Investigation of an Electrospun Nanofibrous, Degradable Polar Hydrophobic Ionic Polyurethane Patch for Cardiac Tissue Regeneration

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Cardiac tissue engineering (CTE) can be used to repair and replace the damaged myocardium due to coronary artery disease. An electrospun nanofibrous, degradable polar hydrophobic ionic polyurethane/polycarbonate polyurethane (D-PHI/PCNU) scaffold was generated and characterized for CTE applications. In addition, induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) were assessed for their compatibility on the D-PHI/PCNU scaffold. D-PHI/PCNU scaffolds had a high cross-linking efficiency, higher surface energy, and lower stiffness relative to PCNU scaffolds. In vivo, the D-PHI/PCNU scaffold degraded relatively slowly, but was well integrated with the surrounding tissue. Furthermore, the aligned D-PHI/PCNU scaffolds were able to support high iPSC-CM viability, adhesion, and expression of functional cardiac markers after a 7-day culture period. The iPSC-CMs had a more elongated and aligned phenotype on aligned D-PHI/PCNU scaffolds as compared to on random D-PHI/PCNU scaffolds and on TCPS. This work demonstrates the potential of using D-PHI/PCNU scaffolds as a platform for CTE.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADO</td>
<td>Anionic dihydroxyl oligomer</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BD</td>
<td>Butane diol</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CM</td>
<td>Cardiomyocyte</td>
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<tr>
<td>CTE</td>
<td>Cardiac tissue engineering</td>
</tr>
<tr>
<td>cTnT</td>
<td>Cardiac troponin-T</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin-43</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>D-PHI</td>
<td>Degradable polar hydrophobic ionic polyurethane</td>
</tr>
<tr>
<td>DVO</td>
<td>Divinyl oligomer</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ESC-CM</td>
<td>Embryonic stem cell derived cardiomyocyte</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>FTIR-ATR</td>
<td>Fourier transform infrared-attenuated total reflection</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HDI</td>
<td>Hexane diisocyanate</td>
</tr>
<tr>
<td>HEMA</td>
<td>Hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cell</td>
</tr>
<tr>
<td>iPSC-CM</td>
<td>Induced pluripotent stem cell derived cardiomyocyte</td>
</tr>
<tr>
<td>LDI</td>
<td>Lysine diisocyanate</td>
</tr>
<tr>
<td>MAA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MMA</td>
<td>Methyl methacrylate</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCN</td>
<td>Poly(hexamethylene carbonate)</td>
</tr>
<tr>
<td>PCNU</td>
<td>Polycarbonate polyurethane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>PEUU</td>
<td>Polyester urethane urea</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PGS</td>
<td>Poly(glycerol sebacate)</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PPy</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>PSC-CM</td>
<td>Pluripotent stem cell derived cardiomyocyte</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vim</td>
<td>Vimentin</td>
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Chapter 1: Introduction

1.1 Background

Cardiovascular disease (CVD) is the leading cause of death in the world. In 2015, CVD was responsible for 17.7 million deaths worldwide, which accounts for approximately 31% of total deaths [1–5]. CVD results in a high impact on society and the economy, costing the Canadian economy more than $21.2 billion dollars yearly [6]. One of the main diseases categorized under CVDs is coronary artery disease (CAD), which occurs after damage to the major blood vessels that supply the heart with blood, oxygen, and nutrients [3]. CAD can lead to myocardial infarction (MI), cardiomyocyte (CM) death, myocardium damage, and ultimately heart failure, due to the limited regenerative potential of the heart [7]. Current treatments for heart failure include heart transplants and ventricular assist devices. However, there is a shortage of organ donors and ventricular assist devices are costly and may not be suitable for all patient types [8–10]. As an alternative, cardiac tissue engineering (CTE) can be used to develop a cell-seeded cardiac patch to repair and replace damaged myocardium. However, current synthetic CTE scaffolds fall short in their ability to generate a biocompatible and biodegradable scaffold with a readily-available cell source. In addition, CTE scaffolds often lack the appropriate mechanical properties required to generate a functionally viable cardiac patch that supports angiogenesis and has a thickness (mm scale) representative of native heart tissue [1,4,7].

Currently, many groups have developed tissue engineered 3D cardiac patches made of both natural and synthetic biomaterials [11–14]. Additionally, stem cells can be seeded onto the cardiac patch to help with tissue reorganization, regeneration, and repair. Ideally, the scaffold needs to be vascularized and integrated with the host tissue while being able to gradually degrade. Thus, it must be able to mimic the structure, mechanical properties, and biochemical function of the heart tissue’s extracellular matrix (ECM) [15]. Natural scaffolds, derived from ECM proteins, provide a physiologically relevant surface for cells to attach, proliferate, and differentiate [16]. Natural scaffolds have the benefits of biocompatibility and biodegradability. However, they have poor mechanical strength, consistency in product is an issue because of batch-to-batch variation, there are immunogenicity concerns with naturally derived products, and lastly, there is a challenge with the inability to often tailor the biochemical and biophysical...
properties [15]. Synthetic scaffolds have the advantage of customizable mechanical and biochemical properties and thus can be tailored for use as cardiac tissue if they are designed to be biocompatible and biodegradable, since synthetic scaffolds are often activators of the foreign body response [15].

Polyurethanes (PU) are becoming more popular synthetic materials for tissue engineering due to their segmented block co-polymeric chemistry which provides the opportunity to tailor the mechanical properties, biocompatibility, and degradation rates [17]. For example, a degradable polar hydrophobic ionic polyurethane (D-PHI) is a biocompatible PU developed in the Santerre Lab which has many properties that address the challenges delineated above for cardiac patches [18]. Currently, D-PHI films have been prepared as a mixture of three monomers: a lysine-based polycarbonate divinyl oligomer (DVO) cross-linker, methacrylic acid (MAA), and methyl methacrylate (MMA) [18]. D-PHI has been shown to have immunomodulatory properties and reduced pro-inflammatory macrophage activation [19]. Moreover, D-PHI has good general compatibility with various cell types including smooth muscle cells, endothelial cells, monocytes, gingival fibroblasts, and more recently stem cells [18,20,21]. In vivo, D-PHI has shown good tissue integration and angiogenesis [20]. Thus, all these benefits provide the possibility of extending the application of D-PHI to CTE.

Electrospinning is a useful technique that uses a high voltage difference to generate an electrically charged polymer solution that is ejected from a nozzle onto a grounded mandrel or collector plate to form a nanofibrous scaffold [22]. Electrospinning can be used to produce natural or synthetic nanofibrous, porous scaffolds that mimic the native ECM [23]. The scaffold resembles the scale and fibrous nature of the ECM with surface features at a nanometer size scale [23]. Also, a high surface area to volume ratio and high porosity help promote tissue biosynthesis and guide cell growth, thus enhancing tissue regeneration [23].

This thesis will attempt to address the current biocompatibility, biodegradability, and mechanical property challenges in cardiac tissue engineered scaffolds by combining the unique properties of D-PHI with the benefits of electrospinning in order to generate an electrospun D-PHI cardiac scaffold which can support CM function and enable eventual cardiac tissue regeneration. The electrospun D-PHI scaffold was characterized to ensure appropriate surface chemistry,
mechanical properties, and in vivo integration and degradation. Subsequently, in vitro biocompatibility studies were performed with CMs.

1.2 Research question

Can electrospinning be used to generate a D-PHI cardiac patch that supports CM function for eventual applications in cardiac tissue regeneration?

1.3 Hypothesis

D-PHI can be electrospun into a highly oriented scaffold, will show biodegradability, and enable the generation of tissue engineered constructs of oriented tissue that have mechanical properties representative of native heart tissue. A sub-hypothesis is that the materials will be sub-toxic to induced pluripotent stem cell derived CMs (iPSC-CMs) (at least 80% viability), and enable CM growth and maturation as measured by the expression of functional CM markers (cardiac troponin-T (cTnT) and myosin light chain 2 (MLC)).

1.3.1 Rationale

The electrospun D-PHI scaffold must mimic the native cardiac ECM and possess a cell compatible character with CMs, while supporting cell maturation, in order to generate a functional cardiac patch for cardiac tissue regeneration.

1.4 Central objective

Electrospin D-PHI to generate a functional cardiac patch which can support CM culture.

1.5 Objective 1

Synthesize and characterize the electrospun D-PHI scaffold to generate a cardiac patch that is biocompatible and biodegradable. The scaffold should have mechanical properties that are representative of native myocardium (0.02-0.5MPa) and have the ability to support CMs [7,24,25]. The electrospinning parameters were optimized using a factorial design experiment. The morphology of nanofibres, cross-linking efficiency, surface energy, mechanical properties (elastic modulus, tensile strength, elongation-at-yield), and in vivo integration and degradation of the scaffold were measured to characterize the electrospun D-PHI scaffold. (Chapter 3)
1.5.1 Rationale

D-PHI is a novel polyurethane elastomer platform that is highly compatible with multiple cell types, enables angiogenesis and tissue regeneration, and has low inflammatory activity with minimal fibrosis post-implantation. The material chemistry and mechanical properties of the scaffold will influence scaffold biodegradability, cell interactions with the biomaterial, and subsequent tissue regeneration [1,7].

1.6 Objective 2

Determine iPSC-CM biocompatibility on the electrospun D-PHI scaffold. The viability of the iPSC-CMs was determined using live/dead viability staining, morphology and adhesion of the cells was determined using scanning electron microscopy (SEM) and DNA assays, iPSC-CM phenotype was determined using immunostaining for functional CM markers, and iPSC-CM elongation and alignment was quantified. (Chapter 4)

1.6.1 Rationale

The electrospun scaffold must support iPSC-CM adhesion and survival, and must not be toxic to cells. The scaffold should also maintain iPSC-CM phenotype and potentially could be used to enhance iPSC-CM maturity. The cell source must be reliable and easily accessible in order to generate mature CMs [26,27].

1.7 Contributions

This work was presented as an oral presentation at the Annual Meeting of the Canadian Biomaterials Society in May 2018 (Chan J, Santerre JP. Investigation of an electrospun, degradable polar hydrophobic ionic polyurethane patch for cardiac tissue regeneration. 2018). In addition, a poster was presented at the Heart Failure Update (May 2018) and at the Institute for Biomaterials and Biomedical Engineering Annual Research Conference (May 2018). Two research papers are in progress (Synthesis and characterization of an electrospun nanofibrous, degradable polyurethane composite scaffold for tissue engineering applications and Induced pluripotent stem cell derived cardiomyocyte compatibility on an electrospun nanofibrous, degradable polyurethane composite scaffold) and will be submitted by Fall 2018.
Chapter 2: Literature review

2.1 The heart

The heart is a muscular organ which plays a key role in the circulatory system by pumping blood through the body’s circulatory system vessels. Oxygen and nutrients are transported through the blood to the body, while carbon dioxide and wastes are removed [28]. Oxygenated blood leaves the heart to the rest of the body, while deoxygenated blood returns to the heart to be transferred to the lungs [28]. The blood vessels that supply the heart with oxygen and nutrients constitute the coronary circulation [29]. The heart is divided into four chambers composed of two upper atria and two lower ventricles, which are separated by a layer of dense connective tissue [29]. The heart wall is made up of three layers: the epicardium, myocardium, and endocardium (Figure 2.1) [28]. The epicardium is the outer most layer of the heart and forms the inner layer of the pericardium, the sac that surrounds the heart [28]. The myocardium is the middle layer which is composed of cardiac cells surrounded by collagen [28]. The endocardium is the inner most layer of the heart made up of a lining of epithelial cells and connects to the endothelium of the arteries and veins of the heart [28]. There are two types of cardiac cells: cardiomyocytes (CMs) and pacemaker cells. CMs are involuntary striated muscle cells which allow for the conduction of electrical impulses generated by pacemakers to control the beating of the heart [30].

Figure 2.1: Layers of the heart wall showing the inner endocardium, middle myocardium, and outer epicardium [31]
2.2 Cardiomyocytes

CMs are striated muscle cells, found in the atria and ventricles of the heart, that are responsible for the contraction of the heart [30]. Human adult CMs are cylindrical in shape and approximately 100 μm long and 10-25 μm in diameter [32,33]. CMs are composed of myofibrils that are in turn made of sarcomeres, the contractile unit of CMs [30]. They are involuntary muscle cells that mainly contain one nucleus and have a high mitochondria density, which is required for the constant generation of energy [30]. CMs are connected by intercalated disks to allow the diffusion of ions between cells, which is essential for cell-cell communication in order to ensure coordinated beating of the heart [30]. CMs undergo an action potential cycle characterized by a polarized rest phase where sodium, potassium, and calcium ions are separated [30]. This is followed by a spontaneous depolarization phase due to the slow influx of sodium ions into the cell, followed by further influx of calcium ions, and then a repolarization phase due to the outward movement of potassium ions [30]. During development, CMs increase in size as the heart grows, but very few cardiomyocytes are able to replicate, leading to a limited regenerative potential of the heart [30].

2.3 Cardiovascular disease

Cardiovascular diseases (CVD) encompass a range of diseases affecting the heart including coronary artery disease (CAD), arrhythmias, and congenital heart defects [29]. CVD is the leading cause of death in the world and accounts for approximately 30% of deaths annually [1–5]. Lifestyle choices, other diseases, and genetics can be contributing factors towards CVD. CAD is caused by atherosclerosis, which is a result of the build-up of fat along the inner wall of arteries [34]. Over time, the fatty plaque can rupture and form a blood clot to block the coronary arteries from supplying blood to the heart [34]. As a result, myocardial infarction (MI), CM death, and myocardium damage could occur [7]. Due to the limited regenerative potential of the heart, once the myocardium is damaged, it cannot be replaced. This can lead to heart failure where the heart is unable to pump enough blood to the body [7].
2.4 Approaches for treating heart failure

Heart failure can be treated with medications to help with the pumping of blood from the heart. Depending on symptoms, a variety of drugs could be taken, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and beta blockers [35]. These drugs aim to control the heart rate, blood vessel diameter, blood pressure, or blood flow to treat heart failure [35]. In some patients, ventricular assist devices can be used as an external pacemaker to supplement the heart’s pumping ability with ventricular assist devices, or completely replace the function of the heart. However, ventricular assist devices are costly and may not be suitable for all patient types [8–10]. Another alternative is to have a complete heart transplant; however, shortage of organ donors and risk of organ rejection make this not the most viable option [8–10]. As an alternative, cardiac tissue regeneration can be used to help repair and regenerate damaged tissue.

2.5 Approaches for cardiac tissue regeneration after myocardium damage

There are many approaches used to regenerate and repair heart tissue after myocardium damage: cell therapy, gene therapy, hydrogel and scaffold approaches, and generation of decellularized heart tissue [11,15,36]. In cell therapy, CMs can be injected into the target site without the use of a delivery vessel. Although this method is simple, allows for the use of autologous cells (if induced pluripotent stem cell derived CMs or adult stem cell derived CMs are used), and does not require genetic modifications, there are problems with low cell retention and survival at the target site [9,36,37]. Also, it is difficult to identify and characterize sub-populations of cells that are responsible for the therapeutic effect [9,36,37]. Another method employs gene therapy to deliver therapeutic genes through a vector to replace missing or defective genes. For example, Vascular Endothelial Growth Factor (VEGF) gene therapy could be used to deliver VEGF to the heart to encourage angiogenesis [36,38]. Gene therapy allows for the transduction of multiple cell types using adenoviruses; however, the gene transfer efficiency is often low and there may only be transient expression of the gene, which is not a viable long term solution [36,38]. In addition, decellularized heart tissue can be used to promote cardiac regeneration through the removal of the native cellular content and repopulation with target cell types. Decellularized tissue has the advantages of an intact cardiac extracellular matrix (ECM), which can allow for
cellular interaction with key proteins; however, the repopulation of the heart with cardiac cells is difficult due to poor cell engraftment [11,39]. Furthermore, there is a lack of human donors and animal hearts have xenogeneic concerns [11,39]. Another option for cardiac tissue engineering is to use hydrogels to encapsulate cells in a 3D matrix to allow for cell spreading and the formation of intercellular connections. These hydrogel systems can be easily miniaturized, naturally occurring hydrogels can help encourage cell spreading, and hydrogels can be gelled in situ [11,40]. However, the mechanical stability of hydrogel systems is usually not as good as scaffold-based techniques, which are becoming an increasingly popular method for cardiac tissue regeneration [11,40]. Using solid, porous matrices seeded with cells, cardiac tissue engineered scaffolds can be engineered to the desired 3D form and can be easily manipulated in vitro [11–15]. Also, they have a high surface area to volume ratio and high porosity [11–15]. The biodegradation rate, mechanical properties, and chemistry of the scaffolds can easily be manipulated as well [11–15]. However, cardiac tissue scaffold may have issues with cell compatibility and toxic degradation by-products, and the mechanical properties need to be properly tailored to match the native tissue [11–15].

