica master, the 10/10 pattern fabrication procedure was identical to that of the other patterns.

The microchannelled films were examined using SEM as described previously.

4.2.2 Cell culture

The SMCs (ATCC, CRL-1444) were routinely cultured and passaged as described in section 3.2.8. Microchannelled films were cut into 1.5cm diameter discs and sterilized in 70% ethanol (30% ddH₂O) for 24h. Before cell seeding, the films were moved to a 24-well culture plate and equilibrated with PBS for 30mins. Then SMCs together with culture medium were added to the plate (10,000 cells/cm²). Medium was refreshed every 3 days.

At certain time points, phase contrast images were taken using a ZEISS inverted microscope. Cell density was assessed by cell counting using hemocytometer after detachment from substrates and trypan blue staining. After 3 days and 7 days in culture SMCs were also examined with SEM. For SEM analysis, the films were rinsed with PBS 3 times, and then the cells were fixed with 2.5% glutaraldehyde for 4h at room temperature. After thorough washing with PBS, the fixed membranes were dehydrated in graded ethanol series (55%, 70%, 80%, 90%, 95% and 100%) each for 30mins. After coating with a thin layer of gold using a sputter coater, the cells were imaged with SEM.

4.2.3 Analysis of SMC morphology and orientation

To quantify cell morphological changes, phase contrast images were firstly taken using a ZEISS inverted microscope (20×), and then the boundaries of SMCs were outlined using AxioVision 4.0 software (Carl Zeiss, Germany). At least twenty cells from 3 fields for each condition were used. Cells on 25/160 pattern and flat films after 3 and 7 days were examined. Cells grown on flat film were used as control. The spreading area (projection area) (S) and perimeter (L) of the cells were measured. The cell shape index (CSI) was calculated as $(4\pi \cdot S/L^2)$. The cell assumes a linear, elongated morphology when CSI approaches 0 and spreads out and becomes more circular in shape when CSI is 1¹⁴.

The degree of orientation of a single cell was assessed by calculating the percent alignment¹⁵. The cell boundaries were outlined using AxioVision 4.0. The percent alignment of the cell with respect to the microchannels was calculated by:

$$\%Alignment = \frac{B - A}{B + A} \times 100 \quad (4-1)$$

where A is the cell width in the Y-axis (perpendicular to the direction of the microchannel), and B is the cell length in the X-axis (parallel to the direction of the microchannel). For flat film, X-axis is parallel to the horizontal edge of the image and Y-axis is perpendicular to X-axis.

Statistical analysis

All data reported are from at least 3 separate experiments. Errors bars on all graphs represent the standard deviation of the mean. Analysis of variance (ANOVA) followed by Tukey's method was performed using MINITAB software to detect

whether a significant difference existing between groups with different treatments. A significant level of $p < 0.05$ is considered significant and the values reported in the text are taken from Tukey's comparison relative to a control level defined respectively in the caption.

4.2.4 Immunostaining of smooth muscle α -actin filaments

SMCs grown on microchannelled or flat PCLLGA films were washed with $1 \times$ PBS 3 times, fixed in 3.7% formaldehyde for 10 mins and permeabilized with 0.5% Triton X-100 for 5 mins. After blocking with 1% bovine serum albumin (BSA) solution for 20mins to minimize background signal, samples were incubated with monoclonal anti-smooth muscle α -actin (Sigma, 1:75) for 1h. After 3 times thoroughly washing with $1 \times$ PBS, 5 μ l 300U Alexa Fluor® 488 (Molecular Probes) in 200 μ l 1% BSA solution was added and samples were incubated for another 1h. After washing with $1 \times$ PBS 3 times, samples were mounted and used for fluorescence LSM510 confocal microscopy (Carl Zeiss, Germany).

4.2.5 Western blotting

SMCs grown on microchannelled films, flat films and tissue culture polystyrene plates were detached by trypsin-EDTA solution after 3 days and 7 days in culture, diluted in complete medium and centrifuged for 5 mins at 2000rpm. Pellets were then lysed in predetermined amount (according to cell number) of sample buffer (0.125M Tris-HCl, pH 6.8, 4% sodium dodecylsulphate (SDS), 2% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). Equal amounts of lysates were then loaded onto a 10% SDS-polyacrylamide gel. The polyacrylamide gel was made by polymerizing the separating gel solution (3.1ml acrylamide, 2.5ml 4 $\times$ separating buffer (1.5M Tris-HCl, pH 8.8), 100 μ l 10% SDS, 4.3ml H₂O, 50 μ l 10% ammonium

persulfate (APS) and 5 μ l TEMED) and subsequent stacking with stacking gel solution (0.67ml acrylamide, 1.25 ml 4 $\times$ stacking gel buffer (10mM Tris-HCl, pH 6.8), 50 μ l 10% SDS, 3ml H₂O, 25 μ l 10% APS, and 2 μ l TEMED). Electrophoresis was performed in running buffer (0.025M Tris-HCl, pH 8.3, 0.192M glycine, 0.1% SDS) at 100V for 1h. The proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) using a semidry electronic transfer system (Bio-Rad). After blocking with 5% nonfat milk powder, in washing buffer (10mM Tris-HCl, pH 7.5, 0.1% Tween 20, 100mM NaCl), the membrane was incubated with anti-smooth muscle α -actin (1/2000) in washing buffer for 1h at room temperature. After thoroughly washing with Tris-HCl buffer, the membrane was incubated with anti-mouse IgG, horseradish peroxidase-linked species-specific whole antibody (from sheep) (Amersham Biosciences, UK; 1/5000) in washing buffer for 1h at room temperature followed by addition of electrogenerated chemiluminescence (ECL) western blotting detection reagents. Membranes were then exposed to hyperfilm (Amersham Biosciences, UK) for periods ranging from 4 s to 30 s and then developed. The blot was scanned with a GS-800 calibrated densitometer (BIO-RAD). The relative levels of detected proteins were assessed by measuring the integrated intensity of all the pixels in each band excluding the local background. Results were percentages of protein levels in SMCs grown on flat films after 3 day culture. Results are from at least 3 separate experiments.

