NANOFIBROUS SURFACE MODIFICATION OF ULTRA-THIN PCL MEMBRANE FOR CARDIOVASCULAR TISSUE ENGINEERING APPLICATION

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PREFACE

This thesis is submitted for the degree of Master of Science in the Graduate Programme in Bioengineering, National University of Singapore. No part of the thesis has been submitted for any other degree or equivalent at another university or institution. As far as the author is aware, all the work in this thesis is original unless reference is made to the other works. Parts of the thesis have been published or presented in the following:

Journal Publications

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International Referred Conference Papers

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ACKNOWLEDGEMENT

The story of cardiovascular tissue engineering in BIOMAT began when the collaboration between Prof Teoh Swee Hin and Prof Lee Chuen Neng got established in August 2004. Prof Teoh comes from a strong engineering background, determined to integrate biomedical engineering research with biological and medical concepts. Prof Lee's clinical perspective favors our group with a strong clinic-driven appetite. Both the two major PI distinguish our research group from many others in this field.

Among the 1st batch of students embarking, the project resembled navigating on uncharted water. This pioneering nature of the project was very challenging, however highly rewarding as well. It is the supervisors' immediate guidance that made us possible to plan my own thesis project and construct roadmap of this group research. Their mission to carry out high quality research enabled me never waste time to realize this unique idea into a true contribution to the science community.

Prof Teoh and Prof Lee's renowned reputation earned this group plenty of collaborative resources. Because of this, I could easily reach out and make contact with top researchers local and international such as Associate Professor Hanry Yu, cell and molecular and former vice Chairman of GPBE, Associate Professor Dietmar Hutmacher, expert in tissue engineering, Professor Michael Raghunath, expert in extra-cellular matrix biology and tissue modeling. I am very grateful to them all for showing me the way of research as

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Singapore is a dynamic society every second with people coming and going. Ephemeral the time we spend together might be; for good our friendship would live.

I have deep sense of thankfulness to my parents and my brother. Family is always where my strength comes from. Their support, advice, caring and love accompanies all my life. Wherever I am, whenever it might be, they are always on my mind.

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LIST OF SYMBOLS

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene diamine tetra-acetic acid
FDA	fluorescein diacetate
NaOH	Soudium Hydoxide
PBS	Phosphate buffered saline
PCL	Poly(e-caprolactone)
PGA	Polyglycolic acid
PLA	Polylactic acid

SUMMARY

Poly (e-caprolactone) (PCL), a FDA approved bioresorbable aliphatic polyester, is an ideal biomaterial for tissue engineering applications. However, the hydrophobic surface of PCL scaffold, which is lack of functional groups, generates an unfavorable cell affinity. This setback hinders PCL for many specific applications, such as cardiovascular tissue engineering applications where a strong attachment between cells and scaffold is required. Hence, it is important to further increase the cell affinity of the PCL for cardiovascular applications. One route is to incorporate a natural extracellular matrix (ECM) mimic surface onto the PCL material. Natural ECM consists of a variety of extracellular macromolecules which are assembled into an organized nanosized fibrillar meshwork. This nano-fibrillar topology, together with the biochemical composition has crucial significance to maintain cellular adhesion, proliferation and function.

The development in electrospinning nanotechnology bears the promise to produce nanofibrous mesh with ECM-mimic architecture. PCL nanofiberous mesh, fabricated by electrospinning technology, could support favorable cellular growth and create a strong cell adhesion. PCL nanofiberous mesh was further reported to support cell proliferation and achieve contract functionality for cardiac tissue replacement, further confirming its huge promise for cardiovascular tissue engineering application.

This thesis reports on a novel hybrid nanofibrous PCL membrane scaffold for cardiovascular tissue engineering application, achieved by surface modification of the PCL membrane with electrospun nanofiberous coating. The purpose was to create a

ECM-mimic nanotopology on the PCL membrane for enhanced cellular attachment. In this study, PCL was successfully fabricated into less than 10μm ultra-thin membrane by using a heated 2-roll-mill process and by bi-axial stretching to improve the mechanical properties. The PCL membrane was further coated with PCL nanofibers via electrospinning process. Finally, sodium hydroxide (NaOH) treatment was employed to enhance the hydrophilicity of the PCL surface.

The nanofibrous PCL membrane scaffold was subjected to a series of characterization from both engineering and biological perspectives. The surfaces were characterized by scanning electron microscopy (SEM), water contact angle, atomic force microscopy (AFM) and scratch test. The results showed that uniform nanofibrous topology were successfully achieved on the surface of the PCL membrane, with increased roughness (more than 17 times) and surface area (almost twice). This nanofibrous topology induced capillary effects after NaOH treatment, causing the water contact angle to drop to almost zero. Scratch tests revealed a strong interaction of PCL nanofiber coating on the PCL membrane. Nanofibrous PCL membrane scaffold was further studied for corresponding cell behaviors, including cell attachment, proliferation and morphology. Alamar Blue assay indicated that NIH 3T3 fibroblast cells proliferated well on the nanofibrous membrane. Confocal Laser Scanning Microscope (CLSM) revealed a dramatically enhanced cell attachment onto the nanofibrous membranes than the untreated membranes. Results from SEM revealed an interesting cell morphology changes due to NaOH treatment on the nanofibrous surfaces: the cells exhibited the spindle-shaped morphology

on the NaOH-treated nanofibrous surface, whilst they remained in isolated spherical shaped entities in the non-treated ones.

This study indicated that the nanofibrous surface modification remarkably enhanced the cell affinity to PCL membrane scaffold. We are optimistic that the present techniques have posed the way for future applications in cardiovascular tissue engineering applications.

Chapter 1:

INTRODUCTION

1.1 Statistics of Heart Diseases

Heart disease is one of the leading causes of morbidity and motility in the modern world. Locally in Singapore alone, heart disease ranked second in principal causes of death in 2004; it is also one of the top three causes of hospitalization in Singapore, causing $ 110million heath coast. [1]

In the rest of the world, heart disease, especially coronary artery disease is also of the leading health problems. Since 1900, it has been the No. 1 killer in the United States, causing more and more death annually. In the recent decades, heart disease claims more lives each year than the next 4 leading cause of death. Nearly 2500 Americans die of heart disease each day, an average of 1 death every 35 seconds. Large amount of funding was invested to the treatment of heart diseases. In 2006, $403.1 billion was incurred for such treatments in the United States. This amount is largely expected to increase rapidly due to an aging population, since the aging process and hereditary predisposition are risk factors that cannot be altered. [2,3]

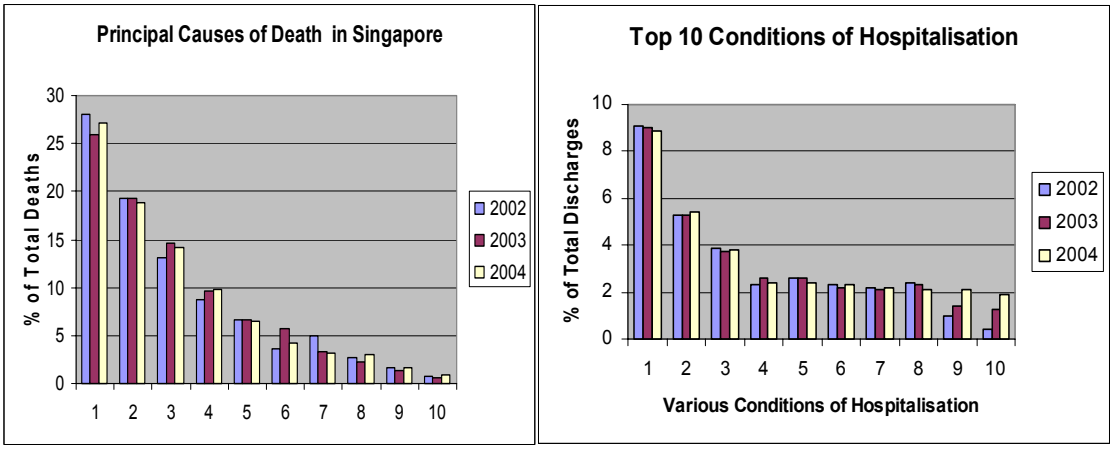

Figure 1-1 Statistics of Heart Disease in Singapore**.** A. Principal Causes of Death in Singapore. [1] 1 Cancer, 2 Ischaemic Heart disease, 3 Pneumonia, 4 Cerebrovascular Disease (including stroke), 5 Accidents, Poisoning & Violence, 6 Other Heart Diseases, 7 Chronic Obstructive Lung Disease, 8 Diabetes Mellitus, 9 Nephritis, Nephrotic Syndrome & Nephrosis and 10 Septicaemia.;

B Top 10 Conditions of Hospitalisation in Singapore**:** 1 Accidents, Poisoning & Violence,2 Cancer,3 Ischaemic Heart Disease,4 Pneumonia,5 Cerebrovascular Disease (including stroke), 6 Other Heart Diseases, 7 Chronic Obstructive Lung Disease, 8 Complications related to Pregnancy, 9 Dengue and 10 Obstetric Complications affecting Fetus or Newborn

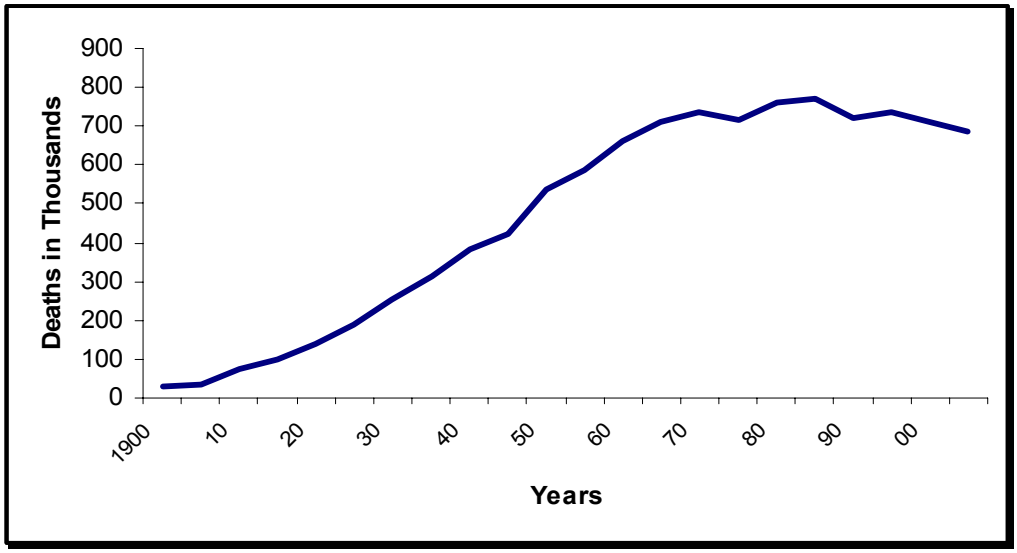

Figure 1-2 Deaths from Diseases of the Heart, data corresponds to United States: from 1900 to 2003 [2]

1.2 Overview of Heart Diseases

1.2.1 Classification of Heart Diseases

Heart disease is a wide-encompassing category that includes all conditions that affect the heart and the blood vessels, including Angina, Atherosclerosis, Cardiac Arrhythmia, Cardiomyopathy, Chronic Venous Insufficiency, Diabetes, Heart Attack, High Cholesterol, High Homocysteine, High Triglycerides, Hypertension, Insulin Resistance Syndrome, Mitral Valve Prolapse, and Stroke.

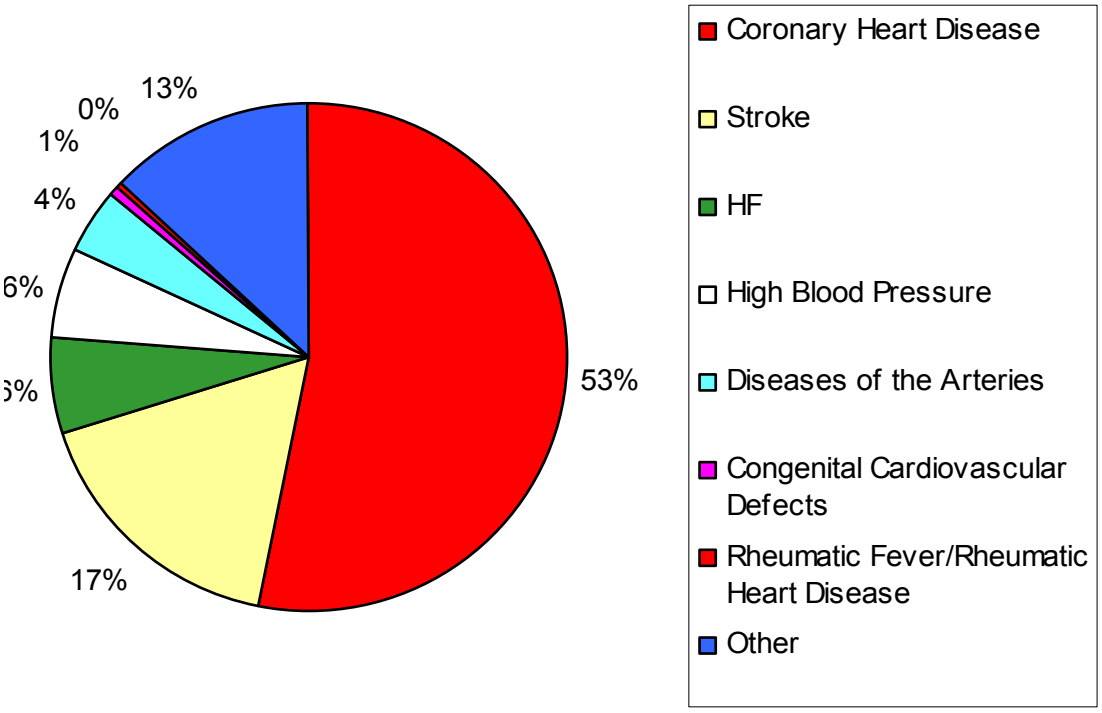

Figure 1-3 Percentage Breakdown of Deaths from Heart Diseases [3]

1.2.2 Symptoms of Heart Diseases

Symptoms of heart diseases vary from individual to individual. People with heart diseases may not have any symptoms, or they may experience difficulty in breathing during exertion or when lying down, fatigue, lightheadedness, dizziness, fainting, depression, memory problems, confusion, frequent waking during sleep, chest pain, an awareness of the heartbeat, sensations of fluttering or pounding in the chest, swelling around the ankles, or a large abdomen. All of these symptoms shed a major impact on patients' quality of life.

1.2.3 Risk Factors of Heart Diseases

Heart disease is found to be associated with many risk factors. Many people with heart diseases have elevated or high cholesterol levels. [4] Low HDL cholesterol (known as the "good" cholesterol) and high LDL cholesterol (known as the "bad" cholesterol) are more specifically linked to cardiovascular disease than is total cholesterol.[5] High LDL cholesterol level would increase the possible of getting atherosclerosis and make the heart more vulnerable to diseases.

Atherosclerosis, or hardening of the arteries, is another common disease in cardiovascular system. It is an inflammatory process in which leukocytes interact with structurally intact but dysfunctional endothelium of the arteries. [6] Atherosclerosis of the vessels that

supply the heart with blood is the most common cause of heart attacks. Atherosclerosis and high cholesterol usually occur together, though cholesterol levels can change quickly and atherosclerosis generally takes decades to develop.

High triglyceride levels might be related to the proposition to heart disease. It was reported that a high triglyceride level is an independent risk factor for heart disease in some people, but the link between high triglyceride levels and heart disease is not as well established as the link between high cholesterol and heart disease. [7]

Hypertension, or high blood pressure, is another leading risk factor for heart disease. Hypertension patients have much higher risk to get coronary heart disease, stroke, peripheral arterial disease, heart failure and end-stage renal disease. [8-9] The risk even increases as blood pressure rises. Hypertension could also work with other risk factors, like glucose intolerance, diabetes and smoking, to contribute larger risk for heart diseases. [6]

Abdominal fat, or a “beer belly,” versus fat that accumulates on the hips, is associated with increased risk of cardiovascular disease and heart attack. Overweight individuals are more likely to have additional risk factors related to heart disease, specifically hypertension, high blood sugar levels, high cholesterol, high triglycerides, and diabetes. [10-11]

1.3 Biology and Physiology of the Heart

The heart is essentially a hollow muscle, which functions as a pump to propel blood throughout the circulation. This function is maintained by cardiac muscle assembled in a complex and highly regulated fashion. The contractions are coordinated by an elaborate electrical system, which serves to maximize mechanical efficiency. Uncoordinated electrical activity is characterized by irregular contractions known as arrhythmias. [12]

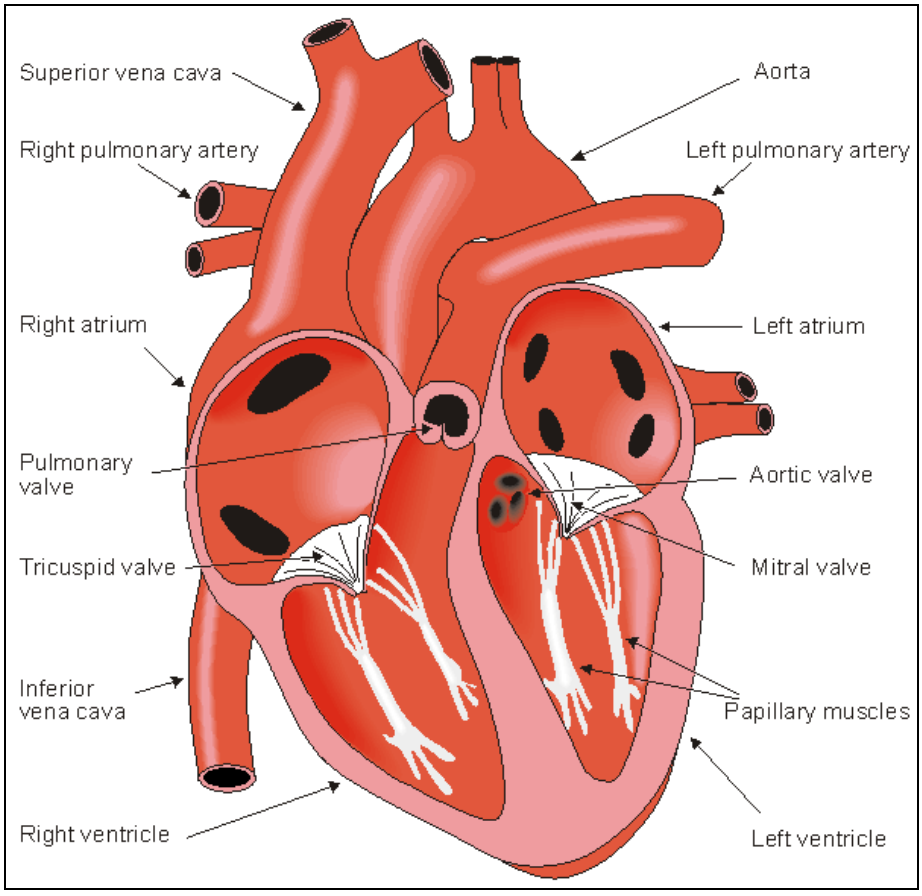

Figure 1-4 Diagram of the heart [12]. Blood flows from the vena cavae into the right atrium, past the tricuspid valve to the right ventricle, past the pulmonary valve to the pulmonary circulation. Oxygenated blood is then return from the lungs to the left.