2.6 Natural vs. synthetic tissue engineered cardiac patches

Currently, many groups have developed tissue engineered 3D cardiac patches made of both natural and synthetic biomaterials. Some common natural materials that have been used are alginate, collagen, and gelatin sponges, while some examples of synthetic materials are polylactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), poly(glycerol sebacate), polyanilines, and polyurethanes [11,13–15]. Furthermore, stem cells can be seeded onto the cardiac patch to help with tissue reorganization, regeneration, and repair. Ideally, the scaffold needs to be vascularized and integrated with the host tissue while being able to gradually degrade. Thus, it must be able to mimic the structure, mechanical properties, and biochemical function of the heart tissue’s ECM [15]. Natural scaffolds, derived from ECM proteins, provide a physiologically relevant surface for cells to attach, proliferate, and differentiate [16]. Natural scaffolds have the benefits of often showing good tissue compatibility and biodegradability; however, they typically have poor mechanical strength, batch-to-batch variation, immunogenicity concerns, and poor biochemical and biophysical customization properties [15]. Synthetic scaffolds have the advantage of customizable mechanical and
biochemical properties and thus can be readily tailored for use as a tissue engineering scaffold. However, synthetic scaffolds may have problems with cell and tissue compatibility, poor ability to promote vascularity vs. fibrosis, and biodegradability [15].

2.7 Synthetic polymeric scaffolds for cardiac tissue regeneration

One of the most commonly used groups of synthetic polymers for cardiac tissue engineering is polyesters. Polymers made of PLA, PGA, or a combination of these two polymers, PLGA, are common polyesters for cardiac tissue engineering due to their biodegradability and cell compatibility [41]. In one study, PLGA electrospun nanofibrous scaffolds were used to culture human induced pluripotent stem cell derived CMs (iPSC-CMs) [42]. The iPSC-CMs were able to align along the fibres and expressed functional cardiac markers α-actinin and cardiac troponin-T (cTnT), while having high expression of gap junction protein connexin-43 (Cx43) [42]. Compared to tissue culture polystyrene controls, iPSC-CMs on the PLGA nanofibres had more efficient calcium cycling [42]. PLGA can also be combined with other polymers to generate a composite scaffold with enhanced properties. Park et al. demonstrated that a composite PLGA/poly(lactide-co-caprolactone)/collagen scaffold led to increased adhesion of neonatal CMs and expression of cardiac markers compared to both collagen and PLGA sponge controls [43]. Although PLGA is a useful material for cardiac tissue regeneration, it degrades into acidic by-products, leading to a decrease in local pH, generation of a pro-inflammatory environment, and reduction in ECM production [44,45].

Another common polyester used for cardiac tissue engineering is poly(glycerol sebacate) (PGS), due to its biocompatibility and biodegradability. Ravichandran et al. showed that a PGS/fibrinogen core-shell nanofibrous scaffold could be used for culturing rat neonatal CMs [46]. The nanofibre core was made of PGS to provide mechanical support to the scaffold while the shell was made of fibrinogen for cell-biomaterial interactions [46]. The CMs expressed enhanced levels of α-actinin, cTnT, myosin heavy chain, and Cx43 as compared to pure fibrinogen nanofibres [46].

Polyurethanes (PU) are another group of degradable, synthetic polymers that are becoming increasingly popular for cardiac tissue engineering. PUs are durable and flexible elastomeric materials that have a segmented block co-polymeric chemistry, which provide the opportunity to
tailor the mechanical properties, biocompatibility, and degradation rates [17]. Furthermore, PUs have good mechanical properties and blood compatibility. The hard and soft segments give the PU a diverse chemistry that can possess both physical and chemical crosslinks [47]. For example, a poly(ester carbonate urethane) urea was co-electrospun with porcine cardiac ECM to form a bi-layered PU patch [48]. The patch was tested in a rat chronic infarction model and was found to alter the tissue remodelling response following myocardial infarction [48]. There was an increase in angiogenesis, while showing a decrease in left ventricle mechanical compliance, functional deterioration, scar formation, and left ventricle wall thinning [48]. The elastomeric PU component of the scaffold helped with providing adequate mechanical support to the ventricle, while the cardiac ECM component helped improve the remodeling response following MI [48].

In another study, polyester urethane urea (PEUU) was blended with gelatin to generate an electrospun nanofibrous scaffold [49]. CMs seeded on the composite PEUU/gelatin scaffolds had enhanced proliferation and integration compared to pure PEUU scaffolds [49]. These studies demonstrate that PUs can be a useful material for generating cardiac tissue engineered scaffolds and their biocompatibility can be further enhanced by the incorporation of ECM proteins.

Since the heart is required to propagate electrical impulses between cells, some groups have investigated the use of electrically conductive biomaterials for generating tissue engineered scaffolds. Polyaniline is a common conducting polymer that has been investigated for cardiac tissue engineering applications. Hsiao et al. showed that aligned polyaniline and PLGA nanofibres could be used to generate an electrically conductive scaffold that led to synchronized beating of CMs [50]. Isolated cell clusters could be seen on the nanofibres with good Cx43 junctions [50]. When an external electrical stimulation was applied to the CMs to mimic the conduction of the heart, the beating of the isolated cell clusters could be synchronized [50]. Similarly, polypyrrole (PPy) is another type of conductive polymer that can be used to mimic the cardiac ECM. A nanofibrous membrane of PPy, poly(e-caprolactone), and gelatin was used to mimic the cardiac ECM and led to CM attachment, proliferation, and expression of cardiac-specific proteins [51]. Another study with a PPy-alginate polymer system showed increased angiogenesis in the infarct area compared to saline-treated animals five weeks post-injection [52]. Although both polyaniline and PPy are useful conductive polymers for cardiac tissue
engineering, they may be limited by their cell compatibility and ultimate biodegradation when used in vivo.

2.8 Degradable polar hydrophobic ionic polyurethane (D-PHI) for CTE

D-PHI is a degradable, biocompatible PU developed in the Santerre Lab which has many properties that make it beneficial for use as a cardiac patch [18]. Currently, D-PHI films have been prepared as a mixture of three monomers: a lysine-based polycarbonate divinyl oligomer (DVO) cross-linker, methacrylic acid (MAA), and methyl methacrylate (MMA) [18]. DVO is synthesized by the reaction of a poly(hexamethylene carbonate) diol (PCN) soft segment and lysine diisocyanate (LDI), 2-hydroxyethyl methacrylate (HEMA) hard segment (Figure 2.2). D-PHI has been shown to have immunomodulatory properties and reduced pro-inflammatory macrophage activation [19]. Moreover, D-PHI has good compatibility with several cell types including smooth muscle cells, endothelial cells (ECs), monocytes, and gingival fibroblasts [18,20,21]. In vivo, D-PHI has shown good tissue integration and angiogenesis [20]. Thus, all these benefits provide the possibility of extending the application of D-PHI to CTE. A comparison of D-PHI to existing natural and synthetic materials is given in Table 2.1.

![Figure 2.2: Synthesis of DVO composed of PCN, LDI, and HEMA](image-url)
Table 2.1: D-PHI overcomes limitations of existing natural and synthetic materials

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Biocompatible</td>
<td>✓</td>
<td>?</td>
<td>✓</td>
</tr>
<tr>
<td>Biodegradable</td>
<td>✓</td>
<td>?</td>
<td>✓</td>
</tr>
<tr>
<td>Appropriate mechanical properties</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Material reproducibility</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Ability to tailor material properties</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

2.9 Polycarbonate polyurethane

Polycarbonate polyurethane (PCNU) is a degradable, linear polymer that has been previously used in the Santerre Lab for generating electrospun nanofibrous scaffolds for tissue regeneration of the spinal disk [53]. PCNU is composed of PCN, hexane diisocyanate, and a butane diol chain extender [53]. PCNU has been shown to be compatible with annulus fibrosus cells of the spinal disk and gingival fibroblasts [53,54].

2.10 Biodegradation of PUs

PUs can undergo surface or bulk erosion, or a combination of these two processes. During surface erosion, the exterior surface of the polymer is degraded and the inside portion does not degrade until all the surrounding outside material is degraded [55]. The erosion rate is proportional to the surface area of the material and the rate of degradation is constant [55]. On the other hand, during bulk erosion, degradation occurs throughout the whole material equally and the erosion rate depends on the volume of the material; the erosion rate decreases over time as the volume of the material decreases [55].

Polymeric cardiovascular devices are affected by hydrolysis, oxidation, environmental stress cracking, and calcification [56]. Each of these factors is dependent on the composition of the polymer. The rate of PU degradation is largely dependent on the chemistry of the soft segment [56]. PUs with polyester soft segments are more vulnerable to hydrolysis due to the ester linkages, and thus are not suitable for long term implantation [57,58]. Polyether PUs are less sensitive to hydrolysis, but are more susceptible to oxidative degradation [56]. Polycarbonate polyurethanes are more resistant to oxidative degradation when compared to both polyester PUs
and polyether PUs and can be designed to be more susceptible to hydrolytic degradation, depending on the chemistry of the hard segment [56]. In vivo, monocytes, macrophages, and foreign body giant cells release reactive oxygen species which initiate PU degradation leading to chain scission and cross-linking of the soft segment [59]. PUs with a higher hard segment content can lead to a higher resistance to hydrolytic degradation, due to hydrogen bonding within the hard segment, which can mask cleavage sites [20,60]. Overall, it is important to match the degradation rate of the PU scaffold to the rate of tissue regeneration [20].

2.10.1 Degradation of D-PHI

D-PHI is degraded through hydrolysis and dissolution of its components. The DVO backbone of D-PHI is hydrolysable, while the carbon chain backbone can be eliminated down through the dissolution of the oligomeric anionic chains that link together the polyurethane components prior to their degradation. Overall, D-PHI degrades into non-toxic by-products of lysine, carbon dioxide, alcohols, and water soluble oligomeric methyl methacrylate and methacrylic acid molecules, which can be metabolized by the body [18,20].

In vitro, D-PHI porous scaffolds degraded relatively slowly with a 13 % mass loss in PBS after 120 days at 37 °C [20]. Using cholesterol esterase, a monocyte derived enzyme, there was a 4 % mass loss after 120 days at 37 °C [20]. In vivo, D-PHI degraded at a more controlled rate with a 7 % mass loss after 7 days post-implant, followed by a linear profile of degradation to lead to a 21 % mass loss by 100 days [20]. The scaffolds degraded more quickly in vivo compared to in vitro due to the presence of both oxidative and hydrolytic degradation occurring and the presence of monocytes and macrophages [20]. There was increased cellular infiltration into the pores of the D-PHI scaffolds over time and increased collagen production within the pores [20]. Throughout the 100 day in vivo experiment, the scaffold size and shape was well maintained, indicating that weight loss and pore expansion was occurring, but there was no collapse in the structure of the scaffold as the formation of new tissue helped to maintain the original structure [20]. This implied that the degradation process could be mainly attributed to a surface degradation phenomenon.
2.10.2 Degradation of PCNU

PCNU is broken down by hydrolytic degradation into carbon dioxide, water, and small molecules [61]. In vitro, electrospun, aligned nanofibrous PCNU scaffolds in cholesterol esterase had a 0.56 mg/week mass loss, leading to a 2 mg total mass loss after four weeks [61]. The mass loss was relatively linear over the four-week period [61]. There was no loss in elastic modulus, despite the mass loss, throughout the study, leading the authors to conclude that the degradation seemed to be mainly surface mediated [61].

2.11 Interaction of macrophages with D-PHI

Macrophages are a type of white blood cell produced by the differentiation of monocytes in the tissue. Two types of macrophages are important in the immune response to foreign substances. M1 macrophages increase the inflammation at the injury site and stimulate the immune system, while playing an important role in the phagocytosis of foreign substances [62]. On the other hand, M2 macrophages play an anti-inflammatory role and can decrease immune reactions through the release of cytokines [62].

D-PHI was designed to possess an immunomodulatory character by reducing the macrophage pro-inflammatory activation [19,62]. In previous studies, the interaction of immunoglobulin G (IgG), a pro-inflammatory protein that supports monocyte-biomaterial interactions, with D-PHI films was investigated [19]. On IgG coated D-PHI surfaces, there was reduced monocyte adhesion and spreading when compared to on IgG-coated tissue culture polystyrene (TCPS) [19]. D-PHI films led to a reduction in the number of exposed IgG-Fab sites, which dominate monocyte-biomaterial interactions, following pre-adsorption compared to IgG adsorbed to TCPS [19]. The monocytes on the IgG-coated TCPS were activated into a pro-inflammatory state with increased spreading and enlargement, which was not seen on D-PHI surfaces [19]. This difference in monocyte response on D-PHI compared to TCPS could be due to differences in protein conformation on the surfaces that subsequently lead to exposure of different ligands for protein-cell interactions [19].
2.12 Using electrospinning for cardiac tissue engineering

Electrospinning is a technique that uses a high voltage difference to generate an electrically charged polymer solution that is ejected from a nozzle onto a grounded mandrel or collector plate to form a nanofibrous scaffold [22]. Electrospinning can be used to produce natural or synthetic nanofibrous, porous scaffolds that mimic the native ECM [23]. The scaffold resembles the scale and fibrous nature of the ECM with surface features at a nanometer size scale [23]. Also, a high surface area to volume ratio and high porosity help promote tissue biosynthesis and guide cell growth, thus enhancing tissue regeneration [23].

Reactive electrospinning is used to provide in situ cross-linking of polymers while electrospinning [63]. After cross-linking a polymer, the polymer is no longer soluble in solution, and thus, cross-linking needs to be performed during the electrospinning process [63]. Often, UV light, heat, or chemical reactions are used to facilitate in situ cross-linking (refer to Table 2.2 for comparison of methods) [64]. In UV curing, the UV lamp is either located under the collecting mandrel, to cure nanofibres after contacting the mandrel, or positioned to provide in-flight curing while the charged polymer solution travels from the nozzle to the mandrel [63,65–69]. Polymers can be lightly cross-linked before electrospinning, as shown with a HEMA-co-MAA polymer and 2,2’-azobis(isobutyronitrile) thermal initiator that was used to initiate polymerization of monomers into oligomers and slightly cross-linked chains [63]. Following thermal polymerization, the solution was quenched and electrospun with in-flight UV curing [63]. The photo-cross-linkable reaction is extremely fast and produces high cross-linking efficiency to minimize the amount of toxic chemical residues [67]. Other reactive electrospinning setups have employed the use of a long heated capillary belt for cross-linking poly(vinyl alcohol) nanofibres and a dual syringe system to combine a hyaluronic acid derivative with a poly(ethylene glycol) diacrylate cross-linker immediately before electrospinning [70,71].
Table 2.2: Advantages and disadvantages of different reactive electrospinning methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV light cure</td>
<td>UV light reacts with photoinitiator to cross-link polymer in-flight or immediately after electrospinning</td>
<td>Simple, can be used for a wide range of polymers with addition of photoinitiator to system</td>
<td>Requires external UV lamp, cross-linking needs to be fast enough for in-flight curing</td>
<td>[63, 65–69, 72, 73]</td>
</tr>
<tr>
<td>Heat cure</td>
<td>Heat used to generate cross-links</td>
<td>Can be used to pre-cure monomers into oligomers and slightly cross-linked polymer chains, can have a heated syringe tip</td>
<td>Need to control viscosity of polymer solution to ensure that polymer can still be electrospun after curing</td>
<td>[63, 70]</td>
</tr>
<tr>
<td>Chemical reaction</td>
<td>Dual syringe system where monomer and cross-linker contained in two separate syringes and combined immediately before electrospinning, chemical reaction forms cross-links</td>
<td>Does not require external UV lamp or heating system</td>
<td>Requires specific chemistries of monomers, only applicable to specific polymer systems</td>
<td>[64, 71]</td>
</tr>
</tbody>
</table>

2.12.1 Effect of electrospinning parameters

The electrospinning process is dependent on several parameters: solution composition, electrospinning settings, and environmental conditions.

It is important for the solvent to have sufficient conductivity, in order to be able to form an electrically conductive solution [74]. Additionally, organic or inorganic salts can be added to the solution to increase conductivity, but this could lead to impurities in the resultant product [74]. If the solution conductivity is too high, it could lead to reduced fibre diameter and bead formation during the electrospinning process [74]. Another crucial solution parameter for electrospinning is the solution viscosity [74]. The solution viscosity needs to be sufficient to prevent the polymer jet from breaking into droplets as it is drawn out from the needle to the collecting surface.
A higher molecular weight polymer and a higher solution concentration will lead to a higher viscosity, smoother polymer jet, and larger fibre diameter [22,74]. However, if the viscosity is too high, the electrical charges may not be strong enough to stretch the solution to form fibres [22,74]. Moreover, the solvent has to possess high volatility in order to evaporate in-flight during the electrospinning process before the fibres contact the collecting surface [74]. If the solvent volatility is too low, wet or fused fibres, or no fibres, may be formed. If the solvent volatility is too high, there could be intermittent spinning, leading to solidification of the polymer at the needle tip [74].