4.3 Results

4.3.1 Dimensional fidelity of microstructured polymeric films

Large area (5cm $\times$ 5cm) patterned films with nine groups of microstructures (specifically 10/40, 10/80, 10/120, 10/160, 25/10, 25/40, 25/80, 25/120, 25/160) were

successfully prepared (Fig 4.3(a)). Capillary action facilitated microchannel filling by the PCLGLA diacrylate. The ϵ -CL content (60 mole %) of the copolymer also facilitated the mold wetting and demolding; unlike previous embossing studies¹⁵³, no contaminating release additive was needed. The patterned flexible film can be rolled into a tubular structure and sealed using the PCLGLA diacrylate as an adhesive and cured under UV (Fig 4.3(b)) for tissue engineering of small diameter blood vessels as well as other organs such as esophagus or intestine.

Figure 4.3 UV-embossed biodegradable patterns. (a) Large area patterning (5cm×5cm); (b) tubular structure with diameter of about 5mm.

Figure 4.4 SEM imaging of patterns. Isometric view of UV embossed pattern (a) 10/10; (b) 10/160.

The mold and the UV embossed patterns were examined with SEM (shown in Fig 4.4 and 4.5) and the cross-sections were also examined to measure the dimensions of each pattern (Fig4. 5).

Figure 4.5 SEM imaging of patterns. (a) Cross-section of 10/10 pattern. 1, UV embossed pattern; 2, Si mold; 3, pairing of neighboring walls of PDMS mold; (b) cross-section of 10/160 pattern. 1, UV embossed polymer pattern; 2, PDMS mold.

The dimensions of the replicated PCLGA microstructures were compared with those of the PDMS mold or silicon master mold (Table 4.1). The 10/10 pattern dimensions were relatively accurately replicated from the silicon master mold; the small discrepancy between the mold and embossing dimensions (Δ) was due to resin polymerization shrinkage. For the patterns replicated from the PDMS mold e.g. the 10/160 pattern, there were larger differences between the UV embossed pattern and the corresponding PDMS mold. This was due to the non-wetting nature of the PDMS mold which was essential for easy demolding. However, the replication fidelity was sufficiently good since we are interested in dimensions of several cells' width. An aspect ratio of 8.7 has been achieved for the microwalls by this method and such high aspect ratio has not been reported before in the micromolding of biodegradable polymers.

Table 4.1 Dimension of the patterns (Average dimensions of the patterns measured from 10 points, the results are expressed as average $\pm$ standard deviation.). All dimensions are in micrometers (μm).

Pattern		Wall width	Channel width	Height	Aspect Ratio
10/10	Embossing	10.0 $\pm$ 1.1	8.7 $\pm$ 1.0	62.9 $\pm$ 1.0	6.3
	Δ	0.4	0.0	2.3	
10/160	Embossing	7.0 $\pm$ 0.3	153.3 $\pm$ 0.9	61.2 $\pm$ 1.4	8.7
	Δ	1.6	-2.5	1.9	

$\Delta \equiv \text{dimension(mold)} - \text{dimension(embossing)}$.

4.3.2 SMC response to the micropatterns

We investigated the effects of microchannel geometry on SMC morphology, growth, orientation and smooth muscle α -actin expression. After removal from the polyester carrier substrate used for the embossing, the microchannelled film was soaked in ethanol for 24h for sterilization. The resulting film was composed entirely of biodegradable and cytocompatible material¹⁵⁴⁻¹⁵⁷.

The results show that SMCs aligned along the direction of the microchannel of 10/10 and 25/10 patterns and exhibited an elongated morphology similar to those of SMCs *in vivo*⁴⁸. Representative data are shown for only 25/10 pattern in Fig 4.6(a1), 4.6(a2), 4.7(a1), 4.7(a2), 4.7(b1) and 4.7(b2). Smooth muscle α -actin also aligned along the direction of the microchannels (Fig 4.8 (a1), 4.8(a2)). However, the cell density was low on these patterns with narrow 10 μ m channels even after 7 days ($37.9\% \pm 2.5\%$ of the number of cells grown on flat film, Fig 4.9).

In the wider (40, 80, 120 or 160 μ m) microchannels, the cell growth behavior was similar. The characteristic cell growth in wider channels was demonstrated using the 25/160 pattern. After 3 day culture; the cells grew randomly, showed a fibroblast morphology (Fig 4.6 (b1), Fig 4.7(c1), and 4.7(c2)) and had a CSI comparable to cells grown on flat films characteristic of the synthetic phenotype⁶⁷ (0.41 ± 0.12 versus 0.40 ± 0.10) (Fig 4.10(a)). However, after 7 day culture, the cells aligned along the direction of the microchannel and showed an elongated morphology (Fig 4.6 (b2), 4.7(d1) and 4.7(d2)), which was supported by the significant decrease of CSI (0.17 ± 0.05 after 7 day culture) and the prominent increase of %Alignment of SMCs (from $12.8 \pm 23.8\%$ after 3 day culture to $80.6 \pm 5.3\%$ after 7 day culture) (Fig 4.10). Smooth muscle α -actin staining results also showed randomly oriented actin filaments

with fibroblast-like morphology after 3 day culture (Fig 4.8 (b1)) but elongated and stretched actin filaments along the direction of the microwall after 7 day culture (Fig 4.8 (b2)).