Gross structure of the heart consists of four pumping chambers with the right and left atria forming unit and the right and left ventricles forming another. The right and left sides of the heart pump blood into the systemic and pulmonary circuits of the vascular system. Venous blood enters the right atrium, via the superior and inferior vena cavae, and passes through the tricuspid orifice to the right ventricle, where the blood is then pumped through the pulmonary arteries to the lungs. The oxygenated blood is returned to the left atria via the pulmonary veins, passes through the mitral orifice, where the blood is then pumped through the aorta and its branches for distribution throughout the body. The valves are inserted and supported by a central fibrous skeleton.

The tissue of heart wall mainly consists of 3 layers, namely pericardium, myocardium and endocardium. A double-layered membrane called the pericardium consists of the outermost layer of heart. The outer layer of the pericardium surrounds the roots of the heart's major blood vessels and is attached by ligaments to the spinal column, diaphragm, and other parts of the body. The inner layer of the pericardium is attached to the heart muscle. A coating of fluid separates the two layers of membrane, letting the heart move as it beats, yet still be attached to the body. The endocardium is the innermost layer of the heart. It consists of epithelial tissue and connective tissue. The endocardium lines the inner cavities of the heart, covers heart valves and is continuous with the inner lining of blood vessels. Purkinje fibers are located in the endocardium. They participate in the contraction of the heart muscle. The Myocardium between the endocardium and

pericardium is the muscular layer of the wall of the heart. It is composed of spontaneously contracting cardiac muscle fibers which allow the heart to contract.

Cardiac muscle cells, also called cardiomyocytes, are relatively small, averaging 10–20 μm in diameter and 50–100 μm in length. A typical cardiac muscle cell has a single, centrally placed nucleus, although a few may have two or more. They are short, branched and interconnected. Cardiac muscle cell junction at specialized zones called intercalated discs. These discs allow force to be transmitted from one cell to another. Additionally, they contain gap junctions that allow an action potential in one cell to pass directly to an adjoining cell through these electrical synapses. [13-14] The contractility of heart is mainly contributed by cardiac muscle cells. Damage to cardiac muscle cells might cause pathological hypertrophic growth and remodeling, making the heart function considerably impaired.

1.4 Pathology of the Heart Diseases

Pathology varies from different heart diseases and the mechanisms are often interrelated. For example, heart attack or myocardial infarction is typified by the extensive damage to the heart tissue. During the development of heart attack, atherosclerosis of the coronary arteries plays an important role. In some cases, a blood clot in the coronary arteries blocks blood flow; other times, the narrowing is caused by atherosclerosis alone. The most common cause is the stenosis and closure of the coronary arteries and insufficient blood supply to the downstream heart muscle cells.

Cardiomyocytes, the cells responsible for cardiac contractility, are very sensitive to ischemia.. Minutes of deprival of blood supply might cause catastrophic cell death in the myocardium. Cardiomyocytes have no inherent mechanism for significant self-renewal. Lost cardiomyocytes are replaced by scar, and depending upon the extent of myocardial damage, heart failure can ensue. A significant portion of the deaths attributed to heart disease could be prevented if an adequate means of cardiac muscle repair could be developed. [15]

The first symptom of a heart attack is usually deep aching or pressure-like chest pain that may radiate to the back, jaw, or left arm. Discomfort may be mild or severe. About 20% of heart attacks are silent (i.e., they cause no symptoms and may therefore be missed). Aged people may experience shortness of breath. Nausea and vomiting may also occur. Restlessness, apprehension, pallor, and sweating are common. Heart attack requires timely first aid to prevent the cost of life.

1.5 Medical Options for Heart Disease

1.5.1 Drugs for Heart Disease

The conventional treatment for cardiovascular disease includes specific medication for any underlying causes. Various drugs are involved in the administration aimed at reducing the work load on the heart, as reviewed by Guyatt and Devereaux[16]. These drugs are especially important at the time of sudden infarction outbreak. The counter

aspirin (Bayer Children's Aspirin®, Ecotrin Adult Low Strength®, Halfprin 81®) might be beneficial for reducing recurrent strokes and for reducing the risk of future heart attacks. The use of prescription medications are also involved as therapies, such as Angiotensin-Converting Enzyme (ACE) Inhibitors (e.g., captopril, enalapril, lisinopril), beta-blockers (e.g., propranolol), blood thinners (e.g., aspirin, warfarin), the combination of hydralazine and isosorbide dinitrate, digitalis, nitroglycerin, and diuretics. In general, medicines could simply relieve the symptoms and save life at the first place, however, they are not able to cure the heart disease.

1.5.2 Device and Surgical Treatment of Heart diseases

In some severe cases, surgical treatments, such as angioplasty, Coronary artery bypass graft (CABG), heart valve replacement and pacemaker installation may be recommended.

Angioplasty is a medical procedure in which a balloon is used to open narrowed or blocked blood vessels of the heart (coronary arteries). It is not considered to be a type of surgery. Angioplasty may be performed to treat persistent chest pain (angina), blockage of one or more coronary arteries and residual obstruction in a coronary artery during or after a heart attack. The major risks for any angioplasty surgery are bleeding and infection. Other risks includes complete obstruction of blood flow to an area of the heart, damage to a valve or blood vessel, stroke, arrhythmia, bleeding in the groin, kidney failure, allergic reaction to the X-ray dye and even death.

Coronary artery bypass graft (CABG) is one of the most common and effective procedures to manage blockage of blood to the heart muscle. CABG improves the blood flow to the heart with a new route, or "bypass," around a section of clogged or diseased artery. The surgery involves sewing a section of vein from the leg or artery from the chest or another part of the body to bypass a part of the diseased coronary artery. This creates a new route for blood to flow, so that the heart muscle will get the oxygen-rich blood it needs to work properly. Although the risks and complications related could occur, the long term results of CABG are excellent. The majority of patients obtain excellent relief of their symptoms of angina after surgery. Although symptoms may recur, most patients have sustained relief. A minority of patients will require repeat surgery, usually 10 or more years after their original operation. Unfortunately, the procedure is not applicable to those patients whose veins from the legs are diseased, especially for diabetes patients whose veins legs are suitable for implantation.

Valve replacement, especially aortic valve replacement, is an "open heart" procedure performed by cardithoracic surgeons for treatment of narrowing (stenosis) or leakage (regurgitation) of the aortic valve. Unlike the mitral valve which can often be repaired, the aortic valve usually requires replacement. Both mechanical replacement valves and natural or biological valves are available today. The principle advantage of mechanical valves is their excellent durability. From a practical standpoint, they do not wear out. The principle disadvantage is that there is a tendency for blood to clot on all mechanical valves. Therefore patients with artificial valves must take anticoagulants or "blood thinners" for the rest of their life. There is also a small but definite risk of blood clots

causing stroke. There are a variety of natural or biological valves that can be used to replace an abnormal valve. They all share a reduced risk of blood clots forming but all are less durable than mechanical valves. Given enough time, they will probably all wear out.

Artificial pacemakers are used to treat arrhythmias by replacing the natural electrical impulses created by the S-A node. An artificial pacemaker consists of a lithium battery, a pulse generator, and a wire which connects the pacemaker to the heart. Installation of a pacemaker is a relatively easy and short operation. The entire procedure usually only takes about half an hour and the patient can return home within the next few days. Some minor complications are still associated with the pacemaker implantation. [17] Most pacemakers run on lithium batteries, which need to be replaced about every 10 years.

1.5.3 Device Therapy

Various devices have been explored as an alternative therapy to heart diseases. Many designs of Left Ventricular Assist Devices (LVADs) have also recently become available for a partial restoration of function[18]. In end-stage heart failure, however, the only existing clinical solution is a total replacement of the heart[19]. The Total Artificial Heart (TAH) project was initiated in the 1960's and a few designs, most notably the AbioCor Implantable Replacement Heart System[20], are currently employed in clinical trials. However, various pathological conditions may arise due to systemic complications in implanting such devices, as reviewed by Thompson et al[21]. These complications

mainly include non-ideal blood compatibility, risk of infection and vulnerability of mechanical failure. The pros and cons make device therapy mainly used as bridge treatment to heart transplantation.

1.5.4 Total Heart Transplantation

Allograft heart transplantation has been fully developed for heart disease treatment. It is the only option for the end-stage heart failure patients. Alexis Carrel performed the first heterotopic canine heart transplant with Charles Guthrie in 1905 [22]. In 1967, Christiaan Barnard performed the 1st human heart transplantation; the 1st local heart transplantation in Singapore was performed in 1990 in Singapore General Hospital. The technical progresses in immuno suppression and surgical techniques lowered the rates of acute rejection and infection leading to graft failure. Heart transplantation remains the gold standard among all the therapies up to now.

However, the detection of acute and chronic allograft rejection remains one of the most important yet unsettled matters. [23-24] With a lengthy waiting list, donor scarcity is also one of the major problems associated.

1.6 Objective

Despite years of efforts that have been devoted into the novel treatment, heart diseases still remain one of the major health problems in the world today. Every single one of the current therapies is insufficient to be employed as ultimate treatment. Recent two decades have witnessed a rapid growth in regenerative medicines. Tissue engineering is one of the hottest research fields in regenerative medicines. This technology can perhaps be best defined as the use of a combination of cells, engineering materials, and suitable biochemical factors to improve or replace biological functions in an effort to effect the advancement of medicine.

Classical cardiovascular tissue engineering concepts of is aimed at allowing surgeons to use a patient's own cells, in combination with absorbable biomaterials, to guide the formation of new cardiovascular tissues. Initial targets of the research program will include heart valves, vascular grafts, and cardiovascular patches. Successful development of the technology could eliminate the need for surgeons to harvest blood vessels from adult patients for bypass procedures, and instead allow new vessels to be grown in the laboratory from a patient's own cells. The technology also promises more durable alternatives, and the opportunity to eliminate the need for anticoagulant therapy, currently required for patients receiving mechanical valve replacements. Recent publications on cardiovascular tissue engineering have shown that this is a vibrant field of research. [25]

The ultimate goal of this study is to develop an ideal scaffold for cardiovascular tissue engineering as a regenerative medical treatment for heart diseases. In our previous work

we propose a composite layer-by-layer technique using ultra thin bioresorbable membrane. Figure 1-6 shows schematically the concept as applied to blood vessel and heart valve construct. [26]

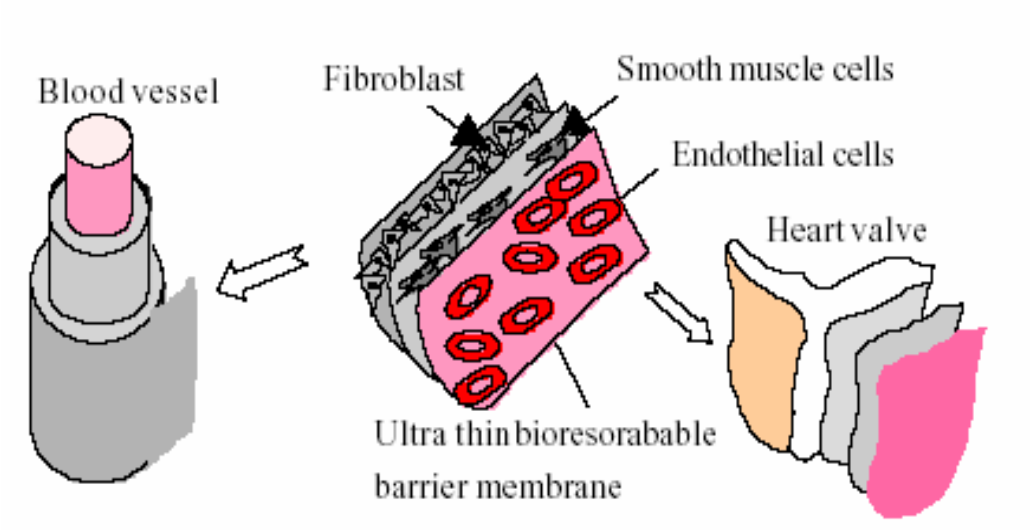

Figure 1-5 Schematic illustration of growing cells layer by layer on an ultra thin bioresorbable barrier membrane scaffold. [26]

This composite layer-by-layer technique is promising together with development of scaffold fabrication technology. However, the material utilized in precious concept was poly (e-caprolactone) (PCL) membrane. Though biaxial stretching PCL membrane own many advantages in soft tissue engineering applications, PCL membrane is far from ideal biomaterials because of its dissatisfactory properties of the synthetic polymeric material surface. This synthetic polymeric material surface is hydrophobic and lack of functional group, which hinders cell adhesion and other further cell behaviors. The drawback makes surface modification of PCL membrane inevitable.

Ideal biocompatible PCL membrane should have a natural extracellular matrix (ECM) mimic surface. The ECM-mimic surface should contain ECM rich protein, such as

collagen. What is equivalently important is the surface topology. . Natural ECM consists of a variety of extracellular macromolecules which are assembled into an organized meshwork. In ECM, various collagens contribute to collagen fibril (10-300nm in diameter) and further to collagen fibers (several micrometer in diameter). [27] This structure has crucial significance to maintain cellular adhesion, proliferation and function. [28]

The objective of this study is to improve PCL membrane biocompatibilities by surface modification for cardiovascular applications. In this study, we explored the possibility of modifying PCL film with the focus of changing the surface topology using electrospinning technology,

1.7 Scope

This thesis reports on a novel surface modification technique of the PCL membrane by coating with electrospun nanofibers. The purpose was to mimic the architecture of the natural extra-cellular matrix and create nanotopography for enhanced cellular attachment.

Chapter 1, introduction, presents the background and motivation of the project, thriving to find an alternative therapy toward curing the number 1 health problem in the world. This chapter reviews the facts of the heart diseases both local and international, the biology and physiology of the heart and the pathology of heart diseases. After introduction of existing medical treatments, the objective finally describes the overall

objective of this thesis project and brief methodology of cardiovascular tissue engineering.

Chapter 2 gives an overview of related literature in terms of regenerative medicine, biomaterials and development of electrospinning nanotechnology. This chapter begins with the introduction of various regenerative medicines mainly including cell therapy and cardiovascular tissue engineering. Biomaterial's application in cardiovascular tissue engineering is then reviewed. Then the story will be narrowed down to the PCL biomaterial in details. With the knowledge of biomaterials, the author would like to present recent data published for the surface modification of PCL, only to clarify the traditional ways to modify the inert surface of PCL. It was pointed that the motivation of surface modification was to mimic the natural extracelluar matrix biochemical composition. The author then introduced the nanotechnology application in the field of tissue engineering, with the focus of electrospinning technology which produces nano-sized nanofibers to mimic the topology of the extracelluar matrix.

In the Chapter 3, materials and methods in nanofibrous surface modification of PCL membrane are presented in details. Ultra-thin PCL membrane was prepared via 2-roll-heated-mill, melt press and biaxial stretching method. The PCL membrane was then coated with PCL nanofiber by electrospinning technology. NaOH treatment was used to enhance the surface hydrophilicity. The nanofibrous surfaces were characterized by scanning electron microscopy (SEM), water contact angle and atomic force microscopy (AFM). Capillary reaction study was used to further investigate the surface hydrophilicity.

The coating was quantitatively analyzed by weighing the sample before and after the coating process. The attaching force between PCL nanofibrous coating and the underlying PCL membrane is evaluated by scratch test. To assess the cell behavior on various PCL membrane scaffold, NIH 3T3 cells were employed. Seeded cells were assessed via Confocal Laser Scanning Microscopy (CLSM), Scanning Electron Microscopy (SEM) and Alamar Blue test.

Chapter 4 presents the results of nanofibrous surface modification of PCL membrane in terms of surface characterization and cell behavior studies. The results showed that uniform nanofibrous topology were successfully achieved on the surface of the PCL membrane, with increased roughness and surface area. This nanofibrous topology induced capillary effects after NaOH treatment, causing the water contact angle to drop to almost zero. Scratch tests revealed a strong interaction of PCL nanofiber coating on the PCL membrane. Alamar Blue assay indicated that 3T3 fibroblast cells proliferated well on the nanofibrous membrane. Confocal Laser Scanning Microscope revealed better cell attachment onto the nanofibrous membranes than the untreated membranes. Results from SEM showed that the cells' spindle-shaped morphology on the NaOH-treated fibrous surface was evident whilst they remained in isolated spherical shaped entities in the non-treated fibrous surfaces.