The electrospinning setup, such as the voltage, flow rate, and tip-to-collector distance, also affect the formation of fibres. The applied voltage needs to be sufficient to draw out the polymer solution and overcome the surface tension of the solution in order to form a steady, charged polymer stream [22,75]. In addition, the flow rate of the solution needs to be compatible with the solution viscosity to allow for the formation of fibres [22]. A lower flow rate allows for more time for solution polymerization and tends to generate smaller diameter fibres with less bead defects [22]. On the other hand, a higher flow rate can lead to beaded fibres with thicker diameters and the nanofibres may be unable to completely dry during the electrospinning process [22]. In addition, the tip-to collector distance can be varied to change the amount of time the polymer spends in-flight as it travels between the needle and collecting surface [22]. In terms of fibre alignment, increasing the speed of the rotating collecting mandrel can help to increase fibre alignment [22].

Moreover, environmental temperature and humidity conditions play an important role in the ability to form fibres [22]. Depending on the solution composition, high humidity and temperature can lead to the inability to form consistent fibres [22].

### 2.12.2 Effect of adding ECM proteins to electrospun scaffolds

The myocardium ECM consists of a matrix of collagen fibres, elastin, fibronectin, laminin, and glycosaminoglycans [51]. To mimic this complex ECM, proteins can be added to the electrospinning solution to help enhance the mechanical properties of the electrospun scaffold, while also promoting cell adhesion, compatibility, and differentiation [16]. Protein molecules can be incorporated as covalently conjugated molecules in the polymer solution or as a protein
coating after electrospinning [76]. One protein that is commonly incorporated into electrospun scaffolds is gelatin. Gelatin is the thermally denatured form of collagen and is affordable, biocompatible, and biodegradable [16,77]. The addition of gelatin to an electrospun fibrinogen nanofibrous scaffold helped increase the tensile strength of the scaffold to 0.0125 – 0.46 MPa, which is representative of native heart myocardium [16]. The fibrinogen-gelatin scaffolds led to higher human CM proliferation when compared to a cross-linked fibrinogen scaffold (33 % increase in cell proliferation) [16]. In another study, electrospun chitosan nanofibrous scaffolds were coated with fibronectin to help with cell adhesion and migration into the fibres [78]. Previously, investigators in the Santerre Lab have shown that a polycarbonate polyurethane (PCNU) coated with fibronectin led to the greatest annulus fibrosus cell attachment compared to collagen and vitronectin surfaces [79].

2.13 Different sources of human CMs for cardiac tissue engineering

There are many different sources of human CMs that can be used for a range of applications in tissue engineering, each with their benefits and limitations. There are three main sources for human CMs: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells [80].

Human ESCs represent an easily accessible cell source; however, there are ethical concerns with obtaining ESCs. Many different ESC cell lines have been successfully differentiated to CMs in suspension culture using either small molecules (CHIR, inhibitor of Wnt Production-4), to modulate the Wnt pathway, or growth factors (Activin A, bone morphogenetic protein 4) [81,82]. In this way, CM purities of greater than 90 % are possible as measured by the percentage of cTnT positive cells [81]. Transplantation of ESC-CMs into small rodent hearts resulted in partial remuscularization of the infarct scar and improved left ventricle contractile function [82]. In addition, grafts were capable of electromechanical integration [82]. However, there were problems with graft related arrhythmias in larger animal primate models [82]. Also, ESC-CMs are faced with problems with cell immaturity and a mixed population of cardiac cells (ventricular, atrial, nodal/pacemaker) [82].
Human iPSCs can be used to generate functional CMs by reprogramming somatic cells [83–87]. Since an autologous cell source can be used to generate iPSCs, there will be reduced issues of immunocompatibility compared to allogenic cell sources [88,89]. However, there are concerns with teratoma formation and the presence of undifferentiated cells [88,89]. iPSCs can be similarly differentiated into CMs through the use of growth factors (basic fibroblast growth factor (bFGF), transforming growth factor beta (TGFβ), activin A, bone morphogenetic protein (BMP)) or small molecules in suspension culture to obtain 70–90 % cTnT positive cells [90]. When these iPSC-CMs were transplanted into a primate subacute MI model, the CMs helped to partially remuscularize the infarct scar and improved contractile function [91]. Grafts survived for up to 3 months, occupied approximately 16 % of the infarct scar, and became electrically coupled with the host myocardium [91]. In these studies, there were also problems with graft-related arrhythmias which peaked 14 days post-transplantation [91].

Both ESC-CMs and iPSC-CMs are limited by the fact that they exhibit a functionally immature, disorganized, fetal-like phenotype that is not representative of adult CMs [92]. Thus, a challenge of tissue engineering strategies is to be able to recapitulate the native cardiac environment to promote CM maturity.

Adult stem cells that are mainly used to differentiate into CMs are mesenchymal stem cells and adipose derived stem cells [82]. In one study, stem cells were co-cultured with CMs and treated with growth factors or 5-azacytidine to induce cardiac differentiation of stem cells [82]. However, the results were questionable since it seemed like the stem cells were fusing with pre-existing cells, rather than differentiating into CMs [82]. There was little direct remuscularization of the infarct scar after transplanting cells in vivo and the main benefits of the stem cell transplantation were through paracrine signalling and secretion of pro-angiogenic factors [82].

2.14 CM attachment to various surfaces

The cardiac ECM provides CMs with structural support, controls cellular processes, and is constantly remodelled in response to stimuli. CM integrins (mainly α1β1, α5β1, α7β1, α6, α9, α10, β1 subunits) interact with the ECM to form connections required for the mechanotransduction between the ECM and cells [93].
In order to enhance CM attachment to surfaces, an ECM protein is often used. Commonly used coatings include collagen, laminin, fibronectin, poly-d-lysine, agarose, synthetic peptides, and synthetic gels [94]. Matrigel is a very common surface coating used for CM attachment made of a gelatinous protein mixture secreted from mouse sarcoma cells [95,96]. It resembles the extracellular environment \textit{in vivo} and is mainly composed of laminin, collagen IV, entactin, and various growth factors [97]. However, since Matrigel is derived from animal origins, there are issues with cross-species reactivity when used for human applications, especially when the exact composition of the material is unknown. Another commonly used surface protein coating is gelatin, which is the hydrolyzed version of collagen [90,98]. Gelatin is a relatively inexpensive protein and can be easily processed for surface coating applications. In addition, fibronectin can be used to enhance CM attachment, but is relatively more expensive compared to gelatin [95,99].

2.15 CM maturity

The morphology and electrophysiological function of fetal and neonatal CMs is quite different from that of mature, adult CMs [92]. Fetal CMs have a rounded morphology and are usually single nucleated. In contrast, mature adult CMs are elongated, rod-shaped, and approximately 30% of CMs are bi-nucleated [92]. In addition, fetal CMs have a much smaller surface area (1000-1300 $\mu$m$^2$) compared to the much larger surface area of adult CMs (10,000-14,000 $\mu$m$^2$) [92,100,101]. Fetal CMs are naturally randomly oriented, neonatal CMs are more aligned, and adult CMs are anisotropic [102,103]. Furthermore, adult CMs have prominent sarcomere striations, which are usually not visible in fetal CMs [102,103]. Most of the contractile proteins found in mature CMs can be found in fetal CMs, but at lower expression levels or different isoforms [104]. For example, fetal CMs express the atrial and ventricular form of myosin light chain (MLC), while adult ventricular CMs only express the ventricular form of MLC [104]. In terms of the proliferative capacity, early fetal CMs are highly proliferative, but this proliferation rate starts to decrease in the late fetal CM stage [105]. Neonatal CMs have the ability to proliferate for 7 days, while adult CMs do not proliferate [103,105].

Human pluripotent stem cell derived CMs (PSC-CMs) exhibit a functionally immature, disorganized, fetal-like phenotype [92]. PSC-CMs have a more rounded morphology, are usually single nucleated, have a lower surface area, are randomly oriented, and have indistinguishable
sarcomere striations [92]. PSC-CMs express low levels of troponin and α-actin isoforms compared to adult CMs [42,100,104]. As a result of the differences in contraction protein isoforms expressed by PSC-CMs, they are unable to contract efficiently [42,100,104]. In 3D culture, the active contraction force of PSC-CMs was 0.1-0.5 mN/mm² and the adult CM contraction was two orders of magnitude higher [106–109]. Moreover, PSC-CMs have a limited proliferative capacity [103,105].

2.15.1 CM electrophysiology and communication

PSC-CMs contract asynchronously, like fetal CMs, due to immature electrical coupling from underdeveloped cell-cell connections [110]. Contrastingly, adult CMs are only excited when they are provided with an electrical stimulus and contract synchronously [110]. CMs are connected by Cx43 gap junctions, N-cadherin adherens junctions, and sodium channels [110]. These cell-cell connections are found within fetal, neonatal, adult, and PSC-CMs at similar amounts, but the spatiotemporal distribution changes during development [110]. Adult CMs have these proteins densely distributed around mature intercalated discs while early fetal CM and PSC-CM proteins are randomly distributed along the cell membrane, due to the indistinguishable intercalated discs [110]. As a result, fetal and PSC-CMs have a 6 to 50 fold lower depolarization velocity compared to adult CMs [101,103,111–113].

2.15.2 Methods to improve CM maturity

In order to use CMs in vivo for therapeutic benefits, they first need to be matured in vitro. CM maturity can be improved using electrical and mechanical stimulation, ECM interactions, and non-CM interactions [92].

2.15.2.1 Effect of mechanical and electrical stimuli on CMs

In the heart, CMs constantly experience mechanical stimuli due to the beating of the heart and blood flow, thus it is important to recapitulate these mechanical stimuli in vitro to induce CM maturity. Many groups have used bioreactor systems to recapitulate the physiological mechanical stimuli in the heart and have shown improvements in CM maturity [92,109,114,115]. In one study, a Flexcell apparatus for engineered heart tissue was made of PSC-CMs and subjected to physiological 5% elongation and 1 Hz strain [109]. After 4 days, there was an upregulation of
cardiac genes and after 5 days, the CMs increased in size and alignment and had synchronized beating [109]. After 7 days there was better sarcomeric organization and the formation of Z disks [109].

Exogenous electrical stimuli can be used to mimic in vivo electrical impulses by directing electrical currents through CMs to improve CM electrophysiological maturity [116,117]. For example, the Biowire uses electrical stimulation of 0 to 3 Hz for 7 days to functionally mature PSC-CMs seeded on collagen/Matrigel scaffolds [116]. The PSC-CMs grown on these constructs had a higher surface area, sarcomere organization, intercalated discs, mitochondria, conduction velocity, and synchronous beating [116]. A combination of both mechanical and electrical stimuli can further enhance CM function and maturity [118,119].

2.15.2.2 Effect of scaffold stiffness on CMs

The stiffness of the ECM influences CM contractility, calcium handling, and cytoskeletal structure. The human myocardium has an elastic modulus of approximately 0.02 to 0.5 MPa [24]. Thus, in order to improve CM maturity, many groups have attempted to generate scaffolds with stiffness similar to that of the native myocardium.

In one study, neonatal rat ventricular CMs were seeded onto polyacrylamide gels with varying stiffness [120]. CMs grown on gels with a stiffness of 10 kPa, similar to the stiffness of the native myocardium, had aligned sarcomeres, while CMs on stiffer gels (50 kPa) had unaligned sarcomeres and stress fibres [120]. In addition, the CMs on the 10 kPa gels generated higher mechanical forces compared to stiffer (50 kPa) or softer (1 kPa) gels [120]. Thus, this suggests the importance of substrate stiffness on enhancing CM maturity.

In another study, Marsano et al. showed that by varying the stiffness of a porous PGS scaffold through changing the cross-linking density, they could alter the contractile function of engineered cardiac constructs [121]. This study found that scaffolds with a lower stiffness (compressive modulus of 2.35 kPa) had a greater contraction amplitude when they were stimulated by an external electrical signal compared to stiffer scaffolds (compressive modulus of 5.99 kPa) [121].
2.15.2.3 Effect of nanotopography on CMs

The cardiac ECM is composed of proteins that form a fibrous, anisotropic network to generate aligned CMs [92]. Muscle fibres are aligned within each layer of the myocardium to produce an anisotropic environment and the layers vary from approximately -70 ° in the epicardium to +80 ° in the endocardium [122]. This anisotropic organization helps with CM contractions and electromechanical coupling as the action potential is directed to propagate in a specific direction, thus helping with CM maturity [92]. In order to recapitulate the nanotopography of the myocardium, many groups have attempted to create surfaces with similar nanopatterning to the native myocardium. One group studied the effect of different sized nanogroove widths (350-2000 nm) on the structural development of iPSC-CMs in vitro and found that iPSC-CMs had the highest amount of alignment, longest sarcomere length, and highest surface area when cultured on 800 nm nanogrooves [95]. Another group assessed the effect of 450 nm grooves with a 100 or 350 nm depth on CM organization and found that the nanotopography affected the organization of the actin cytoskeleton and focal adhesion complexes of cells, thus affecting the morphology, orientation, and contractile function of CMs [123]. The cells became elongated and aligned in the direction of the grooves and the alignment of CMs increased with groove depth [123].

2.15.2.4 Effect of co-culture of CMs with fibroblasts

In the human heart, CMs occupy approximately 75% of normal myocardial tissue volume; however, only 30 to 40% of cells are CMs, while other cells are from the fibroblast, endothelial, or smooth muscle cell lineages [124,125]. Non-CM cells have been shown to support the growth, proliferation, survival, differentiation, and maturation of CMs [26,124,126,127]. In the human heart, fibroblasts outnumber CMs by 3 to 5 times and are the largest cell population by number [128]. A dense network of cardiac fibroblasts surrounds CM clusters of two to four cells in the ventricular myocardium and act as bridges between myocardial tissue layers [125]. Cardiac fibroblasts are important in maintaining normal cardiac function and cardiac remodelling during MI and hypertension [125]. Fibroblasts are responsible for synthesizing and depositing ECM and cell-to-cell communication with CMs, monocytes, ECs, and other fibroblasts [125]. The interaction of fibroblasts with CMs affects cardiac development, myocardial structure, cell signalling, electrophysiological properties of CMs, and secretion of growth factors in the healthy and diseased heart [125]. For example, when cardiac fibroblasts were co-cultured with CMs on
chitosan nanofibres, the CMs had a more polarized morphology during long term culture compared to a CM monoculture [78]. Lancaster et al. showed that transplanting CMs on a 3D fibroblast construct led to improved left ventricle function after heart failure in a rat model [129]. Another group showed that the co-culture of PSC-CMs with cardiac fibroblasts in an electrically stimulated collagen/Matrigel construct increased PSC-CM maturity with an optimal cardiac fibroblast to CM ratio of 3:1 [119].

Specifically after MI, an inflammatory response occurs, followed by a wound healing process where fibroblasts are recruited for scar tissue formation, and thus interact with resident CMs [27,130]. In the diseased heart, the CM to fibroblast ratio changes, which affects the interaction between CMs and fibroblasts, thus contributing to fibrosis and arrhythmogenic activity [131]. During cardiac fibrosis, there is an increased level of ECM deposition and fibroblast accumulation, which leads to an increase in the ECM to CM and fibroblast to CM ratios [132]. In the myocardial remodelling process following MI, fibroblasts play a crucial role by sensing mechanical stresses and inducing growth responses through the release of growth factors. Thus, in order to generate a functional engineered cardiac patch, a co-culture of CMs and fibroblasts should be considered.

2.16 Functional cell markers for CMs and fibroblasts

2.16.1 Functional markers for CMs

There are many functional markers that can be used to identify CMs from other cell types.

One of the most common functional markers for CMs is cardiac troponin-T (cTnT). Troponin is a complex of three regulatory proteins: cTnC, cTnT, and cTnI and is an important component in thin filaments [133]. Troponin is vital for the contraction of cardiac and skeletal muscle, but is not found in smooth muscle [133]. Troponin is attached to the tropomyosin protein, which lies in the groove between actin filaments [133]. When the muscle is relaxed, tropomyosin blocks the attachment of myosin cross-bridges to prevent muscle contraction [133]. During muscle contraction, calcium channels open and release calcium which attaches to the cTnC protein of troponin and causes a conformation change to expose binding sites for myosin on actin filaments [133]. cTnT and cTnI can be found in different forms in cardiac and skeletal muscle [133]. cTnT
is responsible for anchoring the troponin complex onto tropomyosin and has several isoforms [133]. cTnI binds to actin to prevent muscle contraction during the relaxed state and only has one specific isoform expressed in the myocardium [133].