Comparatively, SMCs grown on flat film showed randomly oriented cells (Fig 4.6 (c1) and 4.6 (c2)) and smooth muscle α -actin filaments (4.8 (c1) and 4.8 (c2)) both after 3 day and 7 day culture. Further, although cell growth was inhibited on wide micropatterns initially after 3 days (Fig 4.9), cell density on these micropatterns was almost comparable to that on flat film after 7 day culture except on pattern 25/40; the cell density on pattern 25/160 increased from $67.5\% \pm 5.1\%$ to $99.9\% \pm 17.5\%$ of that on flat film as cell culture duration changed from 3 to 7 days whilst the 7-day cell density on 25/40 was only $57.7\% \pm 1.9\%$ of that on flat film.

Figure 4.6 Phase contrast images of SMCs on (a) pattern 25/10; (b) pattern 25/160; (c) flat film. 1: after 3 day culture; 2: after 7 day culture. All pictures were taken at 20x magnification. SMCs on pattern 25/160 switched from fibroblast-like morphology and random orientation (after 3 day culture) to elongated morphology and unidirectional orientation (after 7 day culture). “c” stands for the microchannel and “w” stands for the microwall.

Figure 4.8 Immunofluorescence image of smooth muscle α -actin staining of SMCs on (a) pattern 25/10; (b) pattern 25/160; (c) flat film. 1, after 3 day culture; 2, after 7 day culture. Scale bar = 20 μ m. All pictures were taken at 40x magnification. For SMCs grown on pattern 25/160, initial randomly organized α -actin (after 3 day culture) reorganized into unidirectional orientation along the microchannels after 7 day culture.

Figure 4.9 Cell growth in different patterns. The results were shown relative to cell number on flat films. Cell seeding density: 10,000cells/cm². The cell density on flat film was around 10,000 cells/cm², 144,000 cells/cm² and 235,000 cells/cm² after 1day, 3 day and 7 day culture respectively. Cells grown on tissue culture polystyrene plate were used as control. Bars represent means $\pm$ STDEV from at least 3 experiments. No significant difference was found after 1 day culture among all samples. After 7 day culture cell density on micropatterns with wider channels (80, 120, and 160 μ m wide) was comparable to that on flat film. ANOVA was performed, followed by Tukey's method for comparison between treatments. *Statistical significance (P<0.05) compared with cell number on flat film on the corresponding day.

Figure 4.10 Morphology and orientation change of SMCs on pattern 25/160 after 3 day and 7 day culture. SMCs grown on flat film after 3 day culture were used as control. (a) Cell shape index (CSI) of SMCs; (b) %Alignment of SMCs. Bars represent means $\pm$ STDEV from at least 20 cells from 3 different fields. CSI of SMCs grown on 25/160 after 7 day culture was significantly lower than those of 25/160 and flat films after 3 day culture. In (b), the %Alignment of SMCs significantly increased on pattern 25/160 after 7 day culture compared with 25/160 and flat films after 3 day culture. For statistical analysis, ANOVA was performed, followed by Tukey's method for comparison between treatments. *Statistical significance ($p < 0.05$) compared with "Flat 3d").

In addition, western blotting results showed that smooth muscle α -actin expression of SMCs grown on micropatterns was obviously enhanced after 7 day culture compared to that after 3 day culture (Fig 4.11 (a)). After 7 day culture, the smooth muscle α -actin expression of SMCs grown on pattern 25/160 was 2.84 ± 0.42 fold that of after 3 day culture (Fig 4.11 (c)). Similar significant increase was also observed for the 25/10 pattern. However, no significant increase of smooth muscle α -actin expression of SMCs grown on flat film or tissue culture polystyrene plates was found (Fig 4.11 (a), 4.11 (c)). In another set of experiments, smooth muscle α -actin expression on different substrates after 3 day culture was compared and no significant

difference was found (Fig 4.11 (b), 4.11 (c)) (1.22 ± 0.14 for pattern 25/10 and 0.80 ± 0.08 for pattern 25/160 relative to that on flat film).

(a)

(b)

(c)

Figure 4.11 Smooth muscle α -actin expression of SMCs in channels of micropatterns (25/10 and 25/160), on flat film (flat) or on tissue culture polystyrene plate (control)

by western blotting (a) after 3 day vs 7 day culture; (b) after 3 day culture; (c) quantitative analysis (results were normalized by cell number and were shown relative to α -actin expression of SMCs on flat film after 3 day culture). Bars represent means $\pm$ STDEV from at least 3 experiments. α -actin expression was significantly increased on micropatterns after 7 day culture compared with that after 3 day culture, which was not found on flat films or control.

4.4 Discussion

In this study, the SMC phenotype shift from synthetic to a more contractile phenotype was observed when SMCs reached confluence on micropatterns with wide microchannels (80, 120, 160 μ m wide) separated by microwalls. In these wide microchannels, SMCs were found to proliferate with a fibroblast morphology and random orientation initially (3 days) when the cell density is low; they switched to elongated morphology, unidirectional orientation and a more contractile phenotype with upregulation of smooth muscle α -actin expression nearing confluence (7 days) achieved with cell density comparable to that of unpatterned film. The switch to a more contractile phenotype is hypothesized to be due to increased unidirectional tensile stress generated within closely packed, aligned and elongated cells^{104, 106, 158}. The synthetic phenotype allows cell proliferation and ECM production thus making vascular tissue engineering feasible. Contractile SMCs are required for normal vessel contraction and dilation stability¹³. Such a phenotype switch with confluence is, thus far, unique to our physical micropattern and has not previously been observed.