Discussion about the experimental results is presented in Chapter 5. The present study showed that nanofibrous PCL membranes generate a dramatic topographic change with increased and surface area. PCL membrane's plane surface was replaced by a fibrous

surface which mimics the natural extracellular matrix, making it promising for tissue engineering applications. Chapter 5 then continues to discuss several problems associated with nanofibrous coating the nanofibrous coating, with the first one the decreased the apparent surface hydropilicity of PCL membrane and the mechanism of NaOH treatment enhancing the hydrophilicity. The attachment between the nanofibrous coating and underlying PCL membrane was also discussed to explore the reason why nanofibrous coating is firmly attached to PCL membrane. Furthermore, different cellular responses to different topographies were brought into discussion. By combination of cell work and surface characterization data, it was derived that the nanofibrous surface modification created a strong cell attachment. The author finally called for attention to the effect of NaOH treatment to cell behaviors, speculating its different effect on cell morphology on the plain PCL and the nanofibrous membranes might be due to the different water contact change in the NaOH treatment.

Chapter 6 covers the conclusion and future recommendation. This chapter first summarized success of nanofibrous surface modification on ultra-thin PCL membrane. With the consequent serial of changes due to the modification, a better ECM-mimic surface was achieved, so was a higher cell affinity. The conclusion was drawn that this nanofibrous PCL membrane scaffold bears promising application in cardiovascular tissue engineering. In the future recommendation, a serial of follow-up works were proposed including collagen nanofibrous grafting, nanofiber guidance deposition, necessity development of a quantitative cell-biomaterial interaction and 3 dimensional system for tissue engineering application.

Chapter 2:

LITERATURE REVIEW

2.1 Cardiovascular Tissue Engineering

The limitation of existing therapies necessitates the need for alternative therapies. A novel method of regenerative medicine is proposed in the form of cardiovascular tissue engineering. This therapy may be broadly classified under two categories: cell transplantation methods, injection of therapeutic cells to the site of injury to promote in vivo tissue regeneration; and scaffold-based tissue engineering approaches, the formation of functional tissue in vitro, followed by implantation and in vivo tissue regeneration.

2.1.1 Cell Transplantation

Cell transplantation essentially consists of harvest of the cell of interest from the patient (or suitable donor), cell expansion in vitro and delivery of the therapeutic cells to the site of injury. In so doing, it is hoped that some measure of contractility and cardiac performance is regained, together with replacement of the scar tissue. [29-30]

Studies involving the transplantation of therapeutic cells on injured and normal myocardium have shown that it is a generally safe procedure. The transplanted cells in animal models quickly form well-vascularized tissue grafts, with partial recovery of function, particularly diastolic contractile function. Clinical trials involving the use of bone marrow stem cells and satellite cells, such as skeletal myoblasts, are currently underway[31].

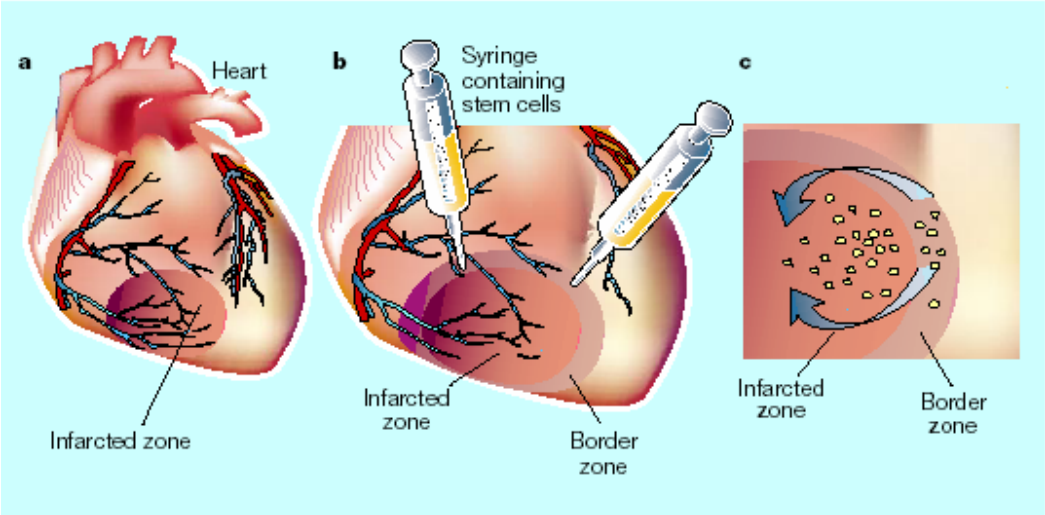

Figure 2-1 Repairing damaged heart tissue with bone marrow cells. A. An infracted heart with stenosis in the coronary arteries. B. Bone marrow stem cell injection to the site. C. Bone marrow stem cells infiltrated and exhibited cardiomyocyte characteristics. Heart function improved. [29-30]

However, it has been observed that the improvement in function was not due to any replacement of scar tissue. Theories have been suggested to explain the benefits, including arrest of ventricular remodeling, improvement of material properties, promotion of angiogenesis, as well as growth factor signaling. However, the specifics of such mechanisms have yet to be derived. Long term studies of cell transplantation therapy are also yet to be carried out, and the possibility of long term arrhythmias remains.

2.1.2 Cell Sheet Technology

The revolutionary application of cell sheets in cardiac tissue engineering was pioneered by Shimizu's group in 2002[32]. Briefly, the technique involve the cultivation of cells into a confluent monolayer on poly (N-isopropylacrylamide) (PIPAAm), or a similar

temperature responsive material. The confluent cell sheets can then be detached in an ezyme-independent manner, and subsequently used in tissue grafts. In their study, Shimizu's group cultivated sheets of neonatal rat cardiomyocytes, which were successively stacked, to get a three-dimensional layered construct. The approach may be further extended to systematically stack cells of different types to get multi-layered organoid constructs.

The cardiac grafts derived as such have been successfully cultured in *in vitro* conditions, with excellent contractility. The main advantage of using this approach is the independence of artificial material. This reduces the host immunological response, especially so if autologous cells are used. More importantly, it has been shown that the contractility of cells is directly related to the volume content of scaffold material[33]. By eliminating the need for a scaffold, contractile function is enhanced.

Many concerns are involved in this technique as well. Monolayer cell sheet constructs, however, tend to display poor mechanical strength. A possible remedy to this would be the inclusion of fibroblastic cell sheets, interspersed with the cardiac constructs, to promote mechanical strength.

2.1.3 Solubilized ECM Protein-Gel Constructs

The use of solubilized ECM proteins, particularly collagen, in cardiovascular tissue engineering was initiated by Eschenhagen in 1997[34]. The technique involves mixing

the cells of interest with collagen solution, and subsequently allowing it to gel in moulds of defined geometries. The gel construct is then implanted into the patient, and the artificial ECM is degraded over time, to be replaced by the host ECM.

Zimmerman et al have since done excellent work in the field of Engineered Heart Tissue (EHT), achieving tissue constructs of thickness up to 1mm without signs of core necrosis[35]. By controlling the volume of collagen used, the group is able to easily control the level of compromise between contractility and mechanical stability. Issues that remain to be resolved in the use of EHT technology include the use of animal-derived collagen. In the absence of synthetic collagen, the acceptable use of animal-derived products remains a question, due both to the increased risk of cross-species infection, as well as variation of compositional make-up between batches.

2.1.4 Scaffold-based Cardiovascular Tissue Engineering

Classical scaffold-based tissue engineering strategies, as proposed envisioned by Langer and Vacanti, involves the construction of an artificial polymeric scaffold capable of supporting cell adhesion, proliferation, growth and achievement of their function as an integrated tissue [36-37]. Cells can be isolated from the patient, or a suitable donor, and cultured onto the scaffold. The cell/scaffold construct can then be cultivated in *in vitro* conditions prior to implantation into the patient. The tissue engineered graft is slowly assimilated into the host, and the polymeric scaffold eventually breaks down into non-toxic by-products over time.

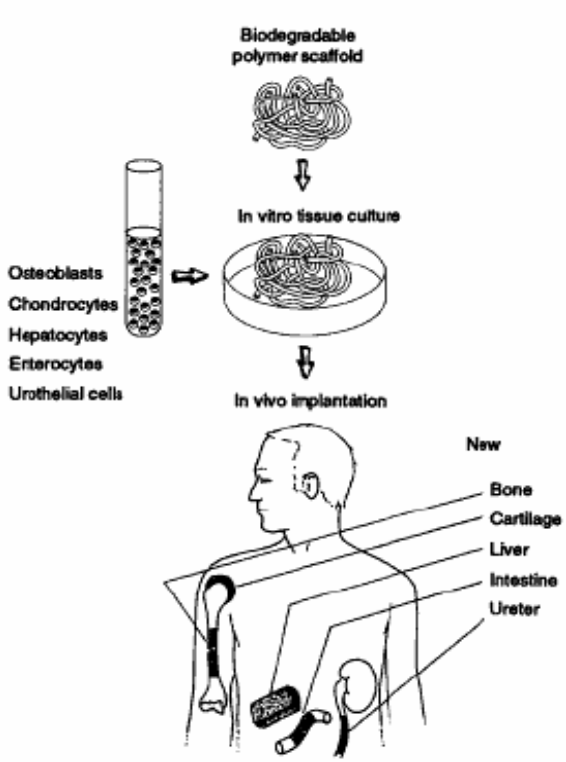

Figure 2-2 Overview of Scaffold-based Tissue Engineering Approach. Scaffold-based tissue engineering approach involves 1) construction of scaffold (Material Selection and Scaffold Design); 2) Cell Selection (Isolation of cells of interest) 3) Seeding of Cells and In vitro culture (Seeding Conditions, In vitro maintenance, differentiation and assessment) 4) Implantation (Animal model selection, In vivo development and Assessment) [36]

To construct scaffold for cardiovascular tissue engineering, various fabrication methods have been proposed, with some of these summarized in the table below.

Matrix Material	*Construct*	*Study*
Polyurethane	Cast	[38],[39]
Chitosan	Cast	[40]
Polycaprolactone (PCL)	Electrospun nanofibers	[41]
	Sponge	[42]
Collagen	Filament extruded	[43]
Polyglycolic acid (PGA)	Mesh	[44], [45]
Polylactic acid (PLA)	Salt leached	[46]

Table 2-1 Summary of available biomaterials and techniques in cardiovascular tissue engineering. In some studies, blending of material is carried out to achieve better properties

The use of preformed scaffolds in tissue engineering offers the advantage of a greater control over geometrical requirements. By introducing the use of advanced manufacturing technologies, the creation of custom-designed scaffolds, with reproducible results will be made possible[47].

However, as mentioned above, the contractile function of cardiac grafts is compromised with a larger scaffold volume. It is thus necessary to select a material with similar properties to that of native heart tissue. Indeed, PGA constructs have largely been unsuccessful because of the high material stiffness. It is thus imperative to match the

stiffness and elasticity of the surrounding tissue. Biomaterials are to be introduced in details in the next section.

2.2 Biomaterials in Tissue Engineering

2.2.1 Criteria for Selection of Biomaterial

Biomaterials play a major role in classical scaffold-based tissue engineering concept. Ideally, we wish to choose a material that satisfies all of the following requirements in order to obtain optimal engineered tissue: [48]

- Biocompatible: the material has to be non-toxic.
- Mechanically strong enough for supporting the cell aggregates and for handling during culture and transplantation [49].
- The degradation characteristics should be such that the rate of degradation of the scaffold in vivo should match that of the regeneration of the tissue. Also the degradation products should be non-toxic and should not elicit unfavorable host tissue reactions.

With the previous criteria, 2 major categories of biomaterials are employed for tissue engineering study, namely natural biomaterials and synthetic biomaterials. Natural biomaterials include collagen [50], gelatin [51], chitosan[52 - 53],polyhydroxy alkanoates[54]. Natural biomaterials have good biocompatibility but lack of good

mechanical property. They are also suspicious of pathological transfer, immune reaction. The second group, synthetic biomaterials, is also popularly studied for tissue engineering application. These materials have known molecular formula, well defined structure and many supreme mechanical properties. These materials include Poly Lactic Acid, Poly Glycolide Aacid, PLGA, Poly (e-caprolactone), Polyphosphazenes.

2.2.2 Natural biomaterials

In general, the utilization of scaffold materials can be divided into two major categories: synthetic materials and biological ones. Natural biomaterials include collagen [55-56], gelatin [57], chitosan[58-59],Alginate[60.].

Natural biomaterials have high cell affinity and biocompatibility, induce mild inflammatory reaction but they usually suffer from batch-to-batch variations, insufficient strength due to quick degradation and unpredictable performance control. Pathological transfer is also among the concerns.

2.2.3 Synthetic biomaterials

Synthetic biomaterials are also popularly studied for tissue engineering application. Synthetic biomaterials include bioceramics such as Bioglass, TCP, HA and biopolymers such as Polyphosphazenes, [61] Polyglycolic acid (PGA) [62], Polylactic acid (PLA) [63], Polycaprolactone (PCL) [64-65] and Polyurethane [66-67].

Synthetic biomaterials generally have known molecular formula and well defined structure. Dependant on the choice of a curtain material, the mechanical property and other performance could be well controlled.

Several drawbacks of synthetic biomaterials cannot be neglected. Synthetic biomaterials often have hydrophobic surface and lack functional groups for cell adhesion which generates inefficient cell affinity. Moreover, for biodegradable materials such as polyester, the acidic degradation products may provoke adverse tissue reaction after implantation [68].

Many researches have been devoted to the improvement of synthetic biomaterials for tissue engineering application. These improvements include manipulation of composition to achieve better properties, surface modification for better cell adhesion and tissue integration. Synthetic biomaterials also bear the potential to incorporate tissue engineering with controlled release of drugs and growth factors. [69- 70]

2.3 Poly (e-caprolactone) (PCL)

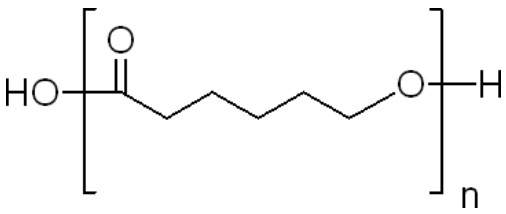

Figure 2-3 Chemical Structure of Poly (e-caprolactone)

Poly (e-caprolactone) (PCL) is one of the most thoroughly studied among various types of biomaterials. The exploration of PCL for biomedical applications dated back to 1970s. PCL is a linear polyeaster, mainly synthesized by ring-opening reaction from e-caprolactone monomer. PCL is well known to be non-toxic, biodegradable and suitable for biomedical applications; it has broad miscibility or mechanical compatibility with many polymers and good adhesion to a broad spectrum of substrates. (Fig 2-2)

melt index	1.0 g/10 min (125°C/44 psi)
impact strength	350 J/m (Izod, ASTM D 256-73A, notched)
Hardness	55 (Shore D, ASTM D 2240-75)
mp	60 °C (lit.)
density	1.145 g/mL at 25 °C
elongation	2 in/min - 800-1000% (ultimate)

Figure 2-4 PCL major Properties, adopted from Sigma-aldrich catalogue

The most immediate advantages of PCL are biocompatibility, easy processability, slow degradation rate and low cost. PCL was approved by FDA for a non-toxic and biocompatible polymer for biomedical application. Degradation of PCL occurs in the form of hydrolysis in physiological conditions. Some of the cross-linked PCL scaffold could also undergo enzymatic degradation. The degradation products, including the low molecular weight pieces, could be engulfed by macrophage cells and get reabsorbed by the host body.

PCL is one of the easiest biopolymers to be processed. This easy processibility lies first in the low melting point of only 60 °C, which enables people to apply engineering know-how to fabricate PCL into required scaffold. PCL degrades at 350 °C, in comparison with other polyeaster of 250 °C. This chemical stability contributes to its easy processibility as well. According to different patients' implantation sites, this easy processibility makes it possible to be shaped in various shape, size and geometry.

The degradation rate of PCL is rather slow, which averages 2 years. Slow degradation rate maintains a stable mechanical structure and help the scaffold to maintain the original volume.

These supreme advantages, together with the lower cost in the market, make PCL one of the promising biomaterials. It has been used for various tissue engineering applications such as bone, skin, nerve and retina. [71, 72,73]

2.4 Ultra-thin PCL Membrane

Ultra-thin PCL membrane, fabricated via 2-roll-heated-mill, melt-press and biaxial stretching method, has been successfully developed by Teoh et al [74,75]. This ultra-thin PCL membrane was reported to have high tensile strengths, suitable gas permeability, and support the cell growth. Ultra-thin PCL membrane provides promising application in soft tissue engineering, i.e. skin, cardiac tissue, and blood vessels.

However, PCL, as a synthetic biomaterial, is known for its poor cell adhesion. Its hydrophobic surface and lack of functional groups make the cell, residing in aqueous medium, different to engage and interact with the membrane. This setback is a major obstacle of PCL for many specific applications, i.e. vascular tissue engineering, where cells are exposed to shear stress in the dynamic physiological environment. Various researches have been devoted to PCL surface modification.