Cardiac myosin light chain (MLC) is part of another useful family of proteins that can be used as markers for CMs. There is a ventricular isoform (myosin light chain-2) that is distinct from that expressed in skeletal muscle, smooth muscle, and cardiac atrial muscle [134]. The MLC protein is non-covalently bound to the myosin head and interacts with the tail region of myosin to regulate myosin motility and function [134]. MLC is an important marker in early embryonic cardiac development and function and is one of the earliest markers of ventricular specification [134].

Other proteins that can be used as markers for CMs are myosin heavy chain, a motor protein of the muscle thick filaments important in muscle contraction, and α-actinin, an actin-binding protein that helps anchor myofibrillar actin filaments to the Z-lines of CMs [135,136].

In addition, CMs need to have good cell-to-cell connections for the propagation of a synchronized electrical pulse throughout all the cells and resultant contraction. The communication between CMs is achieved through gap junctions which facilitate the exchange of low molecular weight molecules, such as small ions, to regulate cell death, proliferation, and differentiation [137]. Connexin-43 (Cx43) is a major protein in heart gap junctions and can be used as a marker for assessing the extent of cell-to-cell connection and communication between CMs [137].

2.16.2 Functional markers for fibroblasts

In order to differentiate fibroblasts from CMs, either vimentin (vim) or fibroblast surface protein markers are often used. Vim is an intermediate filament protein expressed in mesenchymal cells. It is the major cytoskeletal component of mesenchymal cells and is often used as a marker of mesenchymal-derived cells or cells undergoing the epithelial-to-mesenchymal transition [138]. Vim is responsible for supporting and anchoring organelles in the cytosol [138]. Fibroblast surface protein is another marker for fibroblasts and is a glycoprotein produced by connective tissue cells [139].
Chapter 3: Synthesis and characterization of an electrospun nanofibrous, degradable polyurethane composite scaffold for tissue engineering applications

3.1 Foreword

Tissue engineering can be used to generate functional scaffolds that mimic the extracellular matrix of native tissue in order to promote the repair and regeneration of damaged tissue. Engineered tissue scaffolds need to be compatible with local cells and tissues, be biodegradable, be functional with respect to providing cell support, readily accommodate various cell types, and have mechanical properties that enable the simulation of the native tissue. In this study, electrospun degradable polar hydrophobic ionic polyurethane (D-PHI) scaffolds were generated in order to mimic the extracellular matrix structure of tissues for intended tissue engineering applications. Oligomeric divinyl oligomers used in D-PHI synthesis were made, integrated with a degradable linear polycarbonate polyurethane (PCNU), and electrospun with in situ UV cross-linking in order to generate aligned nanofibrous scaffolds. The D-PHI/PCNU scaffold fibre morphology, cross-linking efficiency, surface energy, mechanical properties, in vivo degradation and integration, and in vitro cell compatibility were characterized. The results showed that D-PHI/PCNU scaffolds had a high cross-linking efficiency. The composite scaffold also had a high surface energy and lower stiffness relative to PCNU scaffolds. In vivo, the D-PHI/PCNU scaffold degraded relatively slowly, but was well integrated with the surrounding tissue as shown by greater cellular and protein infiltration and blood vessel formation around the scaffold over time. Based on a preliminary study with A10 vascular smooth muscle cells, the D-PHI/PCNU scaffold was able to support high cell viability, adhesion, and expression of α-smooth muscle actin contractile marker after a 7-day culture period, which was comparable to PCNU scaffolds. These characterization results demonstrate that the unique properties of a D-PHI/PCNU scaffold, combined with the benefits of electrospinning, could allow for the generation of a tissue engineered scaffold that mimics the native extracellular matrix and could be used for functional tissue regeneration.
3.2 Introduction

Tissue engineered scaffolds can be used to repair and regenerate damaged tissue after disease [140]. Additionally, appropriate cell types can be seeded onto the engineered scaffold to aid with tissue reorganization, regeneration, and repair [141]. In order for a tissue engineered scaffold to be effective, it must be biocompatible, meaning that it does not stimulate a foreign body response and supports cell survival [141]. The scaffold must support the adhesion and proliferation of various cell types and enable the cells to express cell markers required for generating functional tissue [141]. Ideally, the scaffold needs to be vascularized and integrated with the host tissue, while being able to gradually biodegrade as new tissues are formed and remodeled over time. In this manner the native tissue can become integrated with the scaffold and gradually replace the area originally occupied by the scaffold [142,143]. Moreover, the mechanical properties of the scaffold must be compliant with the target tissue [143]. Lastly, the scaffold should be reproducible, affordable, and scalable to enable its use in high demand and large tissue (centimeter scale or greater) applications. Thus, all these tissue engineering construct design parameters need to be properly characterized and considered before the scaffold can be used in the intended application.

Tissue engineered scaffolds can be made of both natural and synthetic materials that can be tailored to have physical, topographical, and biochemical properties that mimic the native tissue. Natural scaffolds, derived from extracellular matrix (ECM) proteins, provide a physiologically relevant surface for cells to attach, proliferate, and differentiate [16]. The latter scaffolds have the benefits of often showing good tissue compatibility and biodegradability; however, they typically have poor mechanical strength, batch-to-batch variation, immunogenicity concerns, and the inability to tailor biochemical and biophysical properties [15]. Synthetic scaffolds have the advantage of customizable mechanical and biochemical properties and thus can be tailored for use as a tissue engineering scaffold. However, without appropriate consideration of the innate immune response, synthetic scaffolds may have problems with cell and tissue compatibility, poor ability to promote vascularity vs fibrosis, and biodegradability [15].

Polyurethanes (PU) are popular synthetic co-polymeric materials for tissue engineering due to their segmented block co-polymeric chemistry which provides the opportunity to tailor the
mechanical properties, general biocompatibility, and degradation rates [17]. A degradable polar hydrophobic ionic polyurethane (D-PHI) was previously developed for vascular tissue engineering applications and was shown to have excellent immunomodulatory character [18]. Currently, D-PHI flat films have been prepared as a mixture of three monomers: a lysine-based polycarbonate divinyl oligomer (DVO) cross-linker, methacrylic acid (MAA), and methyl methacrylate (MMA), but these formulations were not amenable to forming fibres because they were heat cured and had viscosities that were not conducive to generating well formed fibres [18]. D-PHI is susceptible to hydrolytic degradation, which makes it suitable for generating a degradable tissue engineering scaffold [20][61]. In addition, the elastic mechanical properties of D-PHI can be tailored for different tissue engineering applications [18]. Furthermore, D-PHI’s immunomodulatory properties favor a reduced pro-inflammatory macrophage activation [19]. Moreover, D-PHI has good compatibility with many types of cell including smooth muscle cells (SMCs), endothelial cells, monocytes, and gingival fibroblasts [18,20,21]. In vivo, D-PHI has shown good tissue integration and angiogenesis [20].

As indicated above, nanofiber topography is an important attribute of a tissue’s ECM and in recent years, electrospinning has been extensively applied to mimic extracellular matrices [22,23]. Electrospinning is a technique that uses a high voltage difference to generate an electrically charged polymer solution that is ejected from a nozzle onto a grounded mandrel or collector plate to form a nanofibrous scaffold [22]. Electrospinning can be used to produce natural or synthetic nanofibrous scaffolds that can be used for a variety of applications, including tissue engineering [23]. Using electrospinning, aligned fibres can be generated on the nanometer size scale to mimic the native ECM [23]. Also, a high surface area to volume ratio and high porosity help promote tissue biosynthesis and guide cell growth, thus enhancing tissue regeneration [23].

In this study, elastomeric polyurethane composites of D-PHI with a high molecular weight degradable linear polycarbonate polyurethane (PCNU) were electrospun in order to generate an aligned nanofibrous scaffold that combines the unique properties of D-PHI with the benefits of electrospinning, in order to investigate whether an electrospun D-PHI-based scaffold could be used for tissue engineering applications. D-PHI was synthesized and integrated with PCNU in order to accommodate viscosity requirements in the electrospinning mixture, and then
electrospun with an in-situ UV cross-linking reaction. Subsequently, the electrospun scaffolds were characterized to assess the cell and tissue compatibility along with biodegradation behaviour and tissue integration. As well, the mechanical properties were assessed for eventual tissue engineering applications. Aligned nanofibres were generated and both the fibre diameter and alignment were measured. In addition, the cross-linking efficiency and surface energy of the scaffold were investigated. A subcutaneous implant of the material was performed on rats to determine \textit{in vivo} integration, vascularity, and degradation of the scaffold, along with the resulting inflammatory response. To determine the cell compatibility of D-PHI/PCNU scaffolds, A10 vascular smooth muscle cells (SMCs) were seeded onto the scaffold and their viability, adhesion, and expression of functional markers was assessed. This work should open up new opportunities to establish biocompatible and biodegradable tissue engineered scaffolds which could have unique sets of topographical, biochemical, and physical cues to address a diverse range of applications.

3.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

3.3.1 Electrospun D-PHI/PCNU scaffold fabrication

D-PHI solutions were generated using a previously established protocol [18]. Briefly, D-PHI solutions were made with a divinyl oligomer (synthesized with lysine diisocyanate (LDI), poly(hexamethylene carbonate) diol (PCN), and 2-hydroxyethyl methacrylate (HEMA)), methacrylic acid (MAA), and methyl methacrylate (MMA) in a 1:5:15 molar ratio [18]. A 3 wt. % Irgacure 1173 photoinitiator (IGM Resins), relative to the resin, was added to the D-PHI polymer solution to initiate the polymerization reaction. The D-PHI solution was protected from light and allowed to mix for a minimum of 12 h prior to use. PCNU solutions were generated using a previously established protocol [53]. Briefly, PCNU was synthesized by the reaction of 1,6-hexane diisocyanate (HDI), PCN, and 1,4-butanediol (BD) in a 3:2:1 molar ratio to have a polystyrene equivalent weight average molecular weight of approximately 140,000 as measured using gel permeation chromatography [53]. PCNU was dissolved in a 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solvent. After achieving a homogeneous D-PHI solution, the D-PHI solution was incorporated into the PCNU solution at a 1:1 ratio to increase solution viscosity and assist
with electrospinning [16]. The HFIP solvent evaporates during the electrospinning process and any residual solvent is removed by vacuum drying and conditioning scaffolds with culture medium prior to cell seeding.

The D-PHI/PCNU polymer solution was fed by a syringe pump into an 18” stainless steel needle for electrospinning. The monomer/PCNU mixture was UV cross-linked in flight (using a Phoseon FE300 365nm UV lamp with an output window of 110 mm x 10 mm and peak irradiance of 3.0 W/cm²) while the mixture traveled from the syringe to the rotating collecting mandrel, generating aligned nanofibers [67,68]. The lamp was placed 15 cm away from the collecting mandrel and the concentration of PCNU in HFIP ranged from 15-16 % w/v in order to obtain a similar solution viscosity between all samples. The viscosity was standardized to the time required for 0.1 mL of polymer solution to flow through a 1 mL syringe with a 22-gauge needle under normal gravitational force (e.g. 140 ± 10 s). The flow rate of the solution was 0.5 mL/h, the tip-to-collector distance was 18 cm, the needle had a positive charge of 18.5 kV, and the collecting mandrel (rotating at 9.25 m/s) had a negative charge of -0.5 kV. Refer to Figure 3.1 for the electrospinning setup.

**Figure 3.1:** (A) Electrospinning setup for electrospinning D-PHI/PCNU scaffolds (B) Schematic for electrospinning setup
3.3.2 Characterization of D-PHI/PCNU scaffolds

3.3.2.1 Scanning electron microscopy (SEM)

To investigate fibre morphology, scaffolds were imaged using a Hitachi VP-SEM SU3500 scanning electron microscope (OCCAM, University of Toronto) (1.50 kV voltage, 30.0 mm spot intensity) after scaffolds were coated with 6 nm of platinum using a SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK). The average fibre diameter, standard deviation of fibre diameter, and fibre alignment distribution were calculated using ImageJ. Two images (1280 x 960 pixels) were taken for each scaffold and the widths of 10 random fibres from each image were measured. The fibre alignment distribution was determined using the fast Fourier transform (FFT) image processing technique in ImageJ [144]. Briefly, a representative image from each scaffold was filtered using the variance processing method to highlight fibre edges and then transformed to graphical representations of their frequency domains using the FFT function to extract directional information. The summation of pixel intensities along a straight line from the centre of the resulting FFT images for all 360° angles around the image was performed using the Oval Profile ImageJ plug-in (authored by Bill O’Connell, http://www.rsb.info.nih.gov/ij/plugins/oval-profile.htm). Pixel intensity as a function of angle was plotted for angles from 0° to 180° in order to provide a graphical representation of the degree of fibre alignment.

3.3.2.2 Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy

To determine the cross-linking efficiency of D-PHI during the reactive electrospinning process, a Thermo Scientific iS50 FTIR-ATR (Analest Lab, University of Toronto) spectrometer with a diamond crystal was used (30 scans per spectra). D-PHI solutions were made up using a 1 wt. % hydroquinone polymerization inhibitor in order to scavenge any free radicals that may form prior to electrospinning. PCNU was added to the D-PHI solution (without the HFIP solvent) and heated briefly to obtain a homogeneous solution of D-PHI and PCNU. FTIR-ATR was conducted on these pre-polymerized solutions and compared with post-electrospun D-PHI/PCNU scaffolds. The results from the electrospun D-PHI/PCNU scaffolds were compared with light cured D-PHI flat films. GRAMS/AI software (Thermo Scientific, version 9.1) was used to perform the deconvolution of the peaks. The peak height centred at an approximate
wavenumber of 810 cm\(^{-1}\) (vinyl C-H deformation) and a CH\(_3\) stable reference peak (~2940 cm\(^{-1}\)) were used to calculate the degree of conversion of double bonded carbons present in the monomers that were converted into single bonds during the chain polymerization reaction in the electrospinning process [145]. The degree of conversion can be directly correlated to the conversion efficiency of the polymer. The formula used to calculate the degree of conversion was:

\[
% \text{degree of conversion} = 100 \times \left(1 - \frac{A(\text{vinyl})_{\text{final}}/A(\text{reference})_{\text{final}}}{A(\text{vinyl})_{\text{initial}}/A(\text{reference})_{\text{initial}}} \right)
\]

3.3.2.3 Contact angle measurements

D-PHI/PCNU and PCNU electrospun scaffold surface energies were evaluated by measuring the advancing and receding contact angle formed between a sessile water droplet and the material surface using a goniometer system (Rame-hart, Model 100, Netcong). Scaffolds were taped onto the goniometer platform to ensure samples were flat. Advancing contact angle measurements were taken after depositing a 6 μL MilliQ water drop. Subsequently, an additional 4 μL of water was added to the existing water drop and 6 μL of water was aspirated to measure the receding contact angle. The equilibrium contact angle was calculated from the advancing and receding contact angle as reported on previously [146,147]. The contact angles of the D-PHI/PCNU electrospun scaffolds were compared to PCNU electrospun scaffolds. The contact angle was used as an indicator of the relative surface energy where a lower contact angle corresponds to a higher surface energy [148].

3.3.2.4 Mechanical testing

Classical stress vs. strain mechanical testing was conducted for dry and wet scaffolds at room temperature in air at a strain rate of 0.1 mm/sec. For wet samples, D-PHI/PCNU and PCNU scaffolds were incubated in PBS for 5 days at 37 °C in order to hydrate the membrane as previously described [18], and then subjected to mechanical testing using a TestResources 840LE2 series tensile tester with a 1.5 kg tension-compression load cell. The data was used to calculate the elastic modulus, tensile strength, and elongation-at-yield. Scaffold thickness was measured using SEM and ImageJ.
3.3.3 Subcutaneous implantation of D-PHI/PCNU scaffolds

Prior to implantation, scaffolds were gamma irradiated (2.5 Mrad Co-60) using a Gammacell 220 (Southern Ontario Centre for Atmospheric Aerosol Research Lab, University of Toronto). Scaffold discs were 8 mm in diameter and approximately 30 μm thick.

Wistar rats (Univ. of Toronto animal protocol #20012047) (250-300 g, all male) were housed in the Division of Comparative Medicine at the University of Toronto and were handled in accordance with the University of Toronto Animal Care Committee and the Canadian Council on Animal Care Guidelines for the Care and Use of Experimental Animals. Rats were given an analgesic (meloxicam 2 mg/kg) and anesthetized with 5 % isoflurane prior to the subcutaneous implantation of electrospun scaffold disks made of D-PHI/PCNU or PCNU. Three scaffolds of each material were implanted per rat and four rats were used per time point (7, 30, 60, and 90 days). Scaffolds were placed flat against the muscle layer under the skin. At each time point, rats were sacrificed with CO₂ euthanasia followed by cervical dislocation and samples were explanted with surrounding tissue (Appendix Figure A 1).