Thaker *et al*¹⁴ showed that on 2-D 20- and 30- μ m collagen strips on glass or petri dish, the vascular SMCs were aligned after 24h. However, these strips were only 2-3 cells wide. With longer term culture, since the surface was not completely non-fouling, cells could deposit matrix and proliferate outside the micro-pattern¹⁵⁹. Chiu *et al* used microfluidic patterning to show that cells can be restricted to grow to confluence in channels¹⁶⁰. However, when the rubber stamp was removed, the cells spread to the initially stamp-contacted area – destroying the micropatterns.

In contrast to the previous study¹⁴ in which 2-3 SMCs were aligned on each of the 10-50 μ m wide 2-D collagen strips, our microchannelled polymeric film with wide

microchannels can align SMCs on a larger proportion of the surface area. The use of 3-D microwalls with wide microchannels guides the cells to grow and align along a defined “tissue axis” nearing confluence. Multilayered elongated and aligned SMCs could be achieved by encapsulation of SMCs within hydrogels (eg., collagen type I) dispensed into the 3-D microchannels¹⁵¹. To achieve a final “tissue-like” continuous multilayered aligned cell sheet, the relatively narrow microwalls should be degraded away. Ideally, the mechanical properties and biodegradability of the microwall should be adjusted to match the SMC growth rate. The degradation and mechanical properties of the copolymer of PCL/LGA copolymer can be adjusted by varying the molecular weight and composition as studied in the Chapter 3. The biodegradability of this particular PCL/LGA is slow since the hydrophobic ϵ -CL content is high (60%). This crosslinked polymer absorbs a small amount of water (7 weight %) and loses a small amount of weight (2.5% of the initial weight) after 10-week immersion in PBS. The biostability of this polymer makes our demonstration of phenotype and alignment shift less complicated but future studies will have to include the effects of biodegradation. Biodegradable aliphatic polyesters based on ϵ -CL, GA and L-LA have been widely used in tissue engineering and implants such as sutures because their degradation products are cytocompatible¹⁵⁴⁻¹⁵⁷ and they are approved by FDA to be used in therapeutic devices^{113, 161}.

L’Heureux *et al*⁴⁷ made a completely biological TE human blood vessel by wrapping cultured SMC sheets around a tubular support to produce the media of the vessel. However, the culture time was long without the use of a scaffold. Our microchannelled film can be wrapped to make a cylindrical tube (Fig 4.3 (b)). Further, similar to the work of Norman *et al*¹⁵¹, SMCs encapsulated in gel can be grown inside the deep 3-D microchannels to achieve a more *in vivo* like 3-D cellular

microenvironment and reconstruction of the relatively thick (few hundred microns) medial layer of small diameter vascular grafts. Our microchannelled scaffold potentially allows the engineered vessels to be implanted before full cellular proliferation for *in vivo* tissue engineering, thereby shortening the time for cell culture in contrast to L'Heureux's approach. Very high aspect ratio (up to 15) UV embossed microwall for other non-biodegradable polymer¹⁶² has also been demonstrated by us. With such a high aspect ratio (e.g. 15) microwall, we can potentially make single-layer microchannels filled with SMC encapsulating hydrogel which are several hundred microns deep separated by narrow (10 to 30 μ m wide) microwalls to emulate the thickness of the medial layer of the natural small diameter blood vessels. Single layer micropatterned film allows for easy nutrient and gas exchange between the encapsulated cells and the medium. Compared to other scaffolds with lower aspect ratio microwalls or narrower microchannels¹⁴, our scaffolds also provide more space for cells to grow to achieve a higher cell density and also relieve the burden of metabolism when the scaffolds degrade since less synthetic materials is used.

Cell-cell contact between adjacent lanes can be achieved by biodegradation of the microwalls or nanoporous, perforated or semi-permeable microwalls. Bipolymer microwalls have been demonstrated by Moran *et al.*¹⁶³ and the method can be extended to multi-skinned or multi-layered microwalls with different biodegradation rates. Nanoporous UV curable materials can also be made by selective removal of one block of a phase separated di- or tri-block copolymer as demonstrated for other polymeric systems^{164, 165}. Perforated microwalls with solid walls separated by small spacings that do not allow through-cell migration can also be used. Alternatively, moderately crosslinked hydrogels that do not swell excessively but yet allowing protein diffusion can also facilitate the lane-to-lane communication.

4.5 Conclusion

We have successfully prepared UV embossed narrow and wide microchannels (10 to 160 μ m wide) separated by high aspect ratio (8.7) and high resolution (10 or 25 μ m) microwalls using a biodegradable and photopolymerizable PCL/LGA diacrylate over a large area (5cm by 5cm). This method of micropatterning is rapid and convenient. We have also shown that the 3-D micropattern with wide microchannels (80 to 160 μ m) allows SMC proliferation at low cell density and causes a shift from synthetic to a more contractile phenotype and random to unidirectional cellular orientation at high cell density nearing confluence. The phenotype shift was confirmed by elongated SMC morphology and enhanced smooth muscle α -actin expression. The unidirectional alignment was confirmed by cellular and α -actin orientation. The wide microchannels also allow cells to grow to a higher cell density comparable to that of flat surfaces. However, the narrower microchannels (10, and 40 μ m wide) inhibited the cell growth leading to a much lower final cell density although they also induced the up-regulation of smooth muscle α -actin expression. These films are flexible and can be folded into tubular and lamellar structures. Such foldable microchannelled films with wide microchannels composed exclusively of biodegradable polymers may be useful for guiding SMCs to grow into functional small diameter vascular grafts and potentially effective scaffolds for engineered functional tissues or organs needing 3-D geometric control.