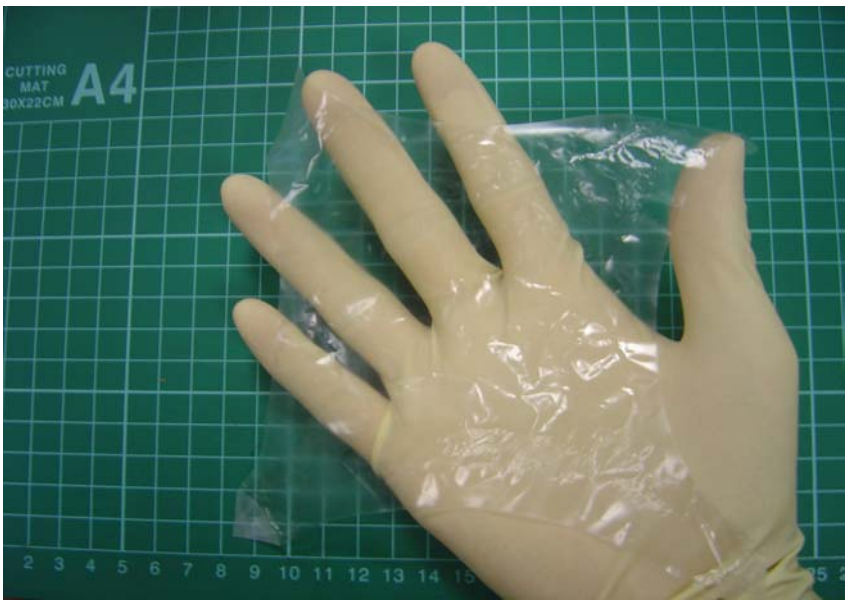

Figure 2-5 Ultra-thin PCL membrane by 2-roll-heated-mill methods. The membrane is transparent, mechanical strong and less than 10 μm in thickness.

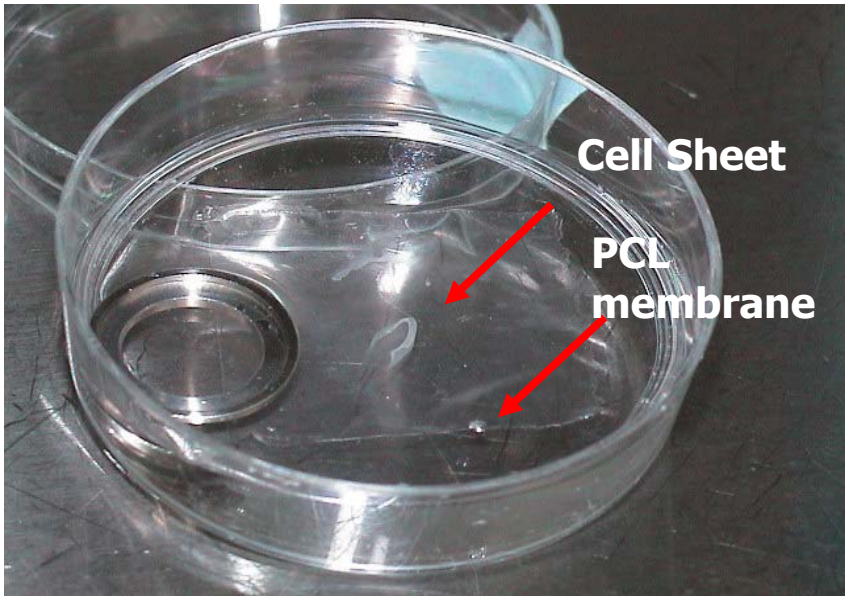

Figure 2-6 Cell sheet detached from PCL membrane. Cell affinity of PCL membrane is insufficient. Cells are prone to be detached from the membrane.

2.5 Surface modification

With the background of insufficient cell affinity on the PCL surface. Surface modification is critically important to make it suitable to tissue engineering application. One route is to incorporate a natural extracellular matrix (ECM) mimic surface onto the PCL material.

2.5.1 Natural Extracellular Matrix (ECM)

Natural ECM consists of a variety of extracellular macromolecules which are assembled into an organized meshwork. Two main classes of extracellular macromolecules make up the meshwork, glycosaminoglycans (GAGs) and fibrous proteins among which collagens is the major one. ECM is the native environment where cells reside in. The major protein

in ECM, collagen, bears RGD domain which is the binding sites for cells attachment. The connections between ECM and cell occur by the trans-membrane protein integrins whose binding sites reside in RGD domain in the collagen protein.

ECM plays an active role in the cell-ECM interaction, instead of a rather passive one. With the strong interaction between each other, ECM and integrins collaborate to regulate gene expression associated with cell growth, differentiation and survival. [76-77]

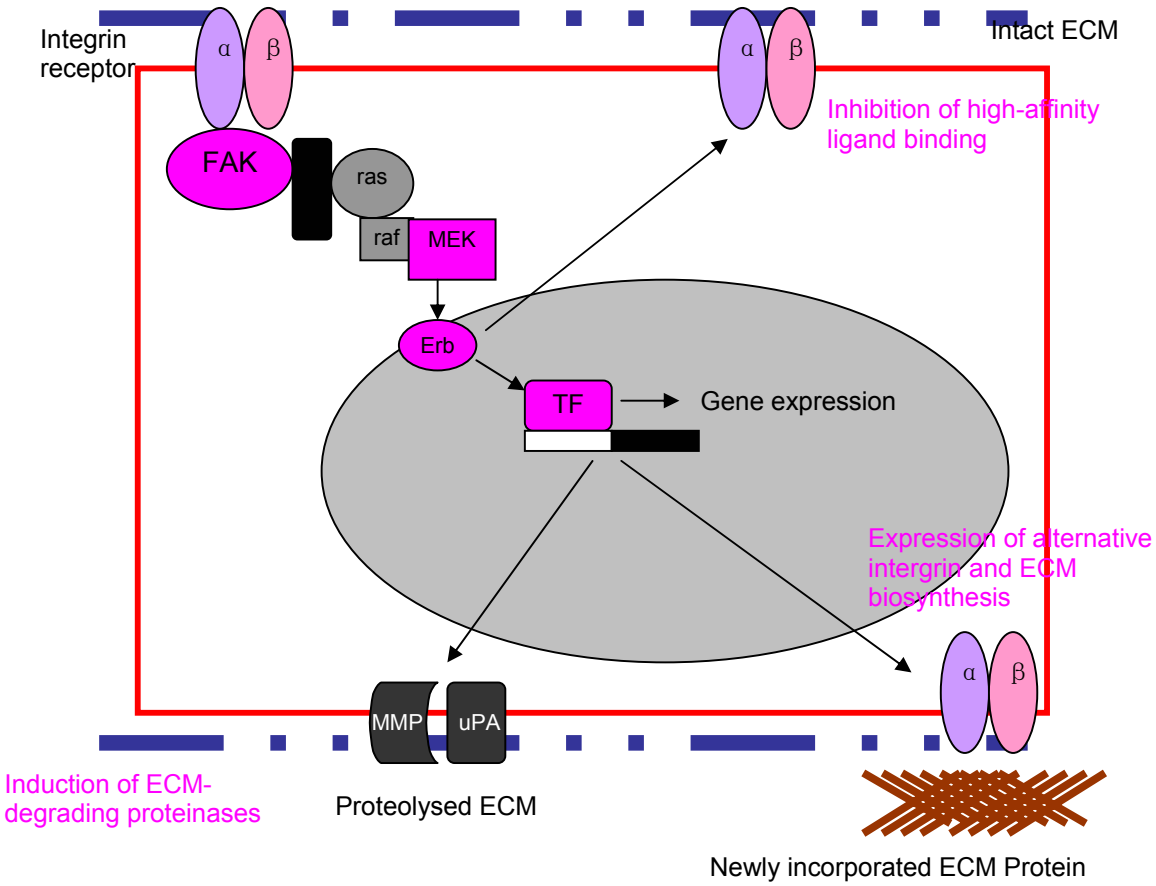

Figure 2-7 A representative picture of cell-ECM interation. The interaction between the integrin receptor and ECM could initiate a cascade of downstream pathway which eventually alters gene transcription and expression. In addition, activation of this pathway may lead to the transcription of target genes that modify cell±ECM

interactions. This mechanism forms the ' Inside-out ' functions of FAK and MAPKs. (TF, transcription factor; P indicates phosphorylation) [77]

Worth noting is the architecture of natural ECM as well. Vvarious collagens contribute to collagen fibril (10-300nm in diameter) and further to collagen fibers (several micrometer in diameter). [78] This structure has crucial significance to maintain cellular adhesion, proliferation and function. [79] Vernon reported a fiberilar collagen membrane, made from freeze drying, which provided favorable cellular support and proliferation, indicating its possible application in tissue engineering.[80]

2.5.2 Traditional Methods of Modification

The motivation to modify the PCL surface has been strongly driven by the understanding of cell-ECM interaction. Incorporation of a natural ECM mimic surface onto the PCL material is one of the traditional routes. Moreover, since surfaces, hydrophobic and lack of functional group, generate poor cell adhesion, multiple methods toward enhancement of hydrophility were also in study.

To increase hydrophilicity, NaOH treatment and plasma treatment [81] were studied. NaOH treatment was useful in increasing the hydrophilicity of the material by creating more OH groups on the surface.[82] Plasma treatment could activate the inert surface of biomaterials, generating free radicals which could further react with oxygen in air atmosphere to produce OH groups [83] . Plasma treatment was also explored to initiate

chemical conjugation of other protein molecules on the biomaterial surface. Both the treatments were reported with enhanced hydrophilicity and further better cell adhesion.

Researchers have further tried to coat extra-cellular (ECM) proteins, like collagen [84], gelatin [85] to enhance cell adhesion. These modified PCL showed excellent cell attachment, favorable cell morphology and faster proliferation rate. However, all of these studies were conducted in static scenarios; it is still in doubt whether the attachment between cells and the modified surface could withstand the shear stress in the dynamic environment in the physiological condition. This issue is more important than ever especially in the field of cardiovascular tissue engineering, where not only a favorable cell adhesion to support cell growth but also mechanically strong attachment is necessary for any practical application.

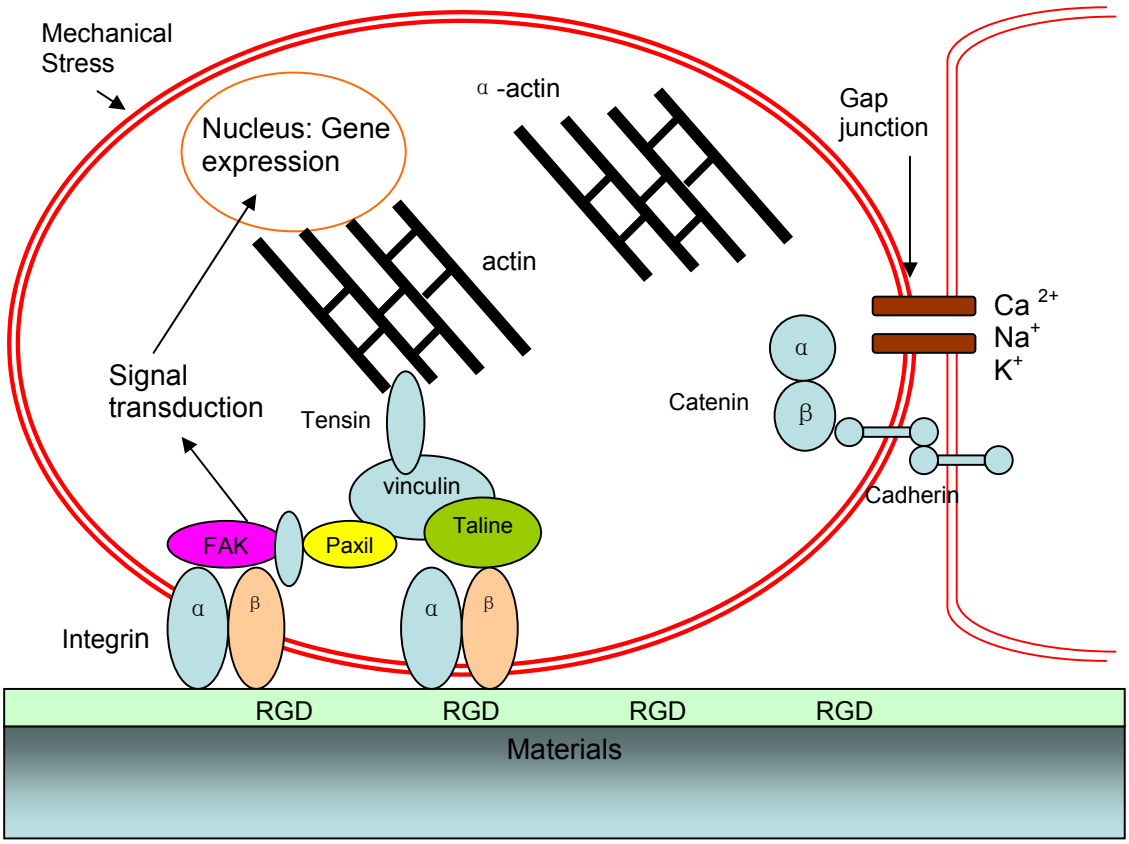

Figure 2-8 Representation of the cell proteins involved in cell adhesion on biomaterial. [86] To create a ECM-mimic surface on the biomaterial is the primary motivation of surface modification.

2.6 Nanotechnology in Tissue Engineering

2.6.1 Development of Nanofiberous Scaffold

The primary motivation to fabricate nanofibrous scaffold is to mimic the fibrous topology of natural ECM so as to enhance cell affinity in a new perspective beside the change of

biochemical composition. Recent development of nanotechnology enables various methods to produce nanoscaled polymeric fibers, including electrospinning, self-assembly, and phase separation.

Self-assembly technology can generate rather small diameter nanofibers in the lowest end of the range of natural ECM collagen; while phase separation, has generated nano-fibers in the same range as natural ECM collagen and allows for the design of macropore structures. However, these two technologies could not be adopted for surface modification.

Electrospinning technology, on the other hand, could produce nanofibers on the upper end of the range of ECM collagen; these attempts at an artificial ECM have the potential to accommodate cells and guide their growth and subsequent tissue regeneration. [87, 88]

2.6.2 Electrospinning Technology

Electrospinning has recently been adopted as a fabrication technique to develop biopolymeric nanofibres. Various synthetic and natural biodegradable polymers have been electrospun into fibres with diameters in the nanometre range (< 1 microm). The fibre diameter, structure and physical properties of the nanofibre matrices can be effectively tuned by controlling various parameters that affect the electrospinning process. [89]

In electrospinning, a polymer solution or melt is injected with an electrical potential to create a charge imbalance and placed in proximity to a grounded target. At a critical

voltage, the charge imbalance begins to overcome the surface tension of the polymer source, forming an electrically charged jet. The jet within the electric field is directed toward the grounded target, during which time the solvent evaporates and fibers are formed. Electrospinning produces a single continuous filament that collects on the grounded target as a nonwoven fabric. Notably, it is possible to fabricate filaments on the nanometer scale with this technique. In the present study, we describe how we have adapted the electrospinning process to fabricate an engineered matrix composed of collagen fibrils for use in tissue engineering. [90]

The advantages of spinning various synthetic and natural materials into ECM-mimic nanofibrous scaffold makes electrospinning one of the hottest research areas right after its introduction into this field. Recently, more and more relevant researches have been devoted various tissue engineering applications [91,92,93,94,95] Nanofibrous scaffold was reported to provide a stronger cell attachment than the conventional scaffold. [96]. The highly porous surface of nanofibrous scaffold generates a higher surface area. This topology tends to enhance protein deposition. Tough no clear mechanism found, protein synthesis was also enhanced with consequent cell adhesion on the electrospun nanofirous scaffold [97]. Another breakthrough with electrospinning is integration of electrospray of cells into nanofibrous mesh, made of poly (ester urethane) urea (PEUU). This so-call microintegration was proved to be possible to maintain smooth muscle cell viability and function in a rather thick construct. [98]

However, electrospinning technology is a rather low efficiency production method. The electrospun nanofrous scaffolds, normally in the form of mesh, suffer from low mechanical strength. This technology has not been studied in surface modification yet.

Chapter 3:

MATERIALS AND METHODS

3.1 PCL Membrane Fabrication

Ultra-thin PCL films were fabricated via 2-roll-heated-mill and biaxial stretch method developed by Teoh et al [99]. PCL pellets (Mn=80 000, Singma-Aldrich Company) were melted and processed into bigger pellets through a 2-roll-heated-mill. (Fig 3-1) These bigger pellets were melt-pressed into thin film at 80 °C with the pressure of 0.4 m Pa for 10min. All the pressed films were cut into 6 × 6 cm^2 for biaxial stretching. Films were preheated for 1 hour in the stretching chamber at 53 ± 1ºC before stretching. Heated PCL films were biaxially stretched 2.5 times its original size. (Fig 3-2) The films were then cut into square 2.2 × 2.2 cm^2, mounted onto microscope coverslip (2.2×2.2 cm^2) and carefully washed with ethanol before coating with nanofibers.