3.3.4 Histology of explanted D-PHI/PCNU scaffolds

Explanted scaffolds were fixed in 10% neutral buffered formalin and dehydrated in an increasing ethanol gradient. Subsequently, scaffolds were paraffin embedded, sliced (~7 μm thick), and stained with hematoxylin and eosin (H&E), May-Grünwald-Giemsa, or Picrosirius red staining kits (Sigma-Aldrich). Scaffold thickness was measured for two slices of each scaffold at five different locations along the scaffold to assess scaffold degradation. H&E histological sections were evaluated using a semi-quantitative scoring system to categorize samples based on the degree of integration, similar to a previously described method [149]. All aspects were evaluated on a scale between 0 and 3 and the cumulative score was reported (Appendix Table A 1). Higher scores were more indicative of a greater degree of integration and constructive remodelling response, while lower scores were more indicative of a lower degree of integration and a more prominent foreign body response. The histological sections were assessed for cellular infiltration into the scaffold, vascularity around the scaffold, and encapsulation of the scaffold. The three parameters were weighted equally as they all contribute to a positive integration response and affect each other. May-Grünwald-Giemsa stained sections were analyzed for the percentage of
macrophages relative to the total number of cells around the scaffold at defined points. Picrosirius red stained sections were analyzed for collagen infiltration into the scaffold over time.

3.3.5 Cell culture and seeding on D-PHI/PCNU scaffolds

Rat A10 smooth muscle cells (SMCs) were cultured in high glucose DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 2% penicillin-streptomycin (Life Technologies) at 37 °C, 5% CO₂. The medium was changed every other day to allow the cells to grow and reach confluence. Cells were passaged following treatment with 0.25% trypsin-EDTA solution.

The SMCs were seeded onto D-PHI/PCNU or PCNU electrospun scaffolds using a modified microfuge tube holder (Figure 3.2A). Prior to cell seeding, D-PHI/PCNU scaffolds were vacuum dried for 24 h at room temperature to remove any residual solvent. Subsequently, scaffolds were loaded into modified 150 μL microfuge tubes. Microfuge tubes were modified by cutting the end tip of the tube as well as the top of the lid (final tube length of ~15 mm) (Figure 3.2B). A rectangular scaffold was placed across the top of the microfuge tube and was held in place by closing the cap of the microfuge tube. The exposed surface area of the scaffold inside the microfuge tube was approximately 28.2 mm². The scaffolds were sterilized by immersing scaffolds in 70% ethanol for 24 h and subsequent air drying of scaffolds in a flow hood at room temperature. Scaffolds were then immersed in PBS (+/+)(Life Technologies), pH 7.0-7.2, overnight to further remove any residual unreacted monomers or solvents in the scaffolds. Scaffolds were preconditioned with 50 μL of media at 37 °C, pH 7.0-7.4 for 1 h prior to seeding cells. Cells were then seeded onto the scaffolds at 1000 cells per scaffold (to obtain confluence after 7 days). As a control, cells were seeded on tissue culture polystyrene (TCPS) petri dishes using a silicone gasket to create wells with an approximate area of 28.2 mm². Media was changed every other day.
Figure 3.2: (A) Procedure for modifying microfuge tube holder for cell seeding; (B) microfuge tube holder for scaffolds

3.3.6 Cell viability

After a 24 h and 7-day incubation period, cell viability was determined using a two-colour fluorescence live/dead viability/cytotoxicity assay kit (Molecular Probes) (calcein AM, ethidium homodimer-1). Briefly, scaffolds were washed once with PBS (+/+), incubated in the live/dead solution (2μM calcein, 4μM ethidium homodimer) for 30 minutes at room temperature, and washed once with PBS (+/+). The cells were imaged using a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility, Toronto Medical Discovery Tower, Toronto, Canada) with a 10x (0.5 NA) Fluar lens. Excitation was performed using the 488 nm and 555 nm lasers.

3.3.7 Cell adhesion

After a 24 h and 7-day culture period, cell adhesion was determined using SEM. Scaffolds were fixed in 3 % glutaraldehyde in PBS (-/-), dehydrated in increasing concentrations of ethanol in water (30, 50, 70, 90, 100%), dried in increasing concentrations of hexamethyldisilazane (HMDS) in ethanol (30, 50, 70, 90, 100%), and sputter coated with 6 nm of platinum using a SC515 SEC coating unit. Samples were imaged at a working voltage of 1.5 kV using a Hitachi 3500 SEM.

3.3.8 Immunofluorescence

Cell-seeded scaffolds were fixed with 4 % paraformaldehyde and immunostained for α-smooth muscle actin (SMA, rabbit polyclonal to SMA, Abcam, 1:100 dilution). The scaffolds were incubated with primary antibody solution (in 0.3 % v/v % Triton X-100 in PBS solution)
overnight at 4 °C. The scaffolds were then treated with 10 % goat serum containing 1:1000 diluted secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG) for 30 min in the dark. Cell nuclei were counter-stained with Hoechst 33258 (1:1000) and scaffolds were imaged with a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility, Toronto Medical Discovery Tower, Toronto, Canada) with a 10x (0.5 NA) Fluar lens. Cell-seeded scaffolds treated with only secondary antibody were used as negative controls to determine the amount of background autofluorescence. The mean fluorescence intensity for SMA in an image was normalized to the cell number in that image.

3.3.9 DNA mass quantification

DNA mass was quantified from cell-seeded scaffolds as previously described using a lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.1 % SDS, pH 8.0) and incubation at 65 °C for 1 h with intermittent vortexing [21]. DNA mass was quantified using Hoechst 33258 and a standard curve prepared from DNA standards of calf thymus DNA [21].

3.3.10 Enzyme-linked immunosorbent assay (ELISAs)

A matrix metalloproteinase-2 (MMP2) ELISA kit (R&D Systems) was used to determine the amount of MMP2 released in the cell culture media at 24 h and 7-day time points, 24 h after a media change, so that the measured levels of MMP2 were the result of 24 h of cellular activity. Media that was incubated with non-seeded scaffolds were used as a control. The ELISA was performed according to the manufacturer’s protocols.

3.3.11 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using the Tukey test for pair-wise comparisons or independent sample t-tests where appropriate. For all analysis, significance was assigned for p < 0.05.
3.4 Results

3.4.1 Fibre morphology of D-PHI/PCNU scaffolds determined using SEM

D-PHI/PCNU scaffolds yielded an average fibre diameter of 410 ± 350 nm and an alignment of 0.60 (perfect alignment = 1, absolute randomness = 0), while PCNU scaffolds had an average fibre diameter of 275 ± 100 nm and alignment of 0.65 (Figure 3.3).

Figure 3.3: SEM images of (A) D-PHI/PCNU electrospun fibres and (B) PCNU electrospun fibres.

3.4.2 Cross-linking efficiency of D-PHI/PCNU scaffolds determined using FTIR

Using the GRAM AI software with the 810 cm⁻¹ vinyl peak, the percentage degree of conversion for vinyl bonds is shown in Figure 3.4A. The degree of conversion for D-PHI/PCNU scaffolds was 94 ± 4 % and was comparable to a value of 96 ± 1 % conversion obtained for D-PHI flat films that were light cured without electrospinning and had gel contents of greater than 96 %. Representative spectra with deconvoluted peaks can be seen in Figure 3.4B to compare the polymer solution before and after electrospinning. In the post-cured scaffold, the vinyl peaks are no longer detectable, thus representing elevated consumption of the vinyl groups.
Figure 3.4: Degree of conversion of vinyl bonds during electrospinning process as determined using FTIR (A) Degree of conversion of vinyl bonds for D-PHI/PCNU electrospun scaffolds compared to D-PHI flat films. Data are mean ± standard deviation. There was no significant difference (p < 0.05) in the degree of conversion between the D-PHI/PCNU electrospun scaffolds and the D-PHI flat films. (n = 3) (B) Representative spectra of D-PHI/PCNU electrospun scaffolds before and after cross-linking with deconvolution of D-PHI/PCNU electrospun scaffold FTIR spectra before and after electrospinning. The red trace shows the FTIR spectra, the green trace shows the deconvoluted peaks, and the blue trace highlights the vinyl C-H peak.
3.4.3 Contact angle measurements of D-PHI scaffolds

Characterization of the advancing, receding, and equilibrium contact angles for PCNU and D-PHI/PCNU electrospun scaffolds are shown in Figure 3.5. Each type of contact angle yielded a significant difference in values for PCNU versus D-PHI/PCNU scaffolds. D-PHI/PCNU scaffolds had a decreased equilibrium contact angle when compared to PCNU scaffolds (44 ± 6° for D-PHI/PCNU scaffolds, 88 ± 7° for PCNU scaffolds), indicating an enhancement in the surface energy of the material by adding D-PHI.

![Figure 3.5: Water contact angle measurements of PCNU and D-PHI/PCNU electrospun scaffolds. Box shows mean ± standard error. The contact angles for PCNU scaffolds were statistically significant from the respective contact angles for D-PHI/PCNU scaffolds. p < 0.05 (n = 12)](image)

3.4.4 Mechanical testing of scaffolds

The elastic moduli, tensile strengths, and elongation-at-yield of D-PHI/PCNU and PCNU wet and dry electrospun scaffolds are summarized in Figure 3.6. The elastic modulus, tensile strength, and elongation-at-yield decreased as D-PHI was added to PCNU scaffolds. Also, wetting the scaffolds decreased the elastic modulus for both the PCNU and D-PHI/PCNU scaffolds. Wetting the scaffolds did not have an effect on the tensile strength. Furthermore, for both the PCNU and D-PHI/PCNU scaffolds, there was an increase in the elongation-at-yield when the scaffolds were wet.
**Figure 3.6:** Mechanical testing of PCNU and D-PHI/PCNU electrospun scaffolds (A) elastic modulus (material effect: p < 0.05, dry/wet effect p < 0.01), (B) tensile strength (material effect: p < 0.01), and (C) elongation-at-yield (material effect: p < 0.01, dry/wet effect p < 0.01). Box shows mean ± standard error. (n = 10)

### 3.4.5 Histology of explanted scaffolds

Both D-PHI/PCNU and PCNU scaffolds degrade relatively slowly *in vivo* as the scaffold form could still be seen after 90 days (**Figure 3.7**). At the later time points, there was increased cellular infiltration into the scaffolds and the formation of blood vessels around the scaffold, showing good integration of the scaffold with the tissue (**Figure 3.7**). In addition, the scaffold boundaries become less distinct with time, indicating signs of degradation and tissue integration occurring (**Figure 3.7**). Picrosirius red staining showed increased collagen infiltration into the scaffolds after 90 days compared to 7 days (**Figure 3.8**).
**Figure 3.7**: Representative cross-sectional histology slices of D-PHI/PCNU and PCNU scaffolds explanted after being implanted subcutaneously for 7, 30, 60, and 90 days and stained with H&E. The scaffolds stain a light purple colour and tissues/cells are a dark purple colour.
Figure 3.8: Representative cross-sectional histology slices of D-PHI/PCNU and PCNU scaffolds explanted after being implanted subcutaneously for 7, 30, 60, and 90 days and stained with Picrosirius red. The scaffolds stain pale yellow, tissue stains dark yellow, and collagen stains red.
From the histological scoring system, a relatively consistent score can be seen for both the PCNU and D-PHI/PCNU scaffolds from 7 to 90 days, indicating good integration of the scaffold and no adverse rejection of the scaffold over time (Figure 3.9). There was no significant difference in score between the PCNU and D-PHI/PCNU scaffolds at the different time points. Representative images for each scoring scale are shown in Appendix Figure A 2.

Figure 3.9: Histological scoring system (range is 0 to 9) results for PCNU and D-PHI/PCNU scaffolds after 7, 30, 60, 90 days. Box shows mean ± standard error. (n = 11-12)

As shown in Figure 3.10, the thickness of the D-PHI/PCNU scaffolds decreased after 7 days compared to the initial scaffold thickness (borderline significant), but did not change significantly from 7 to 90 days, further suggesting slow degradation of the D-PHI/PCNU scaffold and integration with tissue as the synthetic fibres degrade. PCNU scaffolds showed a significant decrease in thickness from 0 to 7 days.
Figure 3.10: Average scaffold thickness for PCNU scaffolds and D-PHI/PCNU scaffolds after 0, 7, 30, 60, 90 days. Box shows mean ± standard error. *p < 0.05 compared to all other PCNU scaffold time points (n = 11-12)

Although the D-PHI/PCNU scaffold degrades relatively slowly, it does not seem like there is an adverse immune response with the host tissue or fibrous capsule formation around the scaffold, as shown by the May-Grünewald-Giemsa stained sections (Figure 3.11). The percentage of macrophage-like cells surrounding the PCNU and D-PHI/PCNU scaffolds decreased over time, but this change was not shown to be statistically significant (Figure 3.12). This indicated that there was still inflammatory cell activity around the scaffold at the 90-day time point. There was no difference between the PCNU and D-PHI/PCNU scaffolds.
Figure 3.11: Representative cross-sectional histology slices of D-PHI/PCNU scaffolds explanted after being implanted for 7 days, 30 days, 60 days, and 90 days and stained with May-Grünwald-Giemsa stain. The scaffolds stain light pink and tissue/cells stain purple.
**Figure 3.12:** Percentage of macrophage-like cells relative to the total number of cells surrounding PCNU and D-PHI/PCNU scaffolds after 7, 30, 60, and 90 days. Box shows mean ± standard error. (n = 10-12)

### 3.4.6 Cell viability

Representative live/dead stained images of the SMCs on the D-PHI/PCNU electrospun scaffolds at 24 h and 7 days post-seeding are shown in **Figure 3.13.** The D-PHI/PCNU scaffolds had a high cell viability (>99%) after 24 h and 7 days, which was comparable to cells grown on PCNU scaffolds (>99%) and TCPS (>99%). Thus, it can be concluded that the D-PHI/PCNU scaffold is non-toxic to cells and can be used to support high cell viability for at least 7 days.
Figure 3.13: Representative live/dead staining of SMCs on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS at 24 h and 7 days post-seeding. Live stain is calcein AM (green) and dead stain is ethidium homodimer-1 (red). Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times.

3.4.7 Cell adhesion

Figure 3.14 shows representative SEM images of the SMCs after 24 h and 7 days post-seeding on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS. Cells can be seen attached and spread out on the fibres with the highly spread out cells aligning parallel to the direction of the fibres.
Figure 3.14: Representative SEM images of SMCs on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS at 24 h and 72 h post-seeding. Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times.

3.4.8 Immunofluorescence

The immunofluorescence images of SMCs stained for SMA on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS are shown in Figure 3.15A. Figure 3.15B shows the mean fluorescence intensity of SMA normalized to cell number on the scaffolds and TCPS. There was a decrease in SMA mean fluorescence intensity from 24h to 7 days for both PCNU and D-PHI/PCNU scaffolds. After 24 h, the SMA intensity was significantly lower on TCPS compared to both
types of scaffolds. Also, at 24 h the SMA intensity was lower on D-PHI/PCNU scaffolds compared to PCNU scaffolds. There was no significant difference in SMA intensity after 7 days.

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3.4.9 DNA assay

There was no significant difference in DNA content on D-PHI/PCNU scaffolds compared to PCNU scaffolds at 24 h and 7 days (Figure 3.16). From 24 h to 7 days, there was a statistically significant increase in DNA content, which indicated that the SMCs were able to proliferate on the scaffolds. The DNA content on the scaffolds was similar to the DNA content on TCPS after 24 h and 7 days as shown in Figure A 6.
Figure 3.16: DNA content on D-PHI/PCNU scaffolds and PCNU scaffolds after 24 h and 7 days. Box shows mean ± standard error. (n = 9)

3.4.10 ELISA

There were significantly higher levels of MMP2 released per cell at 7 days compared to at 24 h when SMCs were cultured on both D-PHI/PCNU and PCNU scaffolds (Figure 3.17). The MMP2 levels at 24 h were extremely low and many samples had MMP2 levels below the sensitivity of the assay. At 7 days, there was no difference in MMP2 levels between D-PHI/PCNU and PCNU scaffolds.

Figure 3.17: Quantification of MMP2 released from SMC cultures normalized to DNA mass after 24 h and 7 days. Box shows mean ± standard error. (n = 9)
3.5 Discussion

3.5.1 Material properties

This study investigated the generation of a uniform, aligned nanofibrous D-PHI/PCNU electrospun elastic scaffold for soft tissue engineering applications. The work demonstrates for the first time the ability to synthesize a cross-linked co-polymeric urethane-elastomer, generated in mid-flight, while fibres are forming. The unique combination of monomers used in the formation of the urethane composite was particularly relevant to the implant field as the combination of chemistries from the monomers have previously been shown to have immunomodulatory character when bulk polymerized [11]. Given the potential for incomplete vinyl group polymerization during air contact with the resins during electrospinning, the D-PHI/PCNU scaffold was characterized for vinyl monomer conversion, and subsequently for preliminary cell compatibility. In order to generate a suitable scaffold to aid in tissue regeneration, the scaffold would preferably mimic the structure and mechanical properties of the *in vivo* extracellular environment [141]. Upon implantation of a scaffold *in vivo*, protein adsorption occurs and can regulate the cellular response and influence tissue generation. Thus, it is important to characterize the material properties of the D-PHI/PCNU scaffold in order to determine if they will have retained the favorable nature of the original bulk heat cured D-PHI materials previously reported on, despite the substantially different nature of material processing and its new composite nature with the PCNU component.