Chapter 5 Effects of cell seeding density on proliferation and phenotype of vascular SMCs on microchannelled PCLLGA films

5.1 Introduction

In the previous chapter, we have demonstrated that our 3-D biodegradable PCLLGA-based microchannelled films with wide microchannels separated by narrow microwalls allowed cell proliferation at low cell density and induced SMC alignment and shift to a more contractile phenotype nearing confluence in 7 days. The cellular and smooth muscle α -actin morphological and orientational details and contractile protein (smooth muscle α -actin) expression confirm these results. Our foldable microchannelled PCLLGA films can be optimized to a clinically viable scaffold for the tissue engineering of small diameter blood vessels. For this, the time required for vessel culture should be as short as possible¹⁶⁶. This is an easily neglected but practically very important problem in view of the frequent perilous condition of patients needing grafts. This is a major reason why present vessel transplant surgery employs synthetic grafts passivated with “biologicals” when autologous grafts are unavailable or unsuitable¹⁶⁷. The main factor causing the long time period necessary for *in vitro* tissue engineering is the low proliferation rate of vascular cells such as SMCs and the slow rate at which cultured cells remodel themselves into the native cell-like microarchitecture. The dynamic culture of SMCs in bioreactors has been widely adopted to quicken engineered vessel maturation. However, the maturation period may be too long for future real clinical application^{18, 168}.

Since the cellular unidirectional alignment and elongation are induced by constrained confluence, we postulate that the culture time required to trigger

elongation and alignment should be influenced by the initial cell seeding density. In fact we have identified this obvious phenomenon in our earlier studies: when cells are seeded in culture wells unequally, the cells with higher initial density will first arrive at the confluence state. This observation suggested to us that the initial cell seeding density may have a significant effect on the SMC alignment rate and on the time needed for *in vitro* regeneration of the vascular wall medial layer. This study seeks to examine the effects of initial cell seeding density on the evolution of SMC morphology and phenotype as a function of culture time on microchannelled scaffolds. Another aim is to optimize the culture conditions for rapid fabrication of large area unidirectional aligned/elongated SMC cell sheets. SMCs at three densities, 10, 100 and 200 thousand cells per cm^2 , were seeded on the microchannelled scaffolds for 1 week. The cell proliferation, morphology, alignment, and quantity and alignment of smooth muscle α -actin were evaluated at different time points. By correlating the seeding density and the phenotypic change of SMCs, we attempt to provide a scientific basis for the choice of seeding density of vascular SMCs in monolayer culture on our PCLLGA-based microchannelled films.

5.2 Materials and Methods

All chemicals were purchased from Sigma and used as received unless otherwise specified. Microchannelled film was fabricated as described in section 4.2.1 using PCLLGA diacrylate (Mn: 12200, molar ratio of CL:LA:GA = 59:21:20) synthesized as previously described in section 3.2.2. The microchannelled film used in this chapter had 160 μm -wide microchannels separated by 25 μm -wide and 60-70 μm -high microwalls.

5.2.1 Cell culture and characterizations

The SMCs (ATCC, CRL-1444) were routinely cultured and passaged as described in section 3.28. Microchannelled films were cut into 1.5cm diameter discs and sterilized in 70% ethanol (30% ddH₂O) for 24h. Before cell seeding, the films were moved to a 24-well culture plate and equilibrated with PBS for 30mins. Then SMCs together with culture medium were added to the plate at different densities (10,000, 100,000 or 200,000 cells/cm²). Medium was refreshed every day. After 0 day, 1 day, 3 day and 7 day culture, phase contrast images were taken using a ZEISS inverted microscope. Cell density was assessed by cell counting using hemocytometer after detachment from substrates and trypan blue staining.

The morphology and orientation of SMCs after 1 day, 3day and 7 day culture were analyzed as described in section 4.2.3. SMCs after 1 day, 3 day and 7 day culture on the microchannelled film were subjected to immunostaining of the smooth muscle α -actin as previously described in section 4.2.4. SMCs grown on microchannelled films were detached after 1 day, 3 day and 7 day culture and used for western blotting analysis for smooth muscle α -actin as described in section 4.2.5. For SMCs seeded with 10,000 cells/cm², the cell density was very low after 1 day culture, therefore, the western blotting analysis was not carried out due to the abundance of samples needed for the analysis. Results were expressed relative to protein levels in SMCs seeded at 10,000 cells/cm² after 3 day culture. Results are from at least 3 separate experiments.

5.3 Results and Discussion

5.3.1 Effects of seeding density on cell morphology and alignment

The effect of seeding density on the initial morphology and alignment was found to be profound. Representative data are shown in Fig 5.1 and 5.2. After 1 day and 3 day

culture, cells seeded with low density (10,000 cells/cm²) grew randomly, exhibited a fibroblast morphology and had a CSI of around 0.4 (Fig 5.2). However, after 7 day culture, the cells aligned along the direction of the microchannel and became elongated, indicated by the significant decrease of CSI and the prominent increase of %Alignment of SMCs. With higher seeding densities of 100,000 and 200,000 cells/cm² after 1 day culture, a more elongated cell morphology indicated by the lower CSI value (0.28 or 0.24 versus 0.39) was observed. Cell alignment was also found to be better with higher seeding densities expressed by the higher %Alignment of 44% and 65% for 100,000 cells/ cm² and 200,000 cells/cm², respectively (p<0.05 compared to 10,000 cells/cm²). With prolonged culture time, cells seeded with both higher densities became more elongated and well aligned as indicated by the %Alignment of 70%-80% after 3 day culture. After 7 day culture, all seeding densities resulted in elongated and well aligned SMCs.