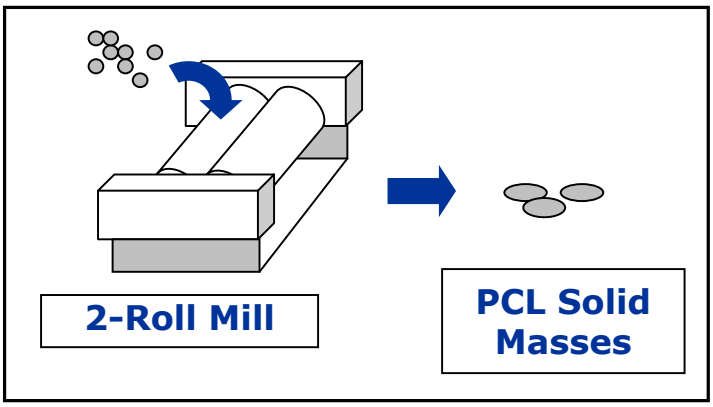

Figure 3-1 2-rill-heated mill: PCL pellets (Mn=80 000, Singma-Aldrich Company) were melted and processed into bigger pellets

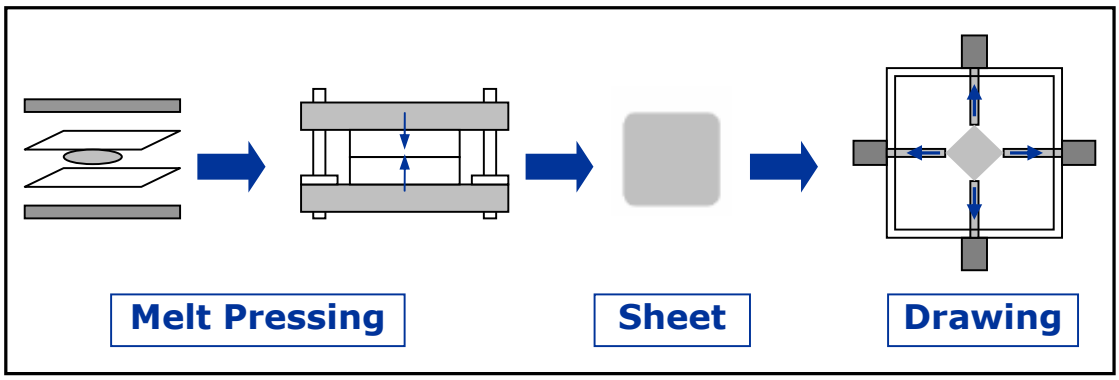

Figure 3-2 Melt-pressing and Biaxially Stretching Process: bigger pellets were melt-pressed into thin film, and then biaxially stretched into ultra-thin PCL films

3.2 PCL Nanofibrous Coating

PCL nanofibers were coated onto PCL films via electrospinning. In this process, PCL pellets (Mn=80 000, Singma-Aldrich Company) was dissolved in a 3:1 mixture of chloroform (J.T Baker) and methanol (Labscan Aisa) to obtain a 7.5 wt% solution. The PCL solution was delivered with a syringe pump (KD Scientific) at a flow rate of 2 ml/min to a blunt-ended 21 Gauge syringe nozzle through a PTFE tubing (Spectra-Teknik, Singapore). Ultra-thin PCL films, mounted on coverslips, were placed 15cm under the nozzle on an aluminum plate. High voltage electric field was generated by a high voltage supplier (Spellman CZE1000) whose positive end was connected to the nozzle and ground terminal to the aluminum plate by crocodile clips connected to the ground. A schematic diagram of the electrospinning device is shown in Fig 3.1. At a voltage of 18 kV, a fluid jet was ejected from the nozzle, and a nanofibrous surface was coated on PCL film eventually. The constructs were stored in a vacuum dessicator for several days.

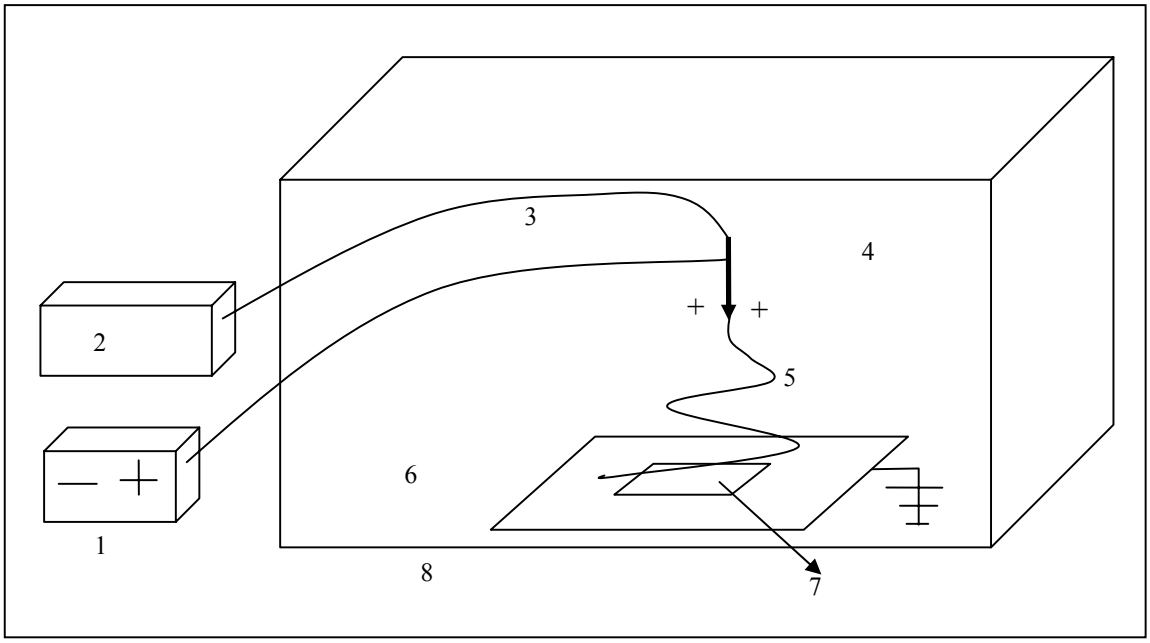

Figure 3-3 Experimental set-up for electrospining: 1 Voltage supplier, 2 Syringe pump, 3 PTFE Tubing, 4 Syringe nozzle supported by clamps, 5 Electrospun fiber, 6 Al collector, 7 Cover slip with film and 8 Hood.

3.3 NaOH Treatement

NaOH treatment was found to enhance the hydrophilicity and further enhance cell adhesion. For NaOH treatment, the nanofiber modified PCL films were soaked in 5 M NaOH in a 50 ml centrifuge tube (Falcon®, Becton Dickinson, NJ, USA) for 3 hours. 200 g of NaOH pellets (J.T. Baker, Phillipsburg, NJ, USA) was dissolved in 1 000 ml of deionized water to achieve the required concentration. The films were washed in deionised water 3 times and soaked overnight to remove the residue NaOH.

3.4 Surface Characterization

3.4.1 Surface Morphology Study by SEM

The surface morphology of the nanofiber coated PCL films (NF - PCL) and PCL Film was captured under scanning electron microscope (SEM) (JSM-5600LV, Japan). NF-PCL was also viewed after 3 hour 5M NaOH treatment by SEM. Before scanning, all the films were first gold sputtered using a current of 30 mA for 60 s with a fine gold coater (JFC-1200, Japan).

3.4.2 Surface Morphology Study by AFM

The surface topology of nanofiber coated PCL films (NF - PCL) and PCL Film was examined in a Dimension 3100 Scanning Probe Microscope (SPM) (Digital Instruments, Veeco Metrology Group) in air. Atomic force microscopy (AFM) images were obtained by scanning surface in a tapping mode (scan size 20 ×20 μm^2) using a silicon nitride probe (model DNP). An arithmetic mean of surface average roughness (Ra) was evaluated directly from the AFM images. Surface area was measured. The surface change was evaluated from the ratio of surface area to scan size.

3.5 Surface Wettability Study

3.5.1 Water Contact Angle Measurement

Membrane scaffold wettability was assessed by water contact angle. Water contact angles of the pristine and nanofiber modified PCL films were measured under laboratory

environment condition (the temperature was 20°C and the humidity was 64~65% RH), using sessile drop method on a goniometer (VCA Optima). Five measurements of each sample were carried out and the resulting values were averaged.

3.5.2 Capillary Reaction Study

PCL nanofibers were electrospun onto 5 × 4 cm frame with a 3 × 2 cm viewing window. Two groups of samples were studied: NF+NaOH (with 5 M NaOH treatment) and NF-NaOH (without 5 M NaOH treatment). Samples were placed into a petridish filled with deionised water for 1 min, under laboratory environment condition (the temperature was 20°C and the humidity was 64~65% RH). The upcoming water front lines of each sample were measured by a normal digital camera (SONY, P92).

3.5.3 Coating Adhesion study by Scratch Test

A custom-made scratch tester [100] (Designed by Material Science Division, Mechanical Engineering, NUS) was used to carry out the scratch tests under laboratory environment condition (the temperature was 20°C and the humidity was 64~65% RH). The purpose was to investigate the adhesion of the nanofiberous layer on the underlying PCL films. The indenter was made of the medium carbon tool steel. The included angle and the tip radius of the conical indenter were 90° and 2~3 μm respectively. The normal load applied on the indenter was set constant 2 N, scratch rate 0.02 mm/s, and scratch length 1mm.

Scratch samples were mounted onto custom designed cell-culture-rings (Fig3-2). An extra supporter, made of 316 L stainless steel and finely grained, was employed to support and flatten the PCL films. Two groups of samples were studied: NF-PCL and physically attached nanofibre mesh using ethanol on to the PCL film. After scratch, samples were viewed under SEM.

Figure 3-4 Cell-culture-ring. 1 Bottom Part 2.Top part 3.with PCL membrane mounted

3.6 Cell Behavior Study

3.6.1 Membrane Scaffold Preparation

Nanofiber-coated PCL film (NF-PCL-NaOH) and PCL film (PCL-NaOH) were wetted with 5 M NaOH for 3 hours, under laboratory environment condition (the temperature was 20°C and the humidity was 64~65% RH); the control groups were nanofiber-coated PCL film without NaOH treatment (NF-NaOH) and PCL film without NaOH treatment (PCL). All the samples were mounted onto culture ring (Fig. 3-2), sterilized in 70% Ethanol overnight and conditioned with cell culture medium for 3 hours, in a incubator at 37ºC in 5% CO2, 95% air, and 99% relative humidity.

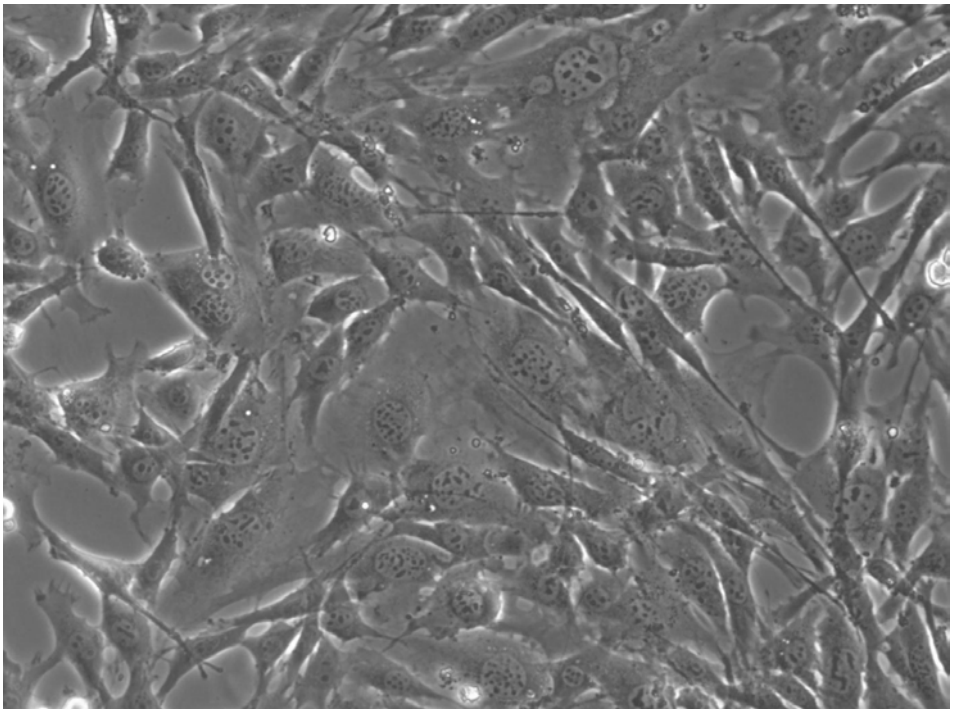

Figure 3-5 NIH 3T3 Fibroblast Cells in Culture

3.6.2 NIH 3T3 Fibroblast Cell Culture and Seeding

NIH 3T3 cells from ATCC (American Type Culture Collection, U.S.A) were used in this study as model cells. Cells were cultured in T-75 tissue culture flask in tissue culture medium, Dulbecco's Modified Eagle Medium (DMEM, NUMI, Singapore) with 10 % FBS (Fetal Bovine Serum, Hyclone, USA) and 1 % PS (penicillin-streptomycin, Sigma, Eschenstr, Germany). All the flasks were placed in an incubator (SANYO, MCO-20 AIC, Japan) with humidified atmosphere at 37ºC and 5% CO_2. Subculture was performed with culture medium replaced every 2 days. For subculture, cells were trypsinized with 0.25% trypsin / EDTA (Hyclone, U.S.A), centrifuged, resuspended in culture medium and aliquot with 1:2 ratio.

4 groups of samples, NF-PCL+NaOH, NF-PCL, PCL, and PCL+NaOH, which had been mounted, sterilized, were placed in 12-well plates. Cells were first trypsinized with 0.25% trypsin/EDTA, centrifuged, resuspended in culture medium and counted with a Neubauer-hemocytometer. A density of 5 000 cells/well were seeded with the loading volume 200µl. After cell seeding, 500 µl of fresh culture medium was added to each culture ring and another 1 ml medium into the well. Samples were further incubated in a humidified atmosphere at 37ºC and 5% CO_2 for 4 weeks with medium changes every 2 days.

3.6.3 Cellular Proliferation Study

A biochemical assay, the AlamarBlue® test was employed to assess cellular viability and cellular proliferation. The AlamarBlue® (Biosource International Inc, Camarillo, USA) reagent is a non-toxic, water-soluble, colorimetric redox indicator that changes color in response to chemical reduction of growth medium as a result of cell growth. The light absorbance reduction indicates the metabolism rate and reflects cell number.

The culture medium of scaffold/cell constructs (n=4) were removed. Samples were rinsed with PBS once, placed in DMEM with 10% AlamarBlue® solution and incubated in a humidified atmosphere at 37ºC and 5% CO2. After 3 hours of incubation, 100 µl of solution from each construct was transferred to 96-well plates in three duplicates and the optical density was measured with a reference filter of 595nm and measurement filter of 560 nm.

The date analysis was to follow the simplified method of calculating percent reduction," The percentage of AlamarBlue® reduction was calculated and co-related to the cell proliferation, as indicated by the supplier (www.biosource.com).

3.6.4 Cellular Attachment Study

Cellular attachment was assessed by Confocal Scanning Laser Microscope (CSLM, Olympus 300, Japan). Samples were stained with fluorescent dyes fluorescein diacetate (FDA), which stains viable cells green.

Culture medium was removed before the staining. The scaffold/cell constructs were rinsed with PBS 3 times. Each sample was loaded with 200 μl FDA solution (2 mg/ml) and incubated for 30 min at in a humidified atmosphere at 37ºC and 5% CO2. The stained samples were then rinsed 3 times in PBS and viewed under a confocal laser scanning microscope.

3.6.5 Cellular Morphology Assay

Scanning electronic microscopy (SEM, JEOL, Japan) assessed cellular morphology. Samples of 12 hours after seeding were fixed in 2.5% gluteraldehyde (Sigma-aldrich Company, U.S.A) under laboratory environment condition (the temperature was 20°C and the humidity was 64~65% RH) for 4 hours. They were then dehydrated in a graded ethanol series of 25%, 50%, 75% and 95% for 5 min for the first one and 10 min for the rest. Samples were further dehydrated in 100% ethanol for 3 times, air-dried and gold sputtered (JFC-1200 Fine-Coater) for 60 s at 30 mA under high vacuum mode. The specimens were further examined with a JEOL JSM 5600LV SEM operating at 10 kV under high vacuum mode.

Chapter 4:

RESULTS

4.1 Surface characterization

4.1.1 Visual Characterization of PCL Membrane

The transparent PCL membrane became opaque in appearance after the nanofibrous coating. (Fig 4-1) The coating is uniform to cover the 22×22 mm sample.

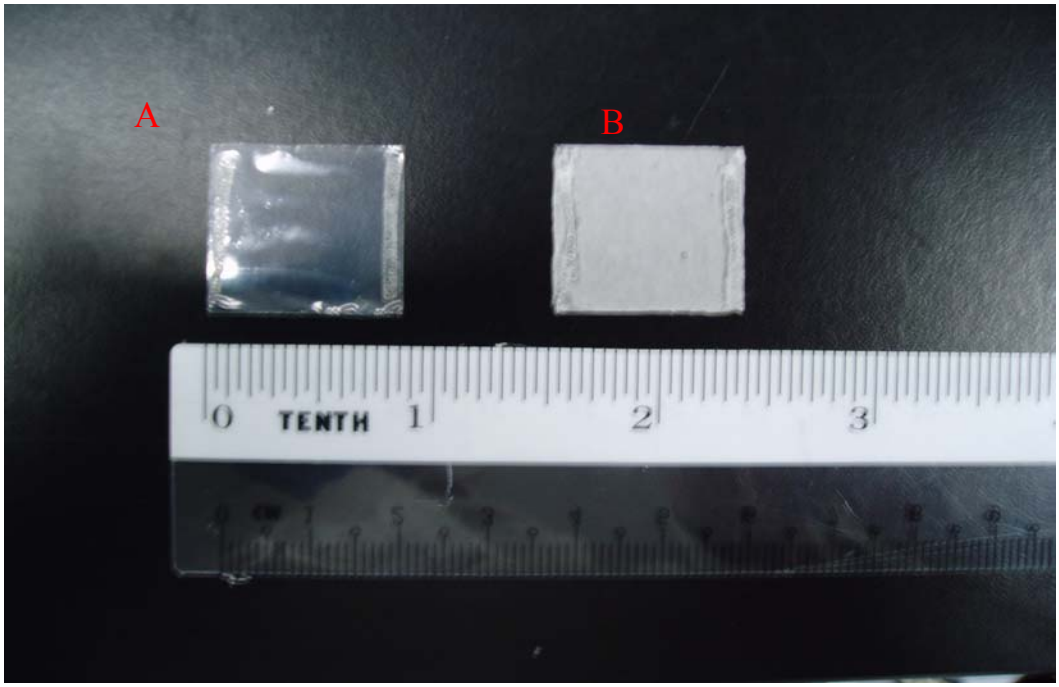

Figure 4-1 PCL membrane (PCL) and Electrospun nanofiber coated PCL membrane (NF-PCL). Pictures of PCL membrane before and after nanofibrous coating were documented by SONY P-92 digital camera. Nanofibrous coating change the PCL membrane from transparent to opaque.