Electrospinning successfully generated aligned D-PHI/PCNU nanofibres with a diameter of 410 ± 349 nm (*Figure 3.3*). The large standard deviation in the fibre diameter reflects the range of thinner fibres interspersed among thicker fibres, which is believed to have been generated from the challenge of controlling each of the two processes associated with the solidification of the fibres during their formation, e.g. light curing and solvent evaporation which are occurring simultaneously. The average fibre diameter of the composite can be varied by modifying the ratio of D-PHI chemistry as well as the D-PHI to PCNU ratio, in addition to the classical electrospinning parameters defined in the methods. This would enable different fibre diameters and composition to be accommodated for a specific application. As mentioned previously, the purpose of electrospinning for tissue engineering applications is to mimic physical aspects of the ECM. In particular, it is desirable to achieve a fibre diameter range and distribution similar to the
ECM fibre diameter (e.g. on the order of the sub-micron to micron scale) [150]. The ECM is composed of a matrix of proteins that provides structural and functional support to cells. Collagen is the major component of the ECM and consists of 10 to 300 nm diameter fibrils that bunch together to form fibres with diameters ranging from 1 to 20 μm [151,152]. These collagen fibrils are comparable in size to the mix of D-PHI/PCNU electrospun fibre diameters achieved in this study. In previous work, PCNU electrospun fibres were used to mimic the complex structure of the annulus fibrosus of the intervertebral disk [53]. The cells in the outer annulus fibrosus are elongated and aligned with collagen fibres, which was mimicked by the electrospun fibre structure [53]. As a comparator, those membranes have fibres with diameters on the order of 275 ± 100 nm (Figure 3.3). The D-PHI/PCNU elastic scaffold could also be used for applications such as recapitulating the native myocardium ECM alignment, similar to what other studies have shown, where cardiomyocytes need to retain an elongated and aligned morphology for appropriate cell function [76].

Since D-PHI is a cross-linked polymer, an in situ UV light to cross-link vinyl units was required to generate cross-linked fibres. Not only is cross-linking important to retain the form of the scaffold during their early degradation, but complete cross-linking is important to determine that the conversion of the vinyl groups was achieved to a high degree. A higher conversion corresponds to fewer residual monomers left in the scaffold. This is important as such residual monomers are highly toxic to cells [153–155]. D-PHI/PCNU scaffolds were shown to have a high degree of conversion of vinyl bonds to single bonds (94 ± 4 %). This was comparable to when D-PHI 2-D flat films (a material already shown to be highly conducive to supporting cells [11,12]) were light cured (96 ±1 %), which translates to a high cross-linking efficiency (Figure 3.4). Hence, the incorporation of the higher molecular weight polyurethane (PCNU), the elevated surface area of the spinning solution contact in air during flight, and the presence of the spinning solvent have not significantly affected the conversion of the D-PHI component of the composite fibres.

Water contact angle measurements showed that D-PHI/PCNU electrospun scaffolds had a significantly lower equilibrium contact angle when compared to that of the PCNU electrospun scaffolds (Figure 3.5). This is a very simple manner by which one can assess the presence of the polar groups super-imposed onto the high surface area of the nanofiber matrix. Since D-PHI
monomers are lower in molecular weight as compared to PCNU, they will have fewer molecular chain entanglements and interactions with the bulk of the material prior to polymerization, thereby potentially facilitating their movement to the surface of the scaffold relative to the PCNU chains [156]. The different components of D-PHI lead to its diverse properties with the hydrophilic (ionic MAA, polar urethane) and hydrophobic (MMA) functional character.

Through the electrospinning process, the ionic MAA molecules and polar groups of the oligo-urethane are anticipated to migrate to the outer surface of the scaffold in order to yield an enhanced polar, hydrophilic character and hence a lower contact angle. The Young’s equation describes the balance of elements at the interface between the solid, liquid, and vapour phases [146,157,158]. It assumes that the surface is chemically homogeneous and smooth; however, most real world samples are not smooth and lead to a range of contact angles between the advancing and receding contact angles instead of an equilibrium contact angle, thus leading to contact angle hysteresis [146,157,158]. Additionally, adding surface roughness to the material increases the wettability generated by the chemistry of the surface [159,160]. This means that hydrophobic surfaces will become more hydrophobic and hydrophilic surface will become more hydrophilic if they are rough. This can possibly explain the lower advancing contact angle of the D-PHI/PCNU electrospun scaffolds (55 ± 7 °) as compared to D-PHI flat films (81.4 ± 1.4 °) that are relatively more smooth [19]. The nanotopography of the D-PHI/PCNU electrospun scaffolds induces more roughness on the material surface, leading to a more hydrophilic surface as compared to smoother D-PHI flat film values previously reported on in literature [19]. Previous studies of electrospun PCNU scaffolds on their own have an advancing contact angle of 109.5 ± 1.9 ° [161], which is comparable to what we report in Figure 3.5. Furthermore, Yang et al. showed that the incorporation of non-polymerizable anionic dihydroxyl oligomer (ADO) into PCNU films could lead to a decrease in the water contact angle [53]. Hence, the polymerized D-PHI component appears to have a similar effect as ADO due to the increased hydrophilic groups introduced into the polymer, but adds the additional feature of being covalently cross-linked into the nanofiber itself, thereby establishing a much more stable bulk and surface chemistry that would not be as susceptible to leaching as the ADO oligomers.

A lower water contact angle translates to a higher surface energy [148]. The surface energy is a measure of the work per unit area done by the force that generates the new surface. When there
are greater attractive forces between the adhering element and the substrate, there will be increased contact between the two entities, a lower contact angle, and a resultant higher surface energy [148]. The surface energy of a material influences protein adsorption as proteins can change conformation when they come in contact with a surface [53,162–164]. A higher surface energy often translates to enhanced protein adsorption, and this change in protein adsorption on the material surface can lead to facilitated cell attachment and growth [162,165,166]. For example, Yang et al. demonstrated that PCNU scaffolds with a greater ADO concentration had a lower contact angle, higher surface energy, and increased annulus fibrosus cell adhesion [53]. The increased concentrations of ADO led to more ionic and polar hydrophilic groups on the surface to yield an enhanced surface polar character, more adhesion of serum proteins, and enhanced cell attachment [53]. In another study, fibroblasts were grown on metal and polymeric surfaces with different surface energies and roughness [165]. It was determined that the surface energy was proportional to cellular adhesion strength and that surface roughness also increased adhesion strength on polymeric materials [165]. Overall, D-PHI/PCNU scaffolds have a lower contact angle when compared to that of PCNU scaffolds despite both having similar physical morphology, and this translates to a higher surface energy, and enhanced cell attachment (Figure 3.5).

Another important material parameter for tissue engineered scaffolds is the mechanical properties of the scaffold. In this study, tensile testing was performed on D-PHI/PCNU and PCNU scaffolds in order to define the scaffold stiffness in the preferred orientation of the fibres. Adding D-PHI to PCNU electrospun scaffolds resulted in a decrease in elastic modulus, tensile strength, and elongation-at-yield (Figure 3.6). In previous studies, PCNU electrospun scaffolds had an elastic modulus and tensile strength of approximately 46 ± 3 MPa and 14 ± 1 MPa respectively when dry [61]. When the scaffolds were wet, the elastic modulus and tensile strength decreased to 9 ± 1 MPa and 6 ± 1 MPa respectively [61]. The blended D-PHI/PCNU electrospun scaffolds incorporates the mechanical properties from both component materials in order to generate a material with a stiffness in between that of D-PHI and PCNU scaffolds [21].

The chemical structure of the hard segment affects the phase separation of the hard and soft domains, which in turn contributes to the mechanical properties [167]. Comparing the hard segments of D-PHI and PCNU, D-PHI incorporates LDI and HEMA, while PCNU incorporates
HDI and BD as a chain extender. HDI is a linear, symmetric isocyanate while LDI is asymmetric. The symmetry of HDI results in both isocyanate groups having similar reactivity, which leads to greater association of the hard segment and thus better hard segment cohesion [167]. The molecular chains can become more closely packed together in a parallel manner to increase PU crystallinity, which increases hard segment interconnectivity and the ability of the hard segment to act as a physical cross-linking site [168]. As a result of increased cross-linking, there will be a restriction in polymer chain movement, which will in turn increase polymer stiffness [169]. On the other hand, the isocyanate groups on LDI have different reactivity, where the isocyanate group attached to the secondary carbon has slightly lower reactivity [167]. This leads to an asymmetric structure at the hard segment borders with the soft segments and would contribute to preventing the ordered packing of the hard segment [167]. In addition, the lysine pendant chain can act as a plasticizer to break up the hard segment polymer chains [167]. Due to the lower hard segment cohesion, there will be lower phase separation and less packing of hard segments compared to HDI, which leads to lower mechanical properties [167,170]. Furthermore, the use of a BD chain extender in PCNU can help promote ordered microphase-separated hard and soft domains stabilized by H-bonding and van der Waals forces respectively [170]. These differences in chemical structure can help to explain in part the higher elastic modulus observed for PCNU scaffolds compared to D-PHI/PCNU scaffolds. Carocciolo et al. have reported similar results where poly(ε-caprolactone) diol based PUs synthesized with HDI had higher elastic moduli compared to those synthesized with LDI [167].

For both D-PHI/PCNU and PCNU scaffolds, there was a resultant decrease in the elastic modulus and increase in elongation-at-yield upon wetting the scaffolds. The decrease in elastic modulus after wetting a polymer is a function of the water absorption of the polymer [171,172]. More hydrophilic polymers were found to have a greater decrease in elastic modulus when wet [171,172]. As more water is absorbed into a polymer, the water molecules disrupt the hydrogen bonding between polymer chains and increases the number of hydrogen bonds with water [171,172]. This has a plasticizing effect to allow polymer chains to slide more easily past each other, leading to a decrease in polymer stiffness and increase in elongation [171,172].

In order to vary the elastic modulus of D-PHI-PCNU scaffolds to match the elastic moduli of different tissues, the ratio of D-PHI to PCNU could be changed or softer/harder polymeric
material components (relative to PCNU) could be co-electrospun with D-PHI or D-PHI/PCNU solutions. For example, PCNU scaffolds were used for annulus fibrosus spinal disk tissue regeneration where the native tissue stiffness is approximately 50 MPa [61]. On the other hand, for softer tissues like in the cardiac myocardium, softer materials could be co-blended with the D-PHI/PCNU scaffolds, e.g. gelatin could be co-blended to achieve this effect [49].

3.5.1 In vivo and in vitro cell studies

The rate of PU degradation is largely dependent on the chemistry of the soft segment, degree of soft segment crystallinity, phase separation, hard segment chemistry, and hard segment concentration [60]. PUs with a polyester soft segment are typically more prone to hydrolysis due to the ester linkages and depending on the nature of the chemistry around the ester group, may not be suitable for long term implantation. Polyether PUs are less sensitive to hydrolysis, but more susceptible to oxidative degradation. PUs with a polycarbonate soft segment are more resistant to oxidative degradation as compared to both polyether and polyester PUs, and they can be designed to be more or less susceptible to hydrolytic degradation depending on hard segment chemistry, which can be used to shield the carbonate chemistry to different degrees [60]. A major advantage of carbonates is that they generate CO₂, a natural pH balancing agent, rather than more acidic organic by-products which have been shown to be pro-inflammatory when accumulated in tissues [44,45]. PUs with a higher hard segment content can lead to higher resistance to hydrolytic degradation, due in part to hydrogen bonding within the hard segment, which can mask cleavage sites [20,60]. For example, PCNUs with a higher hard segment percentage (41 wt. % compared to 20 wt. %) had a higher resistance to hydrolytic degradation due to hydrogen bonding within the hard segment [20,60]. In vivo, both hydrolytic and oxidative degradation processes occur. Reactive oxygen species released by monocytes, macrophages, and foreign body giant cells initiate PU degradation to lead to chain scission and cross-linking of the soft segment [59].

The DVO backbone of D-PHI is hydrolysable, while the carbon chain backbone can be eliminated through the dissolution of the oligomeric anionic chains that will be linking the polyurethane components prior to their degradation. Overall, D-PHI degrades into relatively non-toxic by-products of lysine, carbon dioxide, alcohols, and water soluble oligomeric methyl
methacrylate/methacrylic acid molecules, which can be metabolized by the body [18,20,58,61]. The PCNU linear chain is hydrolysable into carbon dioxide, water, and alcohols [61]. One limitation with the degradation of PCNU scaffolds is that the degradation of hexane diisocyanate leads to hexane diamine as a degradation product, which has been reported to have limited toxicity when present at high levels [54].

Both D-PHI/PCNU and PCNU scaffolds degrade relatively slowly in vivo, as shown by the presence of the scaffold after 90 days in histological sections (Figure 3.7). The histological score of both D-PHI/PCNU and PCNU scaffolds was relatively consistent over time to indicate a good integration response and no adverse rejection of the scaffold over time (Figure 3.9). There was increased cellular and protein infiltration and blood vessel formation around the scaffolds at later time points, indicating good integration and compatibility of the scaffold with surrounding tissue, followed by the area occupied by the fibres becoming replaced with tissue. This was demonstrated by the increased collagen infiltration into the scaffolds at later time points (Figure 3.8). This finding is consistent with previous studies for 3-D D-PHI scaffolds (generated by bulk polymerization with NaHCO₃/polyethylene glycol porogen and extraction to yield porous scaffolds) that had a 7 wt. % loss after 7 days post-implant and a linear profile of degradation that led to a 21 wt. % mass loss by 100 days [20]. That study also saw increased cellular infiltration and collagen production into scaffold pores over time [20]. The size and shape of the scaffold was well maintained during the experiment, which indicated that despite weight loss and pore expansion occurring, there was no collapse in the structure of the scaffold and the form of the new tissue was maintained [20]. A similar process seems to be occurring for the nanofibrous D-PHI/PCNU composite electrospun scaffolds here, as the tissue construct thickness is unchanged throughout the experiment period (Figure 3.10), yet tissue is infiltrating and integrating within the polymeric nanofibers of the constructs.

In terms of PCNU degradation, previous in vitro studies in cholesterol esterase, a monocyte derived enzyme, showed a 0.56 ± 0.05 mg/week mass loss for aligned PCNU electrospun scaffolds alone, leading to a 2 mg mass loss after four weeks [61]. The mass loss was relatively linear over the four-week timeframe, which implied most likely a surface mediated degradation process [61]. Hence, it was not expected to see a sudden collapse in the bulk scaffold structure early on. In the current in vivo study, it was not possible to perform mass loss experiments for the
scaffolds due to the fragile, thin scaffold thickness and its integrated structure with the formed tissue in vivo; however, scaffold thickness was measured as an indicator of the degradation process. While there was an initial contraction of the scaffold from 0 to 7 days, from 7 to 60 days, very little change in PCNU scaffold thickness was observed (Figure 3.10). However, tissue presence within the scaffold was increasing, which implies a gradual surface mediated degradation process, with tissue replacement (Figure 3.10). Note that after 90 days a small but non-significant increase in scaffold thickness was seen, which may be due to the initiation of protein infiltration into the scaffold to lead to scaffold expansion as a result of the elastic nature of the scaffold.

Overall, it would appear that tissue integration into the PCNU scaffolds is occurring at a somewhat faster rate relative to the D-PHI/PCNU scaffolds as shown by the increased collagen infiltration into the PCNU scaffolds after 7 and 30 days (Figure 3.8), but overall scoring of the tissue integration for all histological images suggested a similar integration. The early observations in the histology images may be attributed to the cross-linked structure of D-PHI which should contribute to a slower degradation process. For tissue engineering applications, it is important to match the degradation rate of the scaffold to the rate of tissue regeneration [20][173]. The slower degradation rate of D-PHI/PCNU scaffolds could help with stabilizing new tissue formation and also reduce the burden of synthetic degradation by-products on the local cells. The presence of the neo-vasculature formation and reduced fibrosis over time is a good sign that the cells are accommodating the biodegradation processes and enabling the de novo tissue to develop. The degradation rate is dependent on fibre diameter as an increased surface area would likely lead to increased degradation rates. As well, the degradation rate could be increased by co-electrospinning with faster degrading materials, like gelatin.