Figure 5.1 Phase contrast image of SMCs grown on microchannelled PCLLGA films with different seeding densities (10,000, 100,000 or 200,000 cells/cm²) after 0 day (0d), 1 day (1d), 3 day (3d), or 7 day (7 d) culture. All pictures were taken at 20X magnification.

(a)

(b)

Figure 5.2 (a) Cell shape index (CSI) and (b) %Alignment of SMCs grown on microchannelled (25/160) PCL/LGA films with different seeding densities (10,000, 100,000 and 200,000 cells/cm²). For statistical analysis, ANOVA followed by Tukey's method was performed. *Statistical significance ($p < 0.05$) compared to 1d result of corresponding seeding density for each seeding density. Results were expressed as average $\pm$ STDEV; $n \geq 3$.

5.3.2 Effects of seeding density on cell proliferation

As seen in Fig 5.3, we found that cells seeded at low or high density reached almost the same final density at confluence. A plateau density of around 250,000 cells/cm²

was achieved regardless of the initial cell seeding density. The results were expected since cell proliferation is principally controlled by cell-cell contact inhibition¹⁶⁹, which is essentially density-dependent. Therefore, it could be expected that higher seeding densities will necessarily lead to a more rapid generation of inhibitory contacts and consequently to a more rapid appearance of confluent state.

Figure 5.3 SMC density on microchannelled PCLLGA films with different initial seeding densities after 1 day (1d), 3 day (3d) or 7 day (7d) culture. Results were expressed as average $\pm$ STDEV; $n \geq 3$.

5.3.3 Effects of seeding density on the expression of smooth muscle α -actin

For each of seeding densities, several samples were terminated from culture after 1 day, 3 day or 7 day culture and assigned for immunostaining or western blotting analysis. Western blotting analysis was not carried out for samples seeded with 10,000 cells/cm² after 1 day culture as an abundance of samples are necessary due to the low cell density. After 1 day and 3 day culture, smooth muscle α -actin of the SMCs seeded with low density (10,000 cells/cm²) oriented randomly and became

aligned along the direction of the microchannels after 7 day culture (Fig 5.4). For SMCs seeded at $100,000\text{cells}/\text{cm}^2$, smooth muscle α -actin was randomly oriented after 1 day culture but aligned after 3 day culture. With the highest initial cell seeding density ($200,000\text{cells}/\text{cm}^2$), the SMCs aligned after only 1 day.

Fig 5.5 shows that smooth muscle α -actin expression (measured by western blotting) in SMCs seeded with low density ($10,000\text{cells}/\text{cm}^2$) on microchannelled PCLLGA films was significantly increased only after 7 day culture; 3 day culture was insufficient for the up-regulation of smooth muscle α -actin. With the higher initial seeding densities ($100,000\text{cells}/\text{cm}^2$ and $200,000\text{cells}/\text{cm}^2$), A significant increase in smooth muscle α -actin expression was observed after only 3 day culture (Fig 5.5) and no further increase in smooth muscle α -actin expression was found after 7 day culture with these higher seeding densities. The initial seeding density influenced the rate of increase of smooth muscle α -actin expression of SMCs cultured on microchannelled films: a higher seeding density is associated with a more rapid rate of increase of the smooth muscle α -actin expression.

Figure 5.4 Immunostaining of smooth muscle α -actin of SMCs grown on microchannelled PCLLGA films with different seeding densities (10,000, 100,000 or 200,000 cells/cm²) after 1 day (1d), 3 day (3d) or 7 day (7d) culture. All pictures were taken at 40X magnification.

(a)

(b)

Figure 5.5 (a) Smooth muscle α -actin expression of SMCs with different seeding densities (10,000, 100,000 or 200,000 cells/cm²) in channels of micropatterns (25/160) by western blotting; (b) quantitative analysis (results were normalized by cell number and shown relative to α -actin expression of SMC seeding density of 10,000 cells/cm² after 3 day culture). Bars represent means $\pm$ STDEV from at least 3 experiments.

The switch to a more contractile phenotype is hypothesized to be due to the increased almost unidirectional stresses generated within the closely packed and elongated cells. The physically separating microwalls are hypothesized to induce significant mechanical stresses within the SMCs when cell density becomes high and these are known to regulate SMC phenotype. When SMCs were seeded with medium

or high density (100,000 and 200,000 cells/cm²), smooth muscle α -actin expression was increased after 3 day culture when cells reached confluence as opposed to 7 days needed for low seeding density (10,000 cells/cm²). The smooth muscle α -actin expression saturated to almost the same level, regardless of the initial cell seeding density. Comparing Fig 5.3 and Fig 5.5 (b), the onset for the up-regulation of α -actin coincides with the onset of confluency, regardless of the initial cell seeding densities. The coincidence of the two onsets confirms that confluency and increased intracellular stresses cause the up-regulation of smooth muscle α -actin expression only when the cells become confluent and the mechanical stresses can be transmitted between closely packed and elongated cells. The “saturated” smooth muscle α -actin expression was almost the same for all densities investigated after 7 day culture and this mirrored the saturation trend of cell proliferation. This further confirmed that constrained confluence leads to the switch to a more contractile phenotype. By varying the cell seeding density, the time needed for SMC elongation, orientation and phenotype switch can be modulated, with higher seeding densities leading to more rapid cell elongation, orientation and phenotype switch.