4.1.2 Surface Morphology Study by SEM

SEM image revealed a totally different fibrous surface morphology before and after nanofibrous modification. (Fig 4-2.B) A nanofibrous surface was observed in the group of NF-PCL, while on the pristine PCL film, there was only a surface was plane surface. This image implies that PCL film surface morphology characteristics was thoroughly changed.

The diameter of coated nanofibers was measured at 862 nm. This information is helpful in determining the number of fibers residing on the PCL film later in the discussion.

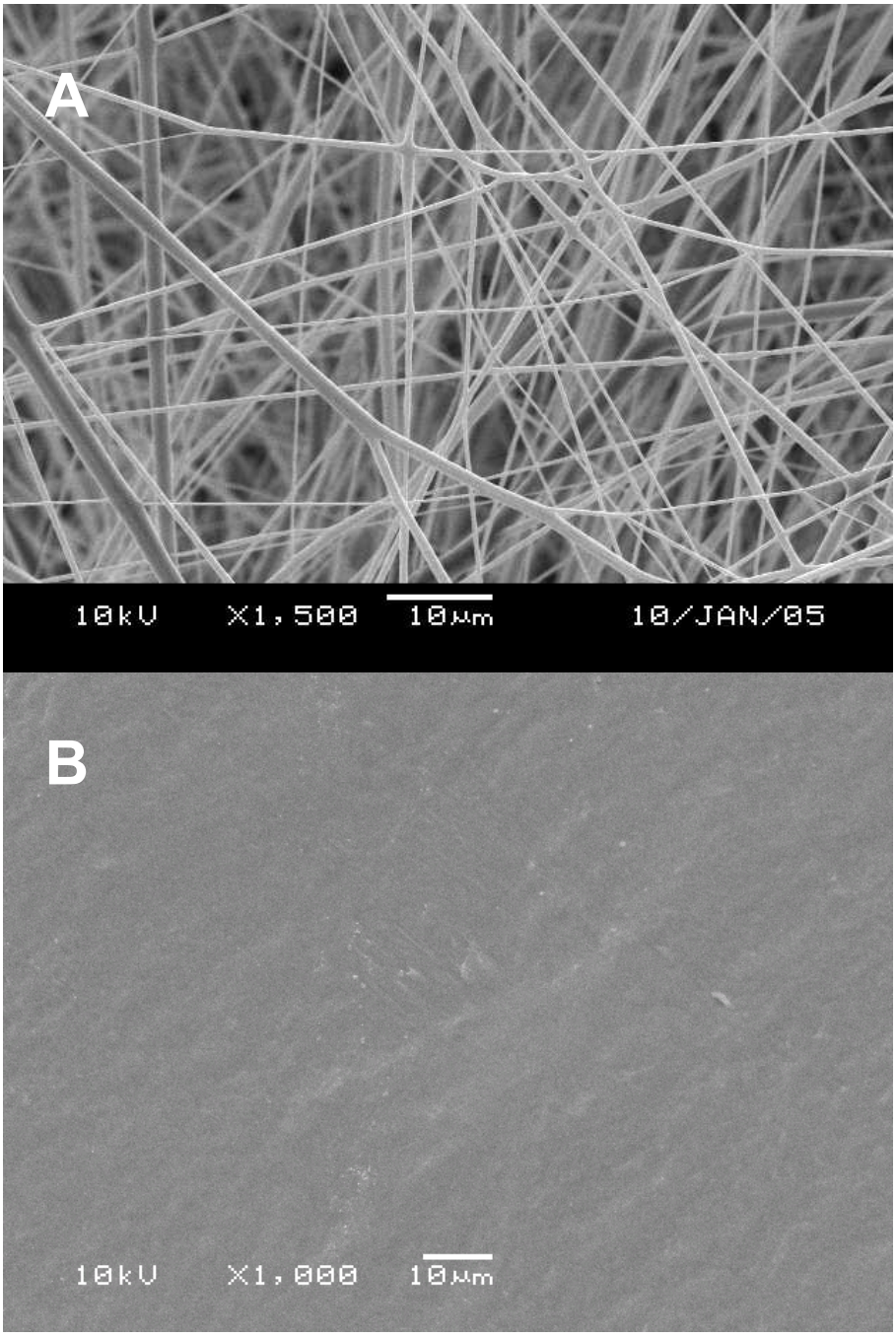

Figure 4-2 SEM image of Surface Morphology. A NF-PCL B. PCL. Pure PCL membrane exhibited a plane surface, while nanofibrous topology was revealed in picture A.

4.1.3 Surface Topology Study by AFM

Nanofibrous surface topology was also studied by AFM (Fig 4-2). A large contrast between the plane pristine PCL film and NF-Film was observed. With the AFM image technology, a 3D structure of surface topology was reconstructed.

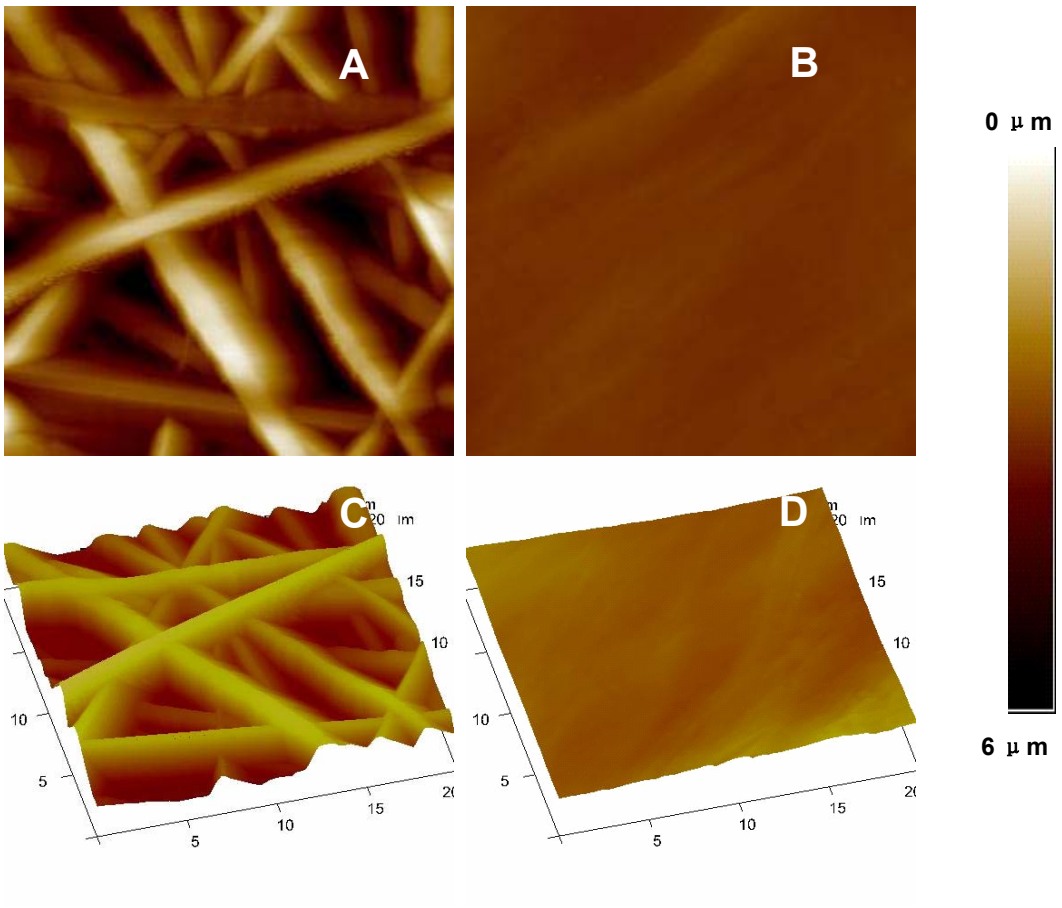

Figure 4-3 AFM image of Surface topology. A and C, NF-PCL; B and D PCL. Pure PCL membrane exhibited a plane surface, while nanofibrous topology was revealed in picture A. The 3D reconstruction revealed the 3D architectures on the nanofibrous scaffold in picture C.

AFM also measured roughness and surface area of the scaffolds. The surface roughness was increased from 53.24(±14.01) nm to 939.21(±57.32) nm (Fig 4-3A). The size of surface area was recorded as well and further evaluated by studying the ratio of absolute surface to the projected surface area or scan size. The ratio was increased from 1.04(±0.10) to 1.88(±0.14) (Fig 4-3B). This is a lower estimate had the basis be made on the nano 3D topography.

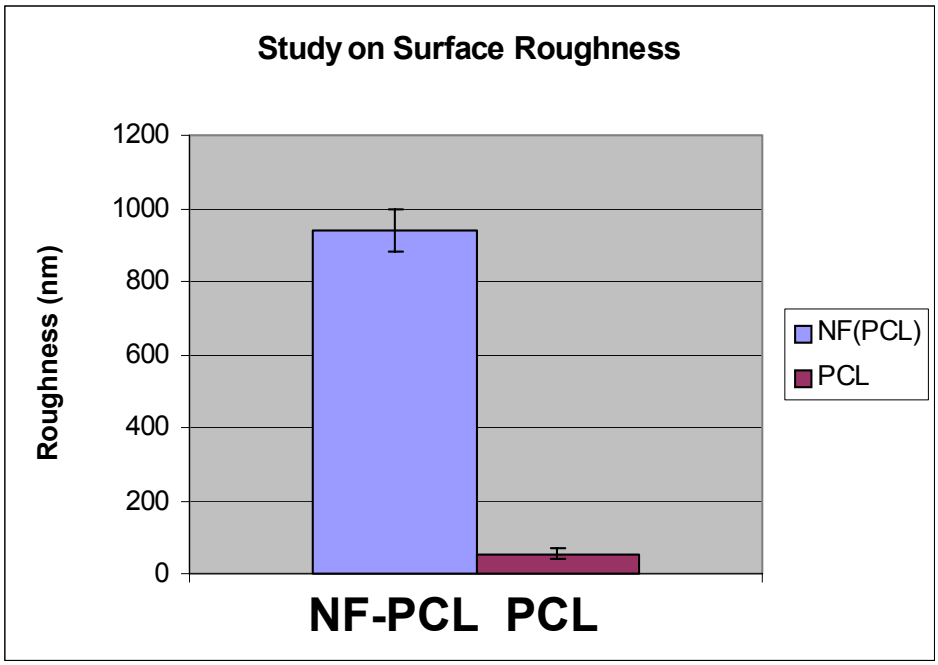

Figure 4-4 Roughness measured by AFM: The surface roughness was increased from 53.24(±14.01) nm to 939.21(±57.32) nm.

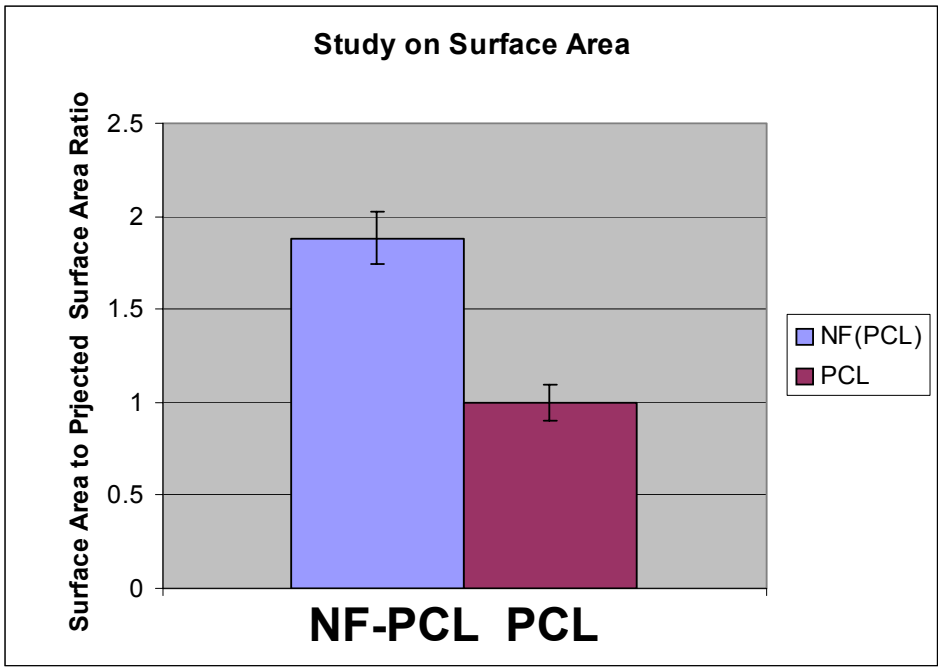

Figure 4-5 Surface Area Data from AFM. Surface area was further evaluated by studying the ratio of absolute surface to the projected surface area or scan size. The ratio was increased from 1.04(±0.10) to 1.88(±0.14)

4.2 Surface Wettability Study

4.2.1 Water Contact Angle Measurement

The water contact angle of each group were measured at Group 1 PCL , 77.42(±0.48) (PCL), Group 2 PCL-NaOH 59.72(±2.70), Group 3 NF-PCL 124.58(±2.42) and Group 0 (±0) (NF-PCL-NaOH). This is graphically shown in Fig 0-6.

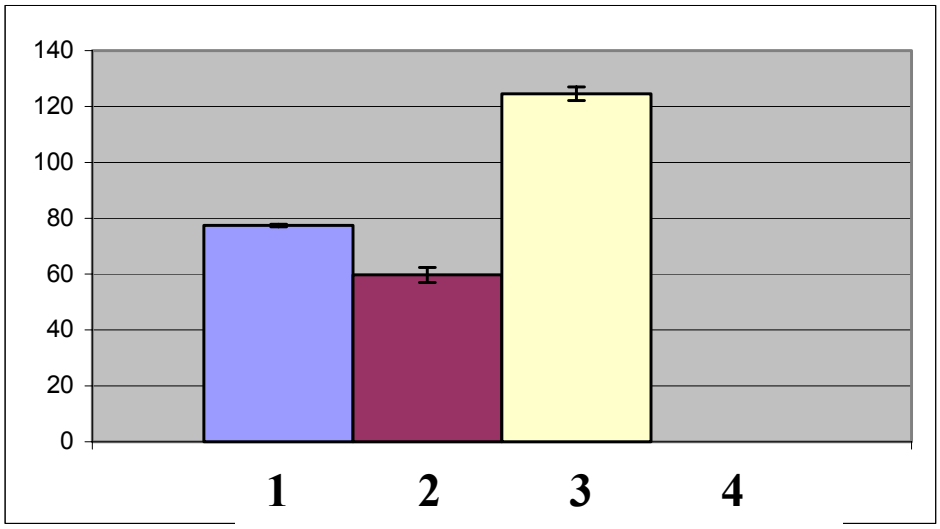

Figure 4-6 Water Contact Angle Measurement Data. Group 1. PCL 77.42(±0.48); Group 2, PCL-NaOH, 59.72(±2.70); Group 3, NF-PCL 124.58(±2.42) and Group 4, NF-PCL-NaOH 0 (±0).

4.2.2 Capillary Reaction Study

In the capillarity reaction study, the waterfront came up very fast in the NF-NaOH film, while the waterfront line remained at the original height in the films without NaOH tratement (NF). After 1 min, the height of the waterfront line went much higher in NF-NaOH than that in the NF group (Fig 7).

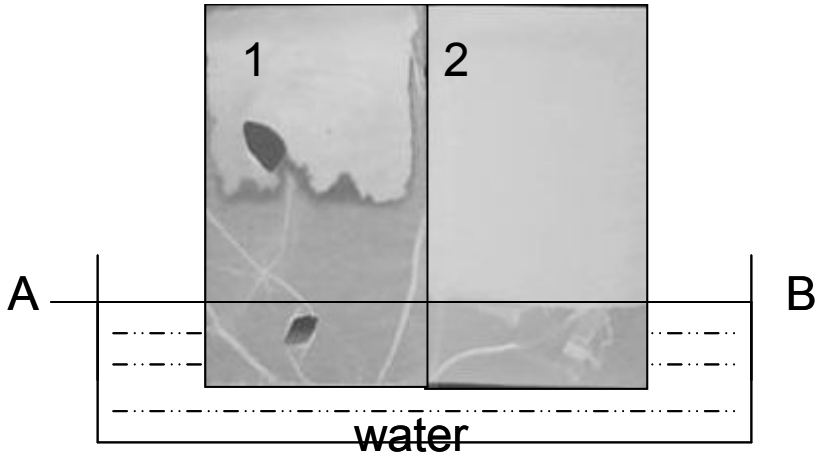

Figure 4-7 Capillarity study: Line AB corresponds to original waterfront line. 1. NF+NaOH 2. NF-NaOH. After 1 min, water front was increased in 1 but maintained at the original level at 2.

4.2.3 Nanofibrous Coating Adhesion Study by Scratch Test

SEM image revealed nanofibrous coating still maintain *in situ* in the NF-PCL group after scratch while nanofibrous coated was delaminated from the PCL membrane in the control group. Meanwhile, scratch affected larger size (> 150μm) in the control group than the NF-PCL group (<30μm).