From these initial in vivo studies, no adverse immune responses were seen around the D-PHI/PCNU scaffolds (Figure 3.11). There was a decrease in the percentage of macrophages relative to the total number of cells surrounding the scaffold from 7 to 90 days, but the difference was not statistically significant (Figure 3.12). There was no observable difference in immune response between the D-PHI/PCNU and PCNU scaffolds. Previous in vitro studies have shown that human monocytes seeded onto D-PHI flat films had a pro wound-healing phenotype compared to poly(lactic-co-glycolic acid) films [62]. Furthermore, McBane et al. showed that in
in vivo, D-PHI scaffolds were able to promote adhesion of a protein layer that attracted monocytes and induced them towards a tissue generation state, rather than an inflammatory state [20]. A similar process would be anticipated to occur over time for D-PHI/PCNU electrospun scaffolds in vivo, and future long-term in vivo work being done for specific tissue regeneration applications should further explore this aspect of immunomodulatory character for the D-PHI/PCNU electrospun scaffolds.

Preliminary cell compatibility studies were performed with A10 vascular SMCs as a model system. Previously, PCNU scaffolds were shown to be compatible with annulus fibrosus cells and gingival fibroblasts [53]. In this work, the behaviour of SMCs on D-PHI/PCNU scaffolds was compared to that on PCNU scaffolds that have been shown to be cell compatible [53,61].

SMCs seeded on D-PHI/PCNU scaffolds had high cell viability that was comparable to that on PCNU scaffolds, thus showing the compatible and non-toxic nature of the D-PHI/PCNU scaffolds (Figure 3.13). From the SEM images, the SMCs can be seen adhered and spread out on the scaffold surfaces (Figure 3.14). The high cell adhesion can be partly attributed to the surface chemistry of D-PHI/PCNU scaffolds which have a high surface energy conducive for protein adsorption and cell adhesion [174–177]. Sharifpoor et al. showed similar results of A10 SMC adhesion on D-PHI porous scaffolds [18].

The nanotopography of the electrospun scaffolds induces cellular alignment in the direction of fibres (Figure 3.14). In the native cellular environment, many cell types adopt an aligned morphology, which is crucial for cellular function in many tissue engineering applications. For example, cardiomyocytes need to be highly elongated and aligned for action potential propagation [96].

When cultured on the D-PHI/PCNU scaffolds, the SMCs expressed the contractile protein SMA after 24 h and 7 days (Figure 3.15). SMA is an important contractile marker protein for SMCs that is important for cellular contraction, motility, and structural integrity [178,179]. SMCs can undergo reversible phenotype changes from a synthetic to contractile phenotype, depending on their environment [180,181]. The synthetic SMC phenotype is characterized by a morphology similar to fibroblasts, cellular proliferation, ECM production, tissue remodelling, and a loss in contractile function [180,181]. On the other hand, the contractile SMC phenotype is
characterized by spindle-shaped cells with low cellular proliferation, well organized ECM, and contractile ability [180,181]. When SMCs are in pathological conditions or when they are cultured in vitro, they tend to lose their contractile ability and adopt a more synthetic phenotype [180,181]. A higher expression level of SMA correlates with a high level of contractile ability, which represents a more contractile phenotype [182].

On both the D-PHI/PCNU and PCNU scaffolds, there was a decrease in SMA intensity from 24 h to 7 days (Figure 3.15). There was a decrease in SMA intensity from seeding to 24 h on D-PHI/PCNU scaffolds and TCPS (Figure A 5). A similar initial decrease in SMA expression was previously seen when human SMCs were cultured in monoculture or co-culture with monocytes on D-PHI porous scaffolds [183]. After 24 h, the intensity of SMA was significantly higher on the two scaffold surfaces compared to on TCPS. In addition, the intensity of SMA on D-PHI/PCNU scaffolds was lower than that on PCNU scaffolds. However, after 7 days, there was no difference in SMA intensity on the scaffolds or on TCPS. The decrease in SMA after 7 days indicates that the SMCs are adopting a more synthetic phenotype compared to after 24 h. Previous studies have shown that the phenotype of SMCs in mono-culture is regulated by the chemistry of the material on which they are grown as the SMCs adhere to the surfaces with different ligands, depending on the material surface chemistry [184]. For example, fibronectin and collagen I surfaces promote a more synthetic phenotype, while laminin, elastin, heparin, and collagen IV promote a more contractile phenotype [180]. The high expression of SMA on the scaffolds after 24 h may imply that the initial cell material interactions with the polyurethane scaffolds keep the cells in a relatively contractile phenotypic state when the SMCs are adjusting to the new scaffold culture conditions, but this is not seen on TCPS. As the SMCs proliferate over time on the scaffold surfaces, the cell-material interactions induce a more synthetic phenotype. For engineering functional vasculature, a synthetic phenotype is initially required for sufficient cellular proliferation and ECM production, while a contractile phenotype is required later on for generating functional vasculature with sufficient vasoactivity and inhibition of neointima formation [180]. Thus, the more synthetic SMC phenotype induced by the scaffolds after 7 days of culture could be beneficial for initial engineering of vasculature. Once sufficient cells and ECM have been produced, other stimuli could be used to induce a phenotypic change to a more contractile SMC phenotype, which is required for further functional maturation of the
vasculature. For example, mechanical strain and the addition of biochemical cues such as transforming growth factor beta (TGFβ) have been shown to induce SMC contractile protein expression and a more contractile phenotype [182].

The amount of DNA detected on D-PHI/PCNU scaffolds was comparable to PCNU scaffolds after 24 h and 7 days (Figure 3.16). In addition, the DNA content on the scaffolds was comparable to typical DNA content on TCPS after 24 h and 7 days (Figure A 6). There was a significant increase in the amount of DNA on the scaffolds from 24 h to 7 days, thus indicating that cellular proliferation was occurring on the scaffolds. These results further demonstrate the ability of the D-PHI/PCNU scaffolds in supporting high cell viability and adhesion.

In terms of MMP2 released by the SMCs, there were higher levels of MMP2 released per cell after 7 days compared to 24 h for both PCNU and D-PHI/PCNU scaffolds (Figure 3.17). The levels of MMP2 produced at 24 h were low, due to the initial low seeding density of the cells. As a result, some sample values were below the sensitivity of the assay. At 7 days, there was no difference in MMP2 levels between the PCNU and D-PHI/PCNU scaffolds. MMP2 is a hydrolytic protease produced by SMCs that is important in ECM degradation and remodelling and is also an early marker of foreign body reactions [178]. SMCs can release MMPs to regulate ECM remodelling during the inflammatory and wound healing process to influence SMC proliferation and migration [178]. Since the levels of MMP2 detected were similar on PCNU scaffolds compared to D-PHI/PCNU scaffolds, this translates to similar levels of ECM degradation and remodelling on both types of scaffolds. These in vitro results parallel the in vivo observations from Figure 3.7 and Figure 3.8 where similar levels of degradation can be seen for the PCNU and D-PHI/PCNU scaffolds.

3.6 Conclusion

This study describes the generation of electrospun D-PHI/PCNU scaffolds with aligned nanofibres for future tissue engineering applications. D-PHI/PCNU scaffolds have a high degree of vinyl conversion, which indicates that there are few unreacted toxic monomers left in the scaffold. Also, D-PHI/PCNU scaffolds have a higher surface energy as compared to PCNU scaffolds or D-PHI films on their own. This should lead to initial enhanced cell attachment. The stiffness of D-PHI/PCNU scaffolds is lower than that of PCNU scaffolds, which would be
beneficial for softer tissue engineering applications. *In vivo*, the D-PHI/PCNU scaffolds degrade relatively slowly, but there was good integration of the scaffold with the surrounding tissue, as shown by increased cellular and protein infiltration and blood vessel formation around the scaffold over time. As a model cell, A10 SMCs showed high viability, adhesion, and expression of SMA contractile marker on the D-PHI/PCNU scaffolds, which was comparable to PCNU scaffolds and standard TCPS after 7 days. This study demonstrates that the electrospun D-PHI/PCNU composite scaffold has a unique combination of surface topography, surface chemistry, stiffness, degradation, anti-inflammatory, and tissue compatibility properties which could be tailored for a diverse range of tissue engineering applications.

### 3.7 Limitations

There are several limitations with the current work which should be considered and further examined for future work. In terms of the material properties of the D-PHI/PCNU scaffold, although D-PHI/PCNU scaffolds have a lower elastic modulus compared to PCNU scaffolds, the stiffness of the D-PHI/PCNU scaffolds would likely have to be further decreased for use in soft tissue engineering applications. This could be accomplished by incorporating softer materials into the bulk of the D-PHI/PCNU scaffold (eg. gelatin) by co-spinning with D-PHI/PCNU. In addition, as shown from the in vivo experiments, the D-PHI/PCNU scaffold degrades relatively slowly. Faster degrading materials could be incorporated into the scaffold (eg. gelatin) to increase the degradation rate if required for specific tissue engineering applications. Furthermore, the histology analysis performed was mainly semi-quantitative or qualitative, which limited the extent of analysis that could be performed. In the future, more quantitative analysis could be explored (eg. staining for specific types of immune cells). Finally, the A10 smooth muscle cell line used for cell compatibility experiments may not be representative for all cell types, especially for more sensitive cells, so further work would be required to investigate the cell compatibility of other cell types with the D-PHI/PCNU scaffold.

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Chapter 4: Induced pluripotent stem cell derived cardiomyocyte compatibility on an electrospun nanofibrous, degradable polyurethane composite scaffold

4.1 Foreword

Cardiac tissue engineering can be used to repair and replace the damaged myocardium that occurs as a result of coronary artery disease. Cardiac tissue engineered scaffolds need to be biodegradable and cell compatible, while being able to mimic the structure and mechanical properties of the native myocardium in order to promote functional tissue regeneration. In this study, induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) were assessed for their compatibility with an electrospun nanofibrous degradable polar hydrophobic ionic polyurethane (D-PHI) composite scaffold intended for use in on-going cardiac tissue engineering. Electrospun D-PHI composite scaffolds exhibit a unique surface chemistry and topography that aims to recapitulate elements of the native extracellular matrix environment. D-PHI was synthesized, integrated with a degradable linear polycarbonate polyurethane (PCNU), and electrospun with in situ UV cross-linking to generate a stable and aligned nanofibrous scaffold. iPSC-CMs were cultured on D-PHI/PCNU scaffolds with different surface protein coatings (Matrigel, gelatin, and culture medium protein) to determine the effect on cell viability, adhesion, expression of functional markers, and cell alignment. The results showed that aligned D-PHI/PCNU scaffolds were able to support high iPSC-CM viability and adhesion on all surface protein coatings. iPSC-CMs expressed high levels of cardiac troponin-T (cTnT) and myosin light chain 2 (MLC) functional markers after a 7-day culture period on the scaffolds. cTnT expression on the scaffolds was similar to when cultured on tissue culture polystyrene (TCPS), but there was a higher expression of MLC on the scaffolds versus TCPS, indicating a higher proportion of ventricular-type iPSC-CMs. iPSC-CMs were more elongated and aligned on aligned D-PHI/PCNU scaffolds as compared to on random D-PHI/PCNU scaffolds and on TCPS. These results demonstrate that the aligned D-PHI/PCNU scaffold is compatible with iPSC-CMs and could be used for undertaking functional cardiac tissue regeneration.
4.2 Introduction

Coronary artery disease is a type of cardiovascular disease that leads to myocardial infarction, myocardium damage, irreversible cardiomyocyte (CM) death, and ultimately heart failure, due to the limited regenerative potential of CMs [7]. In order to help repair and regenerate the damaged myocardium, cardiac tissue engineering can be used to generate a CM seeded cardiac patch scaffold that aims to mimic the native myocardium. CMs used for tissue engineering need to be functionally mature and express CM functional markers, while being capable of generating action potentials and active contractions [185]. Embryonic stem cell derived CMs (ESC-CMs) and induced pluripotent stem cell derived CMs (iPSC-CMs) have been shown to yield functional CMs that express functional markers, have well-organized sarcomeres, and have appropriate electrophysiological properties [186–189]. However, both ESC-CMs and iPSC-CMs are limited by the fact that they exhibit a functionally immature, disorganized, fetal-like phenotype that is not representative of adult CMs [92]. Thus, a challenge of cardiac tissue engineering strategies is to be able to recapitulate the native cardiac environment to promote CM maturity.

Cardiac tissue engineered scaffolds need to be biodegradable and cell compatible to enable cell adhesion and proliferation, while promoting cells to express functional markers required for tissue regeneration. In addition, scaffolds need to mimic the structure and mechanical properties of the native myocardium extracellular environment in order to generate a functional and viable cardiac patch. Natural scaffolds, derived from extracellular matrix (ECM) proteins, provide a physiologically relevant surface for cells to attach, proliferate, and differentiate [16]. These scaffolds have the benefits of often showing good tissue compatibility and biodegradability; however, they typically have poor mechanical strength, batch-to-batch variation, immunogenicity concerns, and the inability to tailor biochemical and biophysical properties [15]. Synthetic scaffolds have the advantage of customizable mechanical and biochemical properties and thus can be tailored for use as a tissue engineering scaffold. However, synthetic scaffolds may have problems with cell and tissue compatibility, poor ability to promote vascularity vs fibrosis, and poor biodegradability [15].

Polyurethanes (PU) are popular synthetic co-polymeric elastic materials for tissue engineering due to their segmented block co-polymeric chemistry which provides the opportunity to tailor the
mechanical properties, general biocompatibility, and degradation rates [17]. Many groups have used different types of PUs for cardiac tissue engineering. For example, a poly(ester carbonate urethane) urea was co-electrospun with porcine cardiac ECM to form a bi-layered PU patch [48]. The patch was tested in a rat chronic infarction model and was found to alter the tissue remodelling response following myocardial infarction [48]. There was an increase in angiogenesis, while showing a decrease in left ventricle mechanical compliance, functional deterioration, scar formation, and left ventricle wall thinning [48]. The elastomeric PU component of the scaffold helped with providing adequate mechanical support to the ventricle, while the cardiac ECM component helped improve the remodeling response following myocardial infarction [48]. In another study, polyester urethane urea (PEUU) was blended with gelatin to generate an electrospun nanofibrous scaffold [49]. Rabbit CMs seeded on the composite PEUU/gelatin scaffolds had enhanced proliferation and integration compared to pure PEUU scaffolds [49]. Furthermore, Parrag et al. demonstrated that aligned electrospun PU scaffolds (composed of polycaprolactone diol, lysine diisocyanate and a phenylalanine-based chain extender) led to the anisotropic organization of mouse ESC-CMs and improved sarcomere formation [190]. These studies demonstrate that PUs can be a useful material for generating cardiac tissue engineered scaffolds and that their cell compatibility can be further enhanced by the incorporation of ECM proteins. However, these studies are in their early phases and the compatibility of the PU material needs to be considered from many perspectives. This includes the inflammatory and foreign body response, and the influence of these processes on human CMs. Such work will likely include more relevant animal models in order to better define these clinically relevant parameters.

In recent years, a degradable polar hydrophobic ionic polyurethane (D-PHI) was developed for vascular tissue engineering applications and was shown to have excellent immunomodulatory character by reducing the activation of pro-inflammatory macrophage markers [18,19]. D-PHI scaffolds are prepared as a mixture of three monomers: a lysine-based polycarbonate divinyl oligomer (DVO) cross-linker, methacrylic acid (MAA), and methyl methacrylate (MMA) [18]. Previously, electrospun nanofibrous D-PHI composite scaffolds were generated for tissue engineering applications (Chapter 3). These nanofibrous D-PHI composite scaffolds combined the unique immunomodulatory properties of the monomers with electrospinning. Electrospinning
of polymers is a useful technique that employs a high voltage difference to generate an electrically charged polymer solution that is ejected from a nozzle onto a grounded mandrel to form a nanofibrous scaffold [22]. Using electrospinning, aligned fibres can be generated on the nanometer scale to mimic the native ECM [23]. Also, electrospun scaffolds have a high surface area to volume ratio and high porosity which can help promote tissue biosynthesis and guide cell growth, thus enhancing tissue regeneration [23].

Previous studies (Chapter 3) have shown that the electrospun nanofibrous D-PHI composite scaffolds have a high cross-linking efficiency, high surface energy, and mechanical properties that could be suitable for general tissue engineering applications. In vivo, these nanofibrous D-PHI composite scaffolds integrated well with the host tissue and slowly degraded due to hydrolytic degradation of their carbonate and urethane linkages [20,61]. In early work, A10 vascular smooth muscle cells (SMCs) were shown to be compatible on the D-PHI composite scaffolds with high cell viability and adhesion, while expressing high levels of alpha smooth muscle actin contractile marker. The current work intends to investigate the extension of these electrospun D-PHI composite scaffolds toward generating a suitable substrate for a much more specialized cell, CMs, which are required for cardiac tissue engineering and have been demonstrated to be challenging cells to maintain in their required phenotypic state [191].