In our lab, a layer-by-layer (LBL) process (Fig 5.6) has been developed to build 3-D tissue¹⁷⁰. The LBL process builds 3-D tissues quickly by overlaying multiple SMC layers with high initial seeding density (150,000 cells/cm²). Briefly, a multilayered structure can be produced by firstly forming a confluent and aligned SMC layer on our microchannelled scaffold and overcoating it with a thin layer of collagen gel followed by overlaying another cell layer, and so forth. A thin layer of collagen gel (type I collagen from rat tail) is added between each SMC layer to provide a substrate for the seeding of the next SMC layer. The time interval between successive SMC seeding for each layer is 1.5 days: 1 day for cell confluence and 0.5 day for collagen

gelation and further contraction. Successive buildup of such layers should lead to a 3-D tissue-like construct with well aligned SMCs possessing the morphology and alignment desired for an engineered vascular medial layer. Serial optical sections of fluorescently dyed F-actin and smooth muscle α -actin filament made by confocal scanning in the Z-direction exhibit different cell layers all consisting of elongated SMCs highly aligned along the long axis of the microchannels. The ability to quickly culture multiple sheets of SMCs implies that the whole period needed for patterning of a 3-D “thick” (by the standards of small vessel engineering) smooth muscle tissue by the LBL process could be markedly shortened, e.g., 5 layers could be rapidly fabricated within 1 week’s time. The cost can be greatly reduced and the implications for clinical application are apparent.

Figure 5.6 (a) Schematic illustration of LBL process.

5.3.4 Conclusion

This study examined the effect of initial SMC seeding density on cell morphology, alignment and phenotype on a microchannelled scaffold. Low, medium and high seeding densities of 10,000, 100,000 and 200,000 cells per cm^2 were investigated. Although the rates of proliferation, alignment and phenotype change were different

with different densities, the cells all became elongated and well aligned along the direction of the microchannels after 7 day culture. The SMCs saturated at confluence at the same final density and with similar smooth muscle α -actin expression levels, regardless of the initial cell seeding density, though a faster increase was observed with higher seeding densities.

In conclusion, for the cell seeding densities investigated, the higher the seeding densities of SMCs on microchannelled PCLLGA films with 160 μm -wide microchannels separated by microwalls (25 μm wide and 60-70 μm -deep), the shorter the time needed for elongation, orientation and increase of smooth muscle α -actin expression. The faster alignment and phenotype change can be exploited in the LBL process to regenerate the 3-D medical tissue layer.

Chapter 6 Conclusion and recommendations

6.1 Design and synthesis of PCLLGA diacrylate

A new series of biodegradable photopolymerizable linear PCLLGA diacrylates based on GA, L-LA and ϵ -CL with low, medium and high molecular weights (1800, 4800 and 9300 Da) and different ϵ -CL contents (20%, 40% and 60%) were successfully synthesized by ring opening polymerization followed by acrylation. The resultant oligomers were amorphous and appeared as viscous liquids at room temperature.

6.2 Mechanical properties and degradation behaviors of UV crosslinked PCLLGA films

Liquid-to-solid polymerization was carried out by UV irradiation in the presence of a photoinitiator. The photocuring yield was high ($> 95\%$) and the photocrosslinked polymers were amorphous and rubbery. Mechanical measurements showed that the polymers can be stretchable or rigid; the high molecular weight/low ϵ -CL network has a strain of 176% and modulus of 1.66 MPa while the low molecular weight/high ϵ -CL network has a strain of 21% and modulus of 12.3 MPa. Films formed from these new polymers have excellent biocompatibility with SMCs. The 10-week *in vitro* biodegradation study showed a two-stage degradation behavior. In the first stage, the polymer weight and strain remained almost constant but a linear decrease in the Young's modulus (E) and ultimate stress (σ) were observed. An almost linear relationship between ϵ -CL content or molecular weight and modulus half-life (time for the modulus to degrade to 50% of initial value) was found; low molecular weight and high ϵ -CL content correlates with longer modulus half-life, suggesting that the lifetime should be predictable. The retained extendibility (maximum strain) during

this stage is an improvement over linear copolymers whose extendibility has been found to continually decline during degradation. The retained extendibility is a desirable feature since partially degraded scaffold must often be elastic during blood vessel or other soft tissue regeneration. The second stage began after the Young's modulus had dropped below 1 MPa and was characterized by rapid weight loss and strain increase. By varying the molecular weight and composition, the crosslinked polymers can be adjusted to obtain high their tensile properties for time periods ranging from several weeks to several months during degradation. This flexibility should make these networks attractive materials for vascular tissue engineering or a wide range of other biomedical applications.

6.3 Fabrication of microchannelled polymeric films

Microchannelled polymeric films with narrow and wide microchannels (10 to 160 μm wide) separated by high aspect ratio (8.7) and high resolution (10 or 25 μm) microwalls were successfully fabricated using the liquid PCLLGA biodegradable diacrylate (with 60% ϵ -CL and Mn of 12200 Da) over a large area (5cm by 5cm) by UV embossing. The replication fidelity of the liquid PCLLGA was good. The liquid oligomer can wet the PDMS mold to fill the microchannels. The resulting microchannelled film was composed entirely of biodegradable and cytocompatible material. The patterned films are flexible and can be rolled into a tubular structure for tissue engineering of small diameter blood vessels as well as other tubular organs such as esophagus or intestine. This method of micropatterning by UV embossing is rapid and convenient.