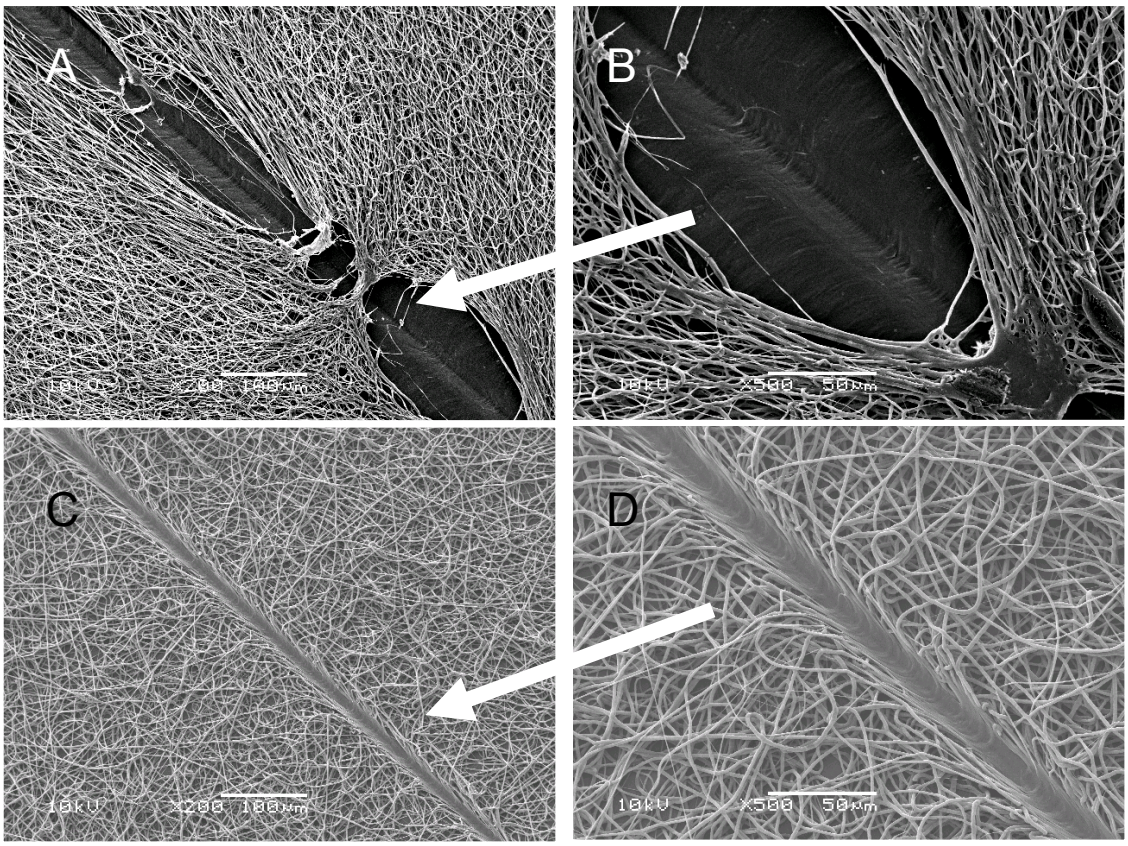

Figure 4-8 Scratch Test. Picture A and B correspond to the control group, nanofibrous layer was physically attached to the PCL film. Picture C and D correspond to NF-PCL, nanofibers were coated on to the PCL film. Greater delaminated surface was observed after scratch on the Picture C and D.

4.3 Cell Behavior Study

4.3.1 Cellular Proliferation Study

NIH 3T3 cell proliferation was observed through all the 4 groups, indicating good biocompatibility of PCL material. (Fig. 4-8) Cells were cultured for one week. They began to migrate and proliferate after 1 day culture. A burst growth was observed after day 3. The proliferation reached a plateau after day 5. The NF-NaOH group showed slightly higher reduction at each of the 5 time points, while the PCL group was recorded

with the lowest reduction. The difference was rather small, indicating that the metabolism rate was not affected so much due to the various substrates.

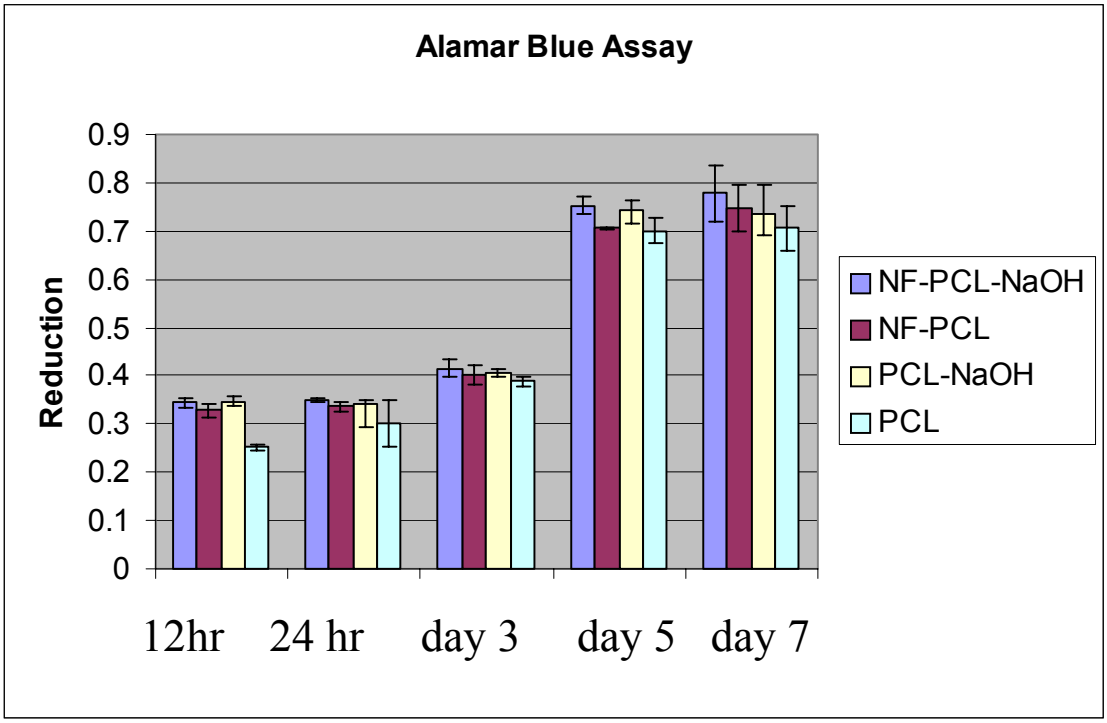

Figure 4-9 Celluar Proliferation by Alama BlueTM Assay: 3T3 cells proliferate well in all 4 groups, indicating good biocompatibility of PCL material. (NF-PCL-NaOH: PCL Nanofiber coated PCL film with NaOH treatment; NF-PCL: PCL Nanofiber coated PCL film without NaOH treatment; PCL-NaOH: Ultra-thin PCL film with NaOH treatment; PCL: Ultra-thin PCL film without NaOH treatment)

4.3.2 Cellular Attachment Study

Better cell attachment was revealed by Confocal Scanning Laser Microscope (Fig. 4-9). Cells were stained green by the FDA and viewed with the incident laser of 543 nm. More cells were observed on Day 6 than Day 1.

On Day 1, NF-PCL-NaOH and NF-PCL groups showed a uniform cell distribution, while in the PCL NaOH group, cells were observed to exist in clusters. The morphology

seemed to well spread in the NF-PCL-NaOH. A more spherical morphology was observed on the NF-PCL. In the group of PCL-NaOH, spherical shaped-cells were obviously observed in the clusters.

On Day 6, cells achieved confluent in the groups of NF-PCL-NaOH and NF-PCL; while few cells were observed on the PCL-NaOH group, indicating a dramatically enhanced cell adhesion due to nanofibrous surface modification.

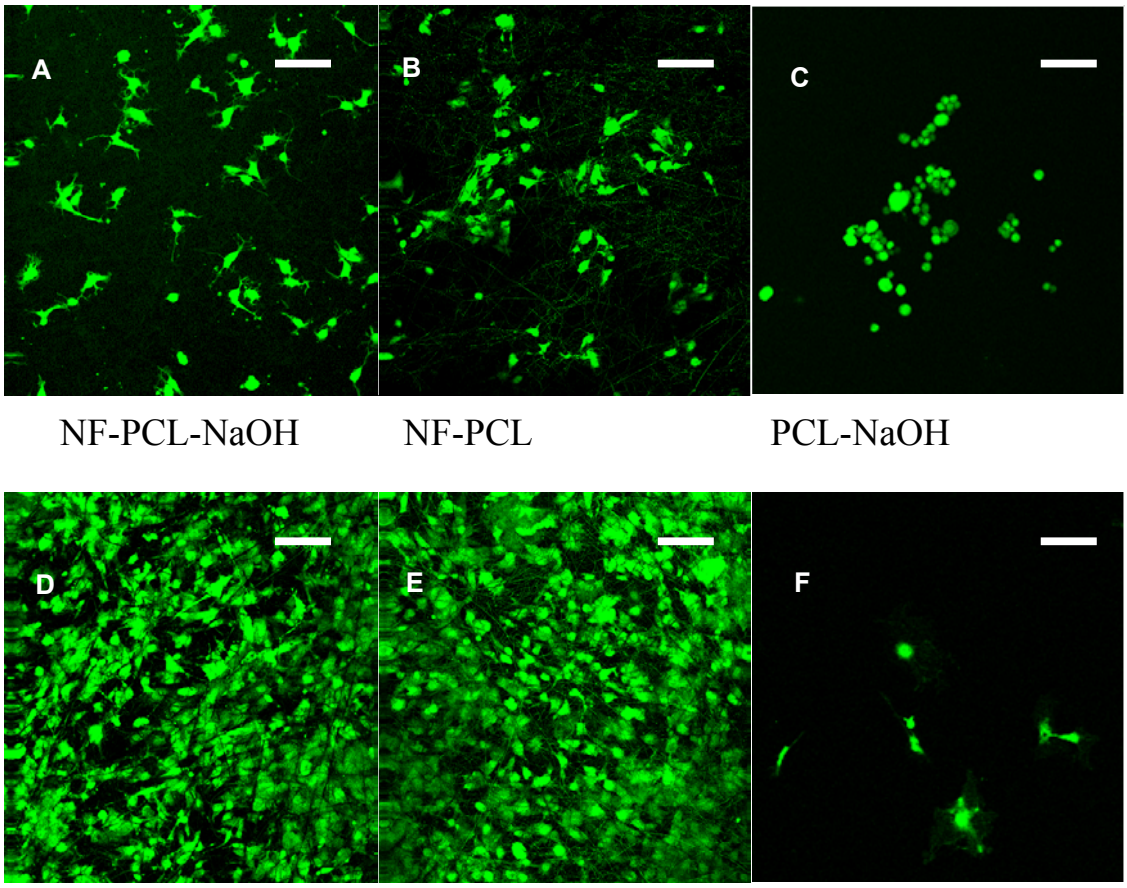

Figure 4-10 Confocal Laser Scanning Microscopy image of cell adhesion: Samples were stained with FDA and rinsed by PBS three times. Large quantity of the cells were detached during washing on C and F, while much more cells remained on nanofiber coated surface, indicating nanoferous topology dramatically enhanced cell adhesion. (A and D: NF-PCL-NaOH at Day 1 and Day 6 respectively; B and E: NF-PCL at Day 1 and Day 6 respectively; C and F: PCL-NaOH at Day 1 and Day 6 respectively. Scale Bar corresponds to 100um)

4.3.3 Cellular Morphology Assay

The cell morphology was further assessed by SEM. SEM results showed that In the PCL-NaOH and PCL groups, cells exhibited a more spread morphology, which is consistent with previous data. However, cells remained in isolated spherical shaped entities in NF-PCL. Fortunately, after NaOH treatment, cells exhibited a favorable spindle-shaped morphology on the NF-PCL-NaOH (Fig 4-10 A and B)

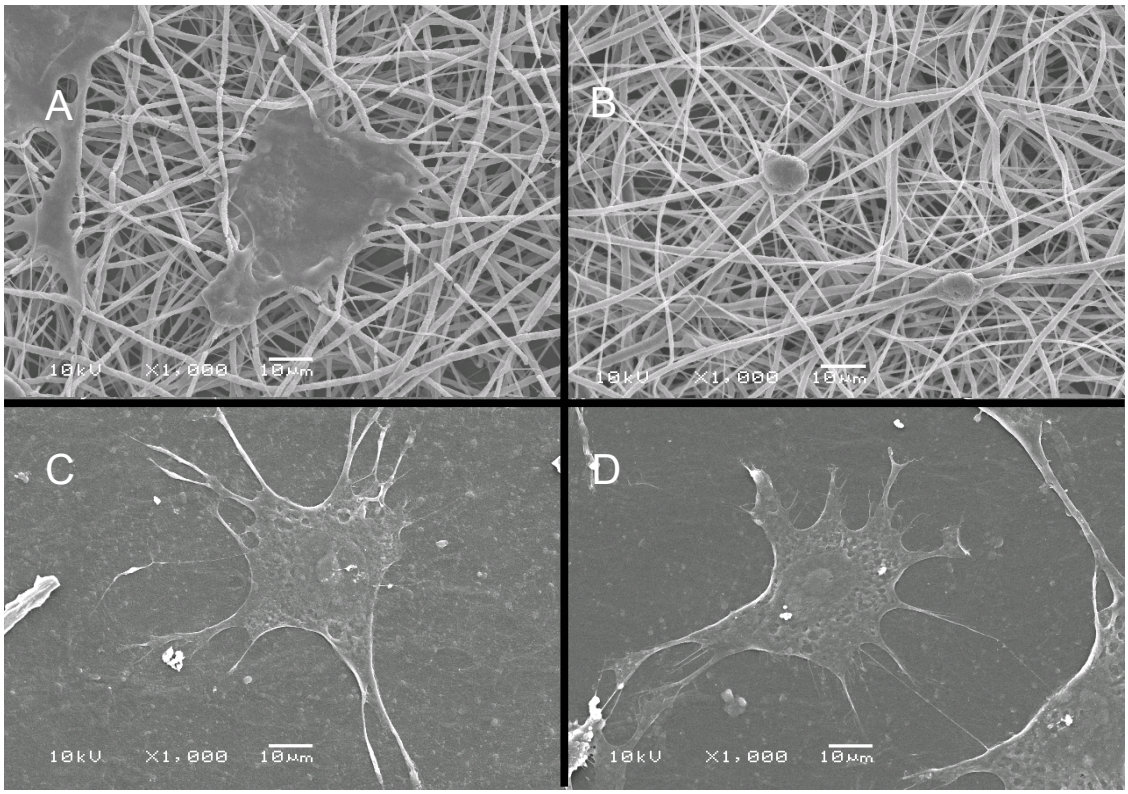

Figure 4-11 SEM image of Cellular Morphology 12 hours after seeding: Cells on NF-PCL+NaOH showed more spherical cellular morphology. Cells spread more easily on other substrates. Picture A NF-PCL+NaOH, B NF-PCL C PCL+NaOH and D PCL respectively.

Chapter 5:

DISCUSSION

Cell affinity, especially cell adhesion, is an important concept in tissue engineering. It is highly dependent on the surface chemistry as well as the surface topology. PCL membrane is a promising scaffold for tissue engineering but suffers from poor cell affinity. In this study, electrospinning technology was employed to modify the surface of ultra thin PCL membrane. The purpose of the study was to enhance cell affinity of ultra thin PCL membrane and make it favorable for cardiovascular tissue engineering application.

5.1 Scaffold Characterization

This novel method generates a dramatic topographic change with the roughness increased by more than 17 times and surface area increased almost twice. The 2D plane surface of PCL film was replaced by a fibrous 3D surface. The increased roughness and surface area provide a better chance for cells to adhere.

Moreover, this nanofibrous 3D surface mimics the natural extracellular matrix. This similarity might be able to initiate biological function for tissue engineering as the ECM in the native tissue, i.e. protein deposition. The similarity makes the novel nanofibrous scaffold promising for tissue engineering application.

This novel nanofibrous membrane scaffold is also supreme to traditional electrospun nanofibers. Although its thickness measures ~10μm, the nanofibrous coating itself is mechanically weak making it unable to survive the in vivo physiological environment. A series of problem might be caused by this mechanical weakness, such as scaffold

swelling or shrink, scaffold dislocation by exposure to blood pressure and even scaffold dislocation caused by cell migration. On the other hand, PCL membrane, after the biaxial stretching, could achieve a strong mechanical strength generating a more stable construct.

Another advantage of the nanofibrous film scaffold lies in the fabrication process. The electrospinning coating is fast and efficient, which takes 2min to achieve a uniform nanofibrous surface. This high efficiency is due to the small amount of the nanofibers needed. With the underlying film as mechanical support, this nanofibrous scaffold much fewer than the traditional electrospun nanofiber scaffold. The number of fibers coated on a single specimen of 22 × 22 mm was calculated to be 50 million. This small amount shortened the production time and makes the fabrication more user-friendly.

However, the nanofibrous modification also involved several problems. It decreased apparent surface hydropilicity of PCL membrane (120ºC water contact angle). This increase in water contact angle is caused by the inherent (CH_3 group) hydrophobic nature of the PCL chemical structure. The CH_3 groups tend to repel the water molecules to the bulk solution. Thus those pores in the porous surface, produced by nanofibrous coating, were filled with highly hydrophobic air. As a result of air's contribution to the surface hydrophilicity, the sharp increase of water contact angle was observed. Since more hydrophilic surface is preferred for cell affinity, this hydrophobic surface is not favorable for tissue engineering [101]. Fortunately, NaOH treatment was proved to be a solution to this problem. NaOH treatment increased the hydrophilicity by creating more OH groups on the nanofibrous surface. Water molecule could easily enter the pores in the

nanofibrous surface, causing capillary reaction. The result turned out to be an almost zero water contact angle. This zero water contact angle surface could suck the growth media and make the cells fully engaged with the scaffold.

Another issue is the attachment between the nanofibrous coating and underlying PCL membrane. We hypothesize the residue solvent chloroform could stick PCL fibers firmly onto the PCL membrane during the electrospinning thus a strong attachment could be formed after the solvent evaporation. This mechanism also speaks for the force to hold the traditional electrospun nanofiber scaffold, where fiberous mesh was stuck together by the residue solvent as well. Scratch test revealed this strong attachment: NF-PCL tended to maintain the position per se and have a smaller devastated area compared with the control group of physically attached ones.

Though the scratch test is rather informative as a qualitative evaluation, it is not useful to test the exact attaching force; other quantitative methods are necessary to further measure this attachment. A pilot study to measure lateral force and co-relate to the scratch SEM image was performed. Unfortunately, the later force was observed to be affected by multiple factors besides the fiber-film attaching force. Other methods are under investigation to make better evaluation.

5.2 Cell behavior study

5.2.1 Cell Proliferation and Cell Attachment

NIH 3T3 cell line is a very popular model to evaluate the biomaterial biocompatibility. This study witnessed various cellular behaviors on the different substrates, indicating different cellular responses to different topologies. The observation was consistent with consensus that surface topology of biomaterials indeed affects cell behaviors. [102] PCL is well-known for its biocompatibility. The AlamaBlue biochemical assay further proved a similar cell proliferation pattern on the various substrates, which bears testimony to this compatibility.

A remarkable observation was made on the dramatically enhanced cell attachment due to nanofibrous surface. On the plane PCL membrane, every few cells were observed on the CLSM image. This CLSM image indicated that cells were not attached firmly, though they proliferated well. It could be derived that the cells must have been detached from the PCL membrane very easily during the staining process. On the other hand, considerably more cells were viewed on the membranes with nanofibrous modification under CLSM. This result could be related to the results of surface characterization data. Larger area after nanofibrous modification provides more binding sites for the cells. The enhanced roughness and fibrous topology also contribute to stronger cell attachment. It was reported nanofirous topology could enhance protein deposition during culture which could further enhance the cell attachment.

5.2.2 NaOH Treatment and the Consequent Cell Behavior

The effect of NaOH treatment is worth noting. No significant changes in cell morphology were viewed on the plane PCL before and after NaOH treatment; while cell morphologies were totally different between the nanofibrous membranes before and after NaOH treatment. One of the possible explanations is the sharp hydrophilicity difference. In the plane PCL membrane, NaOH treatment changed the water contact angle only by 20°; while the water contact angle dropped over 120°. The 20° change in water contact angle was not large enough to induce dramatic morphological change in 3T3 fibroblast cells, the adaptive and fast-growing cell line.

Moreover, untreated nanofibrous topography tends to inhibit cell from spreading, leading to a spherical morphology type on the NF-PCL group (Fig 9 B); NaOH treatment increased the surface energy, induced capillary reaction and fully engaged the water molecules with the nanofibrous membrane. Hence, a more spread morphology was achieved (Fig 9 A). Parts of the spread cells might even extend into the nanofibrous layer, resulting cells got stuck there. All of these contribute to a rather strong attachment mechanically. On the other hand, cells spread nicely on the plane PCL membrane, either with or without NaOH treatment, but the cell adhesion only generates very weak connection with its substrate, which could be easily destroyed by a small shear stress. Even the gentle force generated by PBS rinse in the FDA staining would detach them from the scaffold. CLSM further concluded this detachment of cells. Current data

indicated the necessity of nanofibrous coating and NaOH treatment for soft tissue engineering applications.

5.2.3 Application in Cardiovascular Tissue Engineering

The unique cardiac and vascular physiology necessitates "strength" in scaffold for cardiovascular tissue engineering application. The strength must lie in the cell attachment. A meaningful cardiac patch must survive the vibrant contraction in order to replace the infracted porting of the myocardium. A vascular graft would be exposed to blood pressure after implantation. In either case, the cells' detachment from scaffold will cause serious disaster. The strength is required for scaffold material itself as well. The cardiovascular tissue engineering constructs must maintain the stability in size. The shrinking or swelling of the scaffold would be catastrophic enough to cause leaking in the vessel graft and the collapse of the vibrantly-contracting cardiac patch.

The concept of depositing electrospun nanofiber onto ultra-thin PCL membrane scaffold created a novel hybrid scaffold where the nanofibours coating provides the friendly ECM mimetic interface for cell to anchor while the PCL membrane provides the mechanical strength to maintain the structure. The novel scaffold satisfies the unique requirements of cardiovascular tissue engineering. It is ultimately hoped that the novel nanofibrous scaffold could offer outstanding promise for cardiovascular tissue regeneration.

Chapter 6:

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

An ultra-thin PCL membrane was prepared by two-roll-heated mill, melt-press and biaxial stretch method; a novel modification to this ultra-thin PCL membrane via electrospinning was established. Based on the surface characterization and cell behavior study on this nanofibrous ultra-thin PCL membrane, we could get conclusion as follows:

1) The method of two-roll-heated mill, melt-press and biaxial stretch, which eliminated use of toxic solvent, was proved to be more user-friendly. Ultra thin PCL membrane, fabricated by this method, has very good biocompatibility. PCL material is non-cytotoxic; it supports suitable cell growth.

2) Nanofibrous surface modification is an able and efficient method to create a ECM-mimic nanofibrous topology on PCL membrane. The nanofibrous surface modification increased surface roughness and area. The coating process is very efficient and reliable.

3) NaOH treatment is a useful treatment to enhance biomaterial hydrophilicity. Together with the nanofibrous porous surface, NaOH treatment induced capillary reaction so as to generate an easy engagement of the nanofibous scaffold with water and further with other aqueous solution. This phenomenon might help tissue integration after future implantation in clinical cases.

4) Nanofibrous surface modification results in a better cell affinity on the PCL membrane. Nanofibrous scaffold not only supported cell growth as the pristine PCL membrane, but also produced a strong attachment between the cells and nanofibrous substrate. This nanofibrous PCL film shows promising application in soft tissue engineering.

6.2 Recommendations

This study is the first one to adopt elctrospun nanofibers for surface modification of PCL membrane. Many follow-up works might be continued to explore more area using this study as a beginning.

6.2.1 Work with Cardiomyocytes

NIH 3T3 cell line is a very popularly used cell model for scaffold characterization, but the response of other working cells to this nanofibrous scaffold, such as cardiomyocytes, endothelial cells and smooth muscle cells, could be more informative for cardiovascular application. We successfully extracted human fetal cardiomyocytes (HFCM) from (Fig 6-1) and characterized this cell source for this very study.

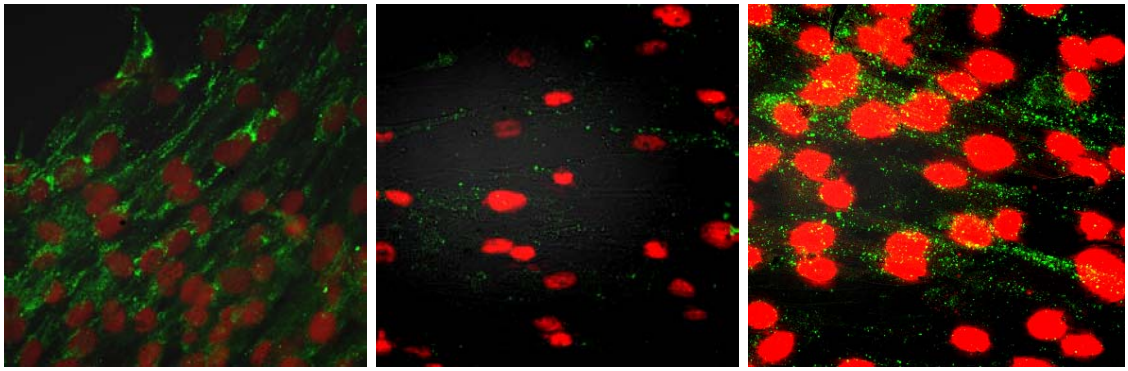

Figure 6-1 Immunophenotyping Human Fetal Cardiomyocyte(HFCM). HFCM were stained positive (green) for (a) Myosin Heavy Chain (b) Cardiac troponin I and (c) Connexin 43. Cell nuclei are counterstained with PI (red). Results indicate that isolated cells are of cardiomyocyte lineage.

6.2.2 Collagen Nanofibrous Coating

Collagen is one of the major proteins in the ECM. The immobilization of collagen onto PCL membrane was achieved through acrylic acid grafting methods but topology of collagen grafting could not be controlled in previous study.

To further mimic the natural ECM, we hypothesize that nanofibrous surface modification by collagen electropun nanofibers might create a new space for improvement of better enhanced cell affinity. Pilot studies have already been done within this thesis research. A SEM picture showed the possibility of nanofibrous collagen coated PCL membrane. (Fig 6-1) The supreme advantages of the collagen nanofiber coated PCL membrane reside in the bioactive surface which is rich in ECM protein and nanofibrous topology. As shown in Fig 6-3, at day 6, more live cells (green) were found on PCL with collagen nanofibers (c) than on pristine PCL films (a) and PCL films with PCL nanobers. The results support the use of PCL films with immobilized collagen nanofibers in myocardial engineering.

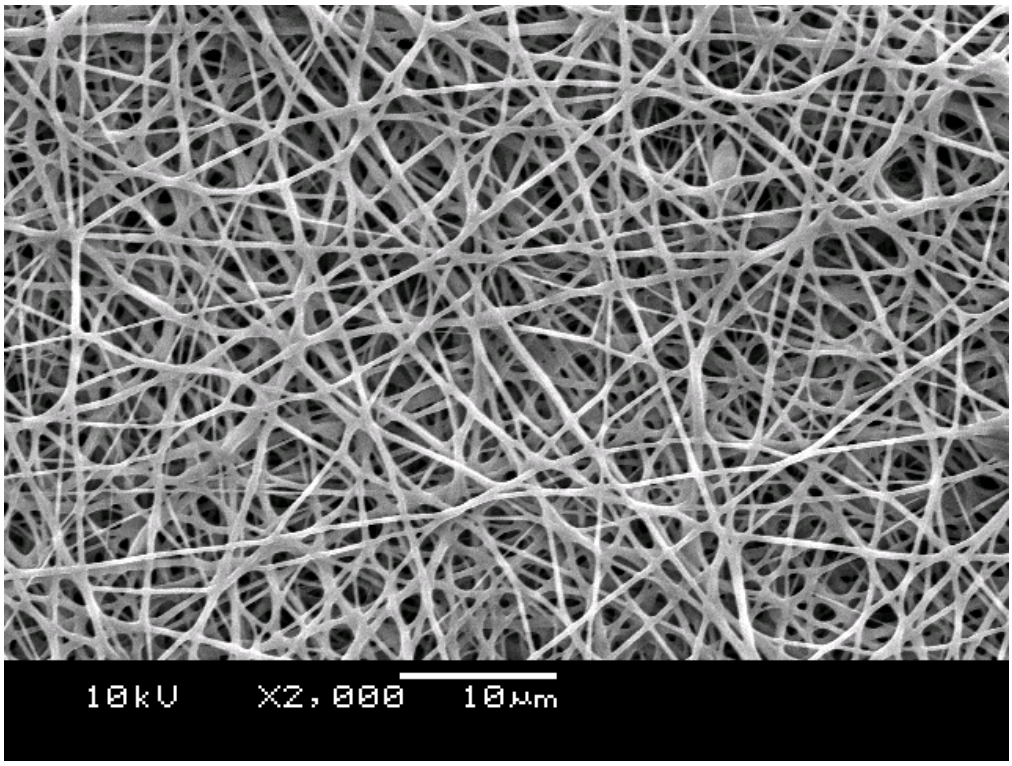

Figure 6-2 SEM image of collagen nanofiber-coated PCL membrane

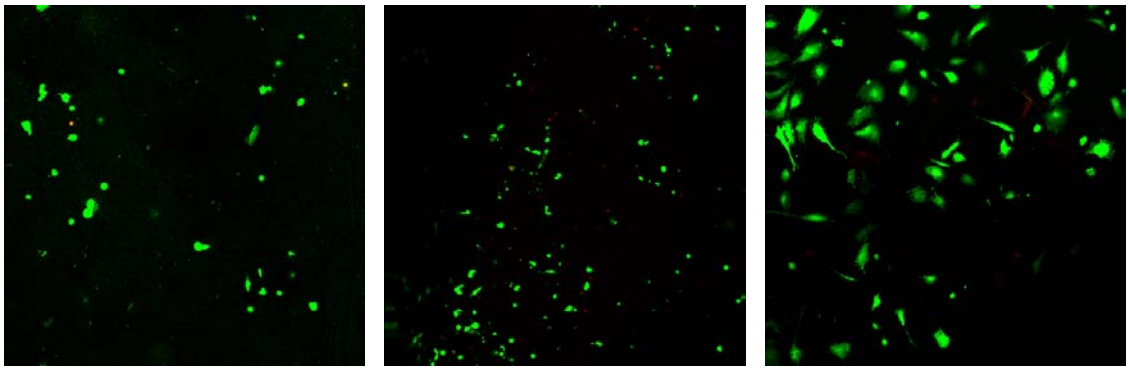

Figure 6-3 CLSM image of Live/Dead Assay of HFCM on collagen nanofiberous PCL membrane. Fluorescein diacetate (FDA)/Propidium Iodide (PI) was used to assess compatibility of film surface with HFCM (10x magnification). At day 6, more live cells (green) were found on PCL with collagen nanofibers (c) than on pristine PCL films (a) and PCL films with PCL nanobers. The cells were also found to attach better, flattening out and adopting typical spindle morphology.

6.2.3 Guidance of Nanofiber Deposition

Guided nanofiber deposition also bears significant important in the future development of nanofibrous scaffold. The great potential is that, by guidance of nanofiber deposition, electrospinning technology could not only create an ECM-mimic surface, but can also form a pre-defined architecture to guide cell growth and development. Hence by controlling the direction of the surface nano-sized architecture, the cell behavior could be in a way controlled.

This hypothesis is supported by the well established ‘‘contact guidance’’ theory. Contact guidance illustrates that a cell’s migration is in a preferred pattern associated with chemical, structural and/or mechanical properties of the substratum [103-104]. Guidance of nanofiber deposition indeed was in hot study in recent years in the electrospinning field. [105-106] We are optimistic that this technology could be incorporated into the nanofibrous surface modification presented in this thesis.

6.2.4 Quantitative Evaluation of Cell-material Interaction

A quantitative system to evaluate cell-material interaction is necessary to make a more accurate analysis of cell-material interaction. Different cell attachment onto various substrates was observed in the cell behavior study in the project. The evaluation was performed by gentle washing followed with confocal laser scanning microscope viewing. Fortunately, the cell attachment differs considerably in this project; however, the

qualitative method is very user dependent and not able to draw solid conclusion in those cases where there are only slight differences in the cell behaviors.

6.2.5 From 2D to 3D System

Ultra-thin PCL membrane is a 2D membrane scaffold while 3D architecture is necessary except few application fields, such as skin, vascular graft, where a monolayer of cell construct is needed. We proposed a layer-by-layer technology to fabricate reasonable thick tissue from PCL membrane. With the aid of nanofibers, the layer-by-layer process might be simplified by integrating cells into the nanofibrous mesh and future stacked into thick tissue. In the system described in Fig 6-2, cells are to be encapsulated in 3D nanofibrous mesh while the nanofibrous mesh links adjacent PCL membranes together. PCL membranes are the major mechanical strength, providing the mechanical strength which keeps the inherent structure from collapsing.

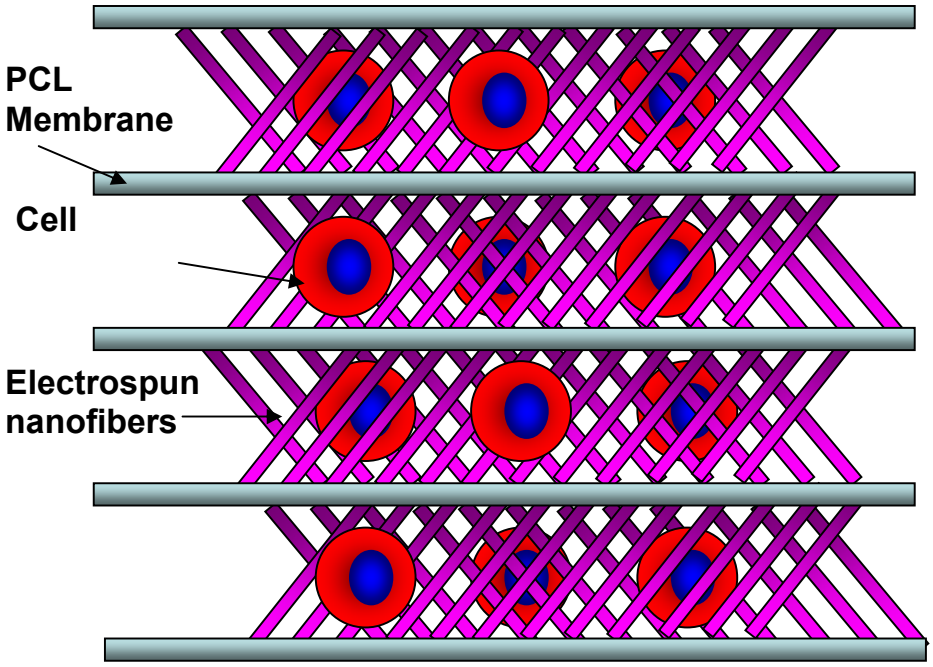

Figure 6-4 A schematic show of 3D system based on ultra-thin PCL membrane

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