6.4 Effect of different widths of the microchannels on SMC response

The widths of the microchannels have significant effects on SMC growth and phenotype. The narrow microchannels (10 and 40 μm wide) have an inhibitory effect on SMC growth although SMC alignment and up-regulation of smooth muscle α -actin expression were observed. The wider microchannels (80, 120, 160 μm wide) allows cell proliferation at low cell densities and causes a shift from synthetic to a more contractile phenotype and random to unidirectional cellular orientation only nearing confluence with achieved cell density comparable to that of flat film. The phenotype shift was confirmed by the change from fibroblast morphology to elongated morphology and the up-regulation of smooth muscle α -actin expression. The synthetic phenotype allows cell proliferation and ECM production thus making vascular tissue engineering feasible whereas contractile quiescent SMCs are required for normal vessel contraction and dilation. Such a phenotype switch with confluence is, thus far, unique to our physical micropattern and has not previously been observed. Our foldable microchannelled films with wide microchannels are potentially effective scaffolds for tissue engineering of functional blood vessels or other organs needing 3-D geometric control.

6.5 Effects of SMC seeding densities on SMCs in the microchannels

Higher seeding densities of SMCs on microchannelled PCLGA films (160 μm -wide microchannels separated by 25 μm -wide and $\sim$ 65 μm -deep microwalls) resulted in faster arrival of SMC elongation, orientation and increase of smooth muscle α -actin expression. A cell seeding density of 100,000 or 200,000 cells/ cm^2 requires less than

3 days for confluence and phenotype switch compared to 7 days needed for that seeded at 10,000 cells/cm². This result brings the development of functional smooth muscle tissue significantly closer to the goal of clinical application.

6.6 Recommendations for future works

This study reports the development and the application possibility of biodegradable microchannelled UV embossed PCLLGA polymeric films for tissue engineering of the tunica media of small diameter blood vessels. A smooth muscle cell line has been used. Primary SMCs and *in vivo* implants are recommended in future studies and some efforts in these directions using these microchannelled polymeric films have been started in our lab. Cell culture with scaffolds in tubular form must be established. SMCs can be aligned and oriented in circumferential microchannels on the outside of a tubular scaffold but this requires a special tubular mold for the micropatterning. The aligned LBL process of SMCs on the microchannelled tubular scaffold would potentially provide an excellent prototype TE medial layer with improved burst strength and vasoactivity similar to that found in native blood vessels. Alternatively, the LBL process could also be performed on microchannelled film and wrapped into a tubular form towards fabricating much thicker vascular medial layer rapidly.

The effect of the degraded products on SMCs is worth further investigation. The effects of the degraded products of the PCLLGA scaffolds on SMC proliferation and differentiation still need to be further evaluated so that the composition and the degradation rate of the scaffold can be optimized for vascular tissue engineering.

The microchannelled film can be made nanoporous, perforated or semi-permeable, which can facilitate the nutrient transfer and adjacent lane-lane communication. Multilayers of cells can be constructed in the microchannels to achieve a final “tissue-

like” multilayered aligned cell sheet. To achieve a more functional smooth muscle tissue, easy medium transfer within the multiple layers of cells and the adjacent lane-lane communication should be developed.

Effects of combined growth factors or mechanical stimulation on SMCs or the engineered tissues should be investigated. Although our microchannelled films has been shown to induce SMC alignment and elongation, and further a more contractile phenotype upon cell confluence, growth factors and/or mechanical stimulation may still be needed to further regulate the cellular growth and differentiation, and ECM formation to achieve a fully functional vascular media.

Co-culture with ECs is worthy to be carried out. As a natural neighbor to SMCs, ECs have marked influence on SMC behavior. Therefore, the co-culture of SMCs with ECs should be established and the effects of ECs on cell growth, differentiation and ECM secretion of SMCs cultured in the microchannels should be explored. The ECs will be cultured on the opposite side of the microchannels. The cell-cell communication can be realized by the nanoporous or semi-permeable scaffold. Alternatively, a coherent layer of ECs can be put on the SMC construct to test the effect on SMC behavior and the function, variability and stability of the system.

The phenotypic changes of SMCs are hypothesized to be induced by mechanical stress as discussed in the text. Relative experimental models should be established and the stress should be measured in order to understand the mechanism behind the microchannel-induced SMC phenotypic changes.

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Appendix: Publication

- (1) Shen JY, Chan-Park MBE, Feng ZO, et al., “UV-embossed microchannel in biocompatible polymeric film: Application to control of cell shape and orientation of muscle cells”, Journal of Biomedical Materials Research Part B-Applied Biomaterials 77B (2): 423-430 MAY 2006
- (2) Shen JY, Chan-Park MBE, He B, et al., “Three-dimensional Microchannels in Biodegradable Polymeric Films for Control Orientation and Phenotype of Vascular Smooth Muscle Cells”, Tissue Engineering, 12(8): 2229-2240 August 2006
- (3) Shen JY, Pan XY, Lim CH, et al., “Synthesis, Characterization and In Vitro Degradation of Biodegradable Photo-linked Film from Liquid Poly(ϵ -caprolactone-co-lactide-co-glycolide) Diacrylate”, Biomacromolecules, In press.
- (4) Chan-Park MB, Zhu AP, Shen JY, Fan AL, “Novel photopolymerizable biodegradable triblock polymers for tissue engineering scaffolds: synthesis and characterization”, Macromolecular Bioscience, 4(7): 665-673
- (5) Feng J, Chan-Park MB, Shen JY and Chan V, “Quick Layer-by-Layer Assembly of Aligned Multi-Layers of Vascular Smooth Muscle Cells in Deep Microchannels”, Tissue Engineering, Accepted.
- (6) Poon YF, Zhu YB, Shen JY, Chan-Park MB and Ng SC, “Cytocompatible Hydrogels based on Photocrosslinkable Methacrylated O-Carboxymethylchitosan with Tunable Charge: Synthesis and Characterization" Advanced Functional Materials, Accepted.