In this study, elastomeric polyurethane composites of D-PHI and a degradable, linear polycarbonate polyurethane (PCNU) were electrospun to generate an aligned nanofibrous scaffold. The potential use of this electrospun D-PHI/PCNU scaffold for cardiac tissue engineering was investigated by determining the compatibility of iPSC-CMs on the scaffold. iPSC-CM viability and adhesion was assessed on D-PHI/PCNU scaffolds coated with different surface protein coatings (Matrigel, gelatin, or media conditioning for 24 h) and on tissue culture polystyrene (TCPS) after a 7-day culture period. These surface protein coatings were chosen as they represent commonly used protein coatings used to promote cell attachment [94,192]. In addition, the expression of cardiac functional markers, cardiac troponin-T (cTnT) and ventricular myosin light chain 2 (MLC), were assessed on the different surface protein coated D-PHI/PCNU scaffolds and compared to classical TCPS substrates that have been used for CM culture [193]. Furthermore, the proportion of iPSC-CMs relative to fibroblasts was determined on the different surface protein coated D-PHI/PCNU scaffolds compared to on TCPS by staining for cTnT and
vimentin (vim). iPSC-CM alignment and elongation on the aligned D-PHI/PCNU scaffolds was quantified relative to random D-PHI/PCNU scaffolds and flat TCPS surfaces to determine whether the nanotopography of the electrospun fibres could induce iPSC-CM alignment more representative of *in vivo* morphology. This work explores the possibility of using a novel electrospun nanofibrous D-PHI composite scaffold to mimic aspects of the native cardiac ECM and provide a compatible scaffold for iPSC-CM growth, which could be used for on-going cardiac tissue engineering applications.

### 4.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

#### 4.3.1 Electrospun D-PHI/PCNU scaffold fabrication

D-PHI solutions were generated using a previously established protocol [18]. Briefly, D-PHI solutions were made with a divinyl oligomer (synthesized from lysine disocyanate, poly(hexamethylene carbonate) diol, and 2-hydroxyethyl methacrylate), methacrylic acid, and methyl methacrylate in a 1:5:15 molar ratio [18]. A 3 wt. % Irgacure 1173 photoinitiator (IGM Resins), relative to the resin, was added to the D-PHI polymer solution to initiate the polymerization reaction [66]. The D-PHI solution was protected from light and allowed to mix for a minimum of 12 h prior to use. PCNU solutions were generated using a previously established protocol [53]. Briefly, PCNU was synthesized by the reaction of 1,6-hexane diisocyanate, poly(hexamethylene carbonate) diol, and 1,4-butanediol in a 3:2:1 molar ratio to yield a polystyrene equivalent weight average molecular weight of approximately 140,000 as measured using gel permeation chromatography [53]. PCNU was dissolved in a 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solvent. After achieving a homogeneous D-PHI solution, the D-PHI solution was incorporated into the PCNU solution at a 1:1 ratio to increase solution viscosity and assist with electrospinning [16]. The HFIP solvent evaporates during the electrospinning process and any residual solvent is removed by vacuum drying and conditioning scaffolds with PBS prior to cell seeding.

The D-PHI/PCNU polymer solution was fed by a syringe pump into an 18” stainless steel needle for electrospinning. The electrospun monomer/PCNU mixture was UV cross-linked in flight.
(using a Phoseon FE300 365nm UV lamp with an output window of 110 mm x 10 mm and peak irradiance of 3.0 W/cm²) while the mixture traveled from the syringe to the rotating collecting mandrel, generating aligned nanofiber [67,68]. The lamp was placed 15 cm away from the collecting mandrel and the concentration of PCNU in HFIP ranged from 15-16 % w/v in order to obtain a similar solution viscosity between all samples. The viscosity was standardized to the time required for 0.1 mL of polymer solution to flow through a 1 mL syringe with a 22-gauge needle under normal gravitational force (e.g. 140 ± 10 s). The flow rate during electrospinning of the solution was 0.5 mL/h, the tip-to-collector distance was 18 cm, the needle had a positive charge of 18.5 kV, and the collecting mandrel had a negative charge of -0.5 kV. Aligned D-PHI/PCNU scaffolds were deposited onto a rotating mandrel (rotating at 9.25 m/s) while random D-PHI/PCNU scaffolds were deposited onto a flat collection plate.

After scaffold fabrication, scaffolds were coated with 6 nm of platinum using a SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK) and imaged using a Hitachi VP-SEM SU3500 scanning electron microscope (SEM) (OCCAM, University of Toronto) (1.50 kV voltage, 30.0 mm spot intensity). The average fibre diameter, standard deviation of fibre diameter, and fibre alignment distribution were calculated using ImageJ (version 1.50). Two images were taken for each scaffold and the widths of 10 random fibres from each image were measured. The fibre alignment distribution was determined using the fast Fourier transform (FFT) image processing technique in ImageJ as previously described [144].

4.3.2 iPSC-CM seeding on D-PHI/PCNU scaffolds

iPSC-CMs were purchased from Cellular Dynamics International (CDI), a company that specializes in iPSC differentiation to various cell types. Specifically, the iCell Cardiomyocytes² (R1017, CDI) were used as they have similar electrophysiological characteristics as the original iCell Cardiomyocytes product, but have been optimized for a more rapid recovery from thaw [194]. iCell iPSC-CMs consist of a mixture of atrial, nodal, and ventricular like myocytes [194]. The cells were characterized by flow cytometry for cTnT and all vials used were greater than 95 % positive for cTnT (remaining cells are mainly from the fibroblast lineage) [194].

The iPSC-CMs were thawed and seeded directly onto D-PHI/PCNU electrospun scaffolds using a modified microfuge tube holder as previously described. Prior to cell seeding, D-PHI/PCNU
scaffolds were vacuum dried for 24 hours at room temperature to remove any residual solvent. Subsequently, scaffolds were loaded into modified 150 μL microfuge tubes. The exposed surface area of the scaffold inside the microfuge tube was approximately 28.2 mm². The scaffolds were treated by immersing scaffolds in 70 % ethanol for 24 hours and subsequent air drying of scaffolds in a flow hood at room temperature. Scaffolds were then immersed in sterile filtered PBS (+/+)] pH 7.0-7.2 (Life Technologies) for 24 h to further remove any residual polymer precursors from the scaffolds. Scaffolds were coated with either Matrigel (human embryonic stem cell-qualified Matrigel Matrix, Corning), gelatin, or conditioned for 24 h with iCell CM Maintenance Media (CDI). For Matrigel coatings, scaffolds were coated for at least 1 h at room temperature and aspirated immediately prior to cell seeding, as described in the product specifications [195]. 0.1% gelatin solutions were prepared from Type A gelatin dissolved in sterile water and autoclaved prior to use. Scaffolds were exposed to the gelatin solution for at least 1 h at 37 °C, and then the gelatin solution was aspirated immediately prior to cell seeding, as described previously [194].

The iPSC-CMs were thawed and seeded onto the scaffolds at 50,000 cells per 28.2 mm² scaffold (as recommended in the product user guide). As a control, cells were seeded on gelatin coated TCPS petri dishes using a silicone gasket to create wells with an approximate area of 28.2 mm². The iPSC-CMs were plated with iCell CM Plating Media (CDI), and media was exchanged 4 h post-seeding to iCell CM Maintenance Media (CDI). After the initial media exchange, media was changed every other day.

4.3.3 Cell viability

After 7 days, cell viability was determined using live/dead staining. Live/dead staining was conducted using a two-colour fluorescence live/dead viability/cytotoxicity assay kit (Molecular Probes) (calcein AM, ethidium homodimer-1). Briefly, scaffolds were washed once with PBS (+/+), incubated in the live/dead solution (2μM calcein, 4μM ethidium homodimer) for 30 minutes at room temperature, and washed once with PBS (+/+). The cells were imaged using a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility, Toronto Medical Discovery Tower, Toronto, Canada) with a 10x (0.5 NA) Fluar lens. Excitation was performed using the 488nm and 555 nm lasers.
4.3.4 Cell adhesion and alignment

Cell adhesion and alignment was determined using SEM, as described above. A separate set of scaffolds were fixed in 3% glutaraldehyde in PBS (-/-), dehydrated in increasing concentrations of ethanol in water (30, 50, 70, 90, 100%), dried in increasing concentrations of hexamethyldisilazane (HMDS) in ethanol (30, 50, 70, 90, 100%), and sputter coated with 6 nm of platinum for SEM imaging using a SC515 SEC Coating Unit (Polaron Equipment, Uckfield, UK).

4.3.5 Immunofluorescence

Cell-seeded scaffolds were fixed with 4% paraformaldehyde and immunostained for cTnT (mouse monoclonal to cTnT, Abcam, 1:200 dilution), MLC (rabbit polyclonal to MLC, Abcam, 1:200 dilution), phalloidin (phalloidin-iFluor 488 Reagent, Abcam, 1:1000 dilution), and vimentin (vim) (rabbit monoclonal to vim, Abcam, 1:200 dilution). The scaffolds were incubated with primary antibody solution (in 0.3% v/v% Triton X-100 in PBS solution) overnight at 4 °C. The scaffolds were then treated with 10% goat serum containing 1:400 diluted secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG) for 30 min in the dark. Cell nuclei were counter-stained with Hoechst 33258 (1:1000) and scaffolds were imaged with a Zeiss LSM700 confocal microscope (Advanced Optical Microscopy Facility, Toronto Medical Discovery Tower, Toronto, Canada) with a 10x (0.5 NA) Fluar lens. Cell-seeded scaffolds treated with only secondary antibodies were used as negative controls to determine the amount of background autofluorescence.

4.3.6 DNA mass quantification

DNA mass was quantified from cell-seeded scaffolds as previously described using a lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, 0.1% SDS, pH 8.0) and incubation at 60 °C for 1 h with intermittent vortexing [21]. DNA mass was quantified using Hoechst 33258 and a standard curve prepared from DNA standards of calf thymus DNA [21].

4.3.7 Cell characterization

cTnT and MLC were used as markers for iPSC-CMs while vim was used as a marker for fibroblasts. The ratios of MLC to cTnT or vim to cTnT positive cells were reported. Cell
morphological properties were determined using ImageJ to determine cell area, aspect ratio (major/minor axis), and circularity (higher circularity closer to 1). Cell alignment was determined similar to fibre alignment using the FFT image processing technique in ImageJ by analyzing the phalloidin stain for actin filaments which were parallel to cell direction.

4.3.8 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using the Tukey test for pair-wise comparisons or independent sample t-tests where appropriate. For all analysis, significance was assigned for $p < 0.05$.

4.4 Results

4.4.1 Electrospun D-PHI/PCNU scaffold fibre morphology

Aligned D-PHI/PCNU scaffolds yielded an average fibre diameter of $410 \pm 350$ nm and an alignment of 0.60 (perfect alignment = 1, absolute randomness = 0) while random D-PHI/PCNU scaffolds yielded an average fibre diameter of $640 \pm 320$ nm and an alignment of 0.49. (Figure 4.1)

![Figure 4.1: SEM images of (A) aligned D-PHI/PCNU electrospun fibres and (B) random D-PHI/PCNU electrospun fibres.](image)

4.4.2 iPSC-CM viability

An iPSC-CM viability of greater than 95% was achieved on all protein coated D-PHI/PCNU scaffolds and the values were comparable to iPSC-CMs seeded on TCPS. Representative
live/dead stained images of the iPSC-CMs, 7 days post-seeding on the aligned D-PHI/PCNU electrospun scaffolds, are shown in Figure 4.2A-C and on TCPS in Figure 4.2D.

**Figure 4.2:** Cell viability of iPSC-CMs on (A) Matrigel coated D-PHI/PCNU aligned scaffolds, (B) gelatin coated D-PHI/PCNU aligned scaffolds, (C) media conditioned D-PHI/PCNU aligned scaffolds, and (D) gelatin coated TCPS after 7 days. Live stain is calcein AM (green) and dead stain is ethidium homodimer-1 (red). Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times.

### 4.4.3 iPSC-CM adhesion and alignment

**Figure 4.3** shows representative SEM images of the iPSC-CMs on gelatin coated aligned and random D-PHI/PCNU scaffolds, compared to gelatin coated TCPS, after 24 h and 7 days post-seeding. On the aligned D-PHI/PCNU scaffolds, cells can be seen attached and spread out on the fibres with cells aligned parallel to the direction of the fibres. Contrastingly, on the random D-PHI/PCNU scaffolds and on TCPS, the cells are randomly distributed. After 24 h, the iPSC-CMs have not fully spread out to form a confluent monolayer and individual cells can be seen; however, after 7 days, a confluent monolayer of iPSC-CMs can be seen.
Figure 4.3: SEM images of iPSC-CMs adhered on gelatin coated aligned D-PHI/PCNU scaffolds, gelatin coated random D-PHI/PCNU scaffolds, and gelatin coated TCPS after 24 h and 7 days.

4.4.4 DNA mass quantification

The DNA assay showed that the iPSC-CMs had good adhesion on all protein coated aligned D-PHI/PCNU scaffolds 7 days post-seeding (Figure 4.4). The iPSC-CMs appeared to be slightly better adhered on Matrigel, followed by gelatin, and then media conditioned D-PHI/PCNU electrospun scaffolds; however, there was not a significant difference between the conditions.
Figure 4.4: DNA content on aligned D-PHI/PCNU scaffolds with different surface protein coatings, 7 days post adhesion. Box shows mean ± standard error. (n = 6)

4.4.5 iPSC-CM characterization

4.4.5.1 Expression of cTnT and MLC functional markers

The immunofluorescence images of iPSC-CMs stained for cTnT and MLC on aligned and random D-PHI/PCNU scaffolds and TCPS are shown in Figure 4.5A-E. Figure 4.5F shows the ratio of MLC to cTnT positive cells on aligned and random D-PHI/PCNU scaffolds and TCPS. The iPSC-CMs expressed high levels of cTnT and MLC on all protein coated D-PHI/PCNU scaffolds after 7 days and no statistical difference could be detected between the different surface protein coatings. The ratio of MLC to cTnT positive cells was significantly higher on all surface protein coated D-PHI/PCNU scaffolds when compared to TCPS.
Figure 4.5: Immunofluorescence staining of iPSC-CMs for cTnT (green) and MLC (red) on (A) Matrigel coated aligned D-PHI/PCNU scaffolds, (B) gelatin coated aligned D-PHI/PCNU scaffolds, (C) media conditioned aligned D-PHI/PCNU scaffolds, (D) gelatin coated random D-PHI/PCNU scaffolds, and (E) gelatin coated TCPS after 7 days; (F) ratio of MLC to cTnT positive cells on aligned and random D-PHI/PCNU scaffolds and TCPS. Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times. Box shows mean ± standard error. *p < 0.01 significant MLC intensity when TCPS compared to scaffolds.
4.4.5.2 Proportion of iPSC-CMs relative to fibroblasts

The immunofluorescence images of iPSC-CMs stained for cTnT and vim on aligned and random D-PHI/PCNU scaffolds and TCPS are shown in Figure 4.6A-E. Figure 4.6F shows the ratio of vim to cTnT positive cells on aligned and random D-PHI/PCNU scaffolds and TCPS. Comparing cTnT and vim expression, there was high expression of cTnT relative to vim on aligned and random D-PHI/PCNU scaffolds after 7 days, indicating a high proportion of iPSC-CMs compared to fibroblasts. The ratio of vim to cTnT positive cells was significantly lower on TCPS when compared to all surface protein coated D-PHI/PCNU scaffolds, indicating a lower proportion of fibroblasts on TCPS compared to on the scaffolds.

A
Matrigel coated aligned D-PHI/PCNU scaffold

B
Gelatin coated aligned D-PHI/PCNU scaffold

C
Media conditioned aligned D-PHI/PCNU scaffold

D
Gelatin coated random D-PHI/PCNU scaffold

E
Gelatin coated TCPS

Hoechst
**Figure 4.6:** Immunofluorescence staining of iPSC-CMs for cTnT (green) and fibroblasts for vim (red) on (A) Matrigel coated aligned D-PHI/PCNU aligned scaffolds, (B) gelatin coated aligned D-PHI/PCNU scaffolds, (C) media conditioned aligned D-PHI/PCNU scaffolds, (D) gelatin coated random D-PHI/PCNU scaffolds, and (E) gelatin coated TCPS after 7 days; (F) ratio of vim to cTnT positive cells on aligned and random D-PHI/PCNU scaffolds and TCPS. Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times. Box shows mean ± standard error. *p < 0.01 significant vim intensity when TCPS compared to grouped scaffolds.

### 4.4.5.3 iPSC-CM alignment

iPSC-CMs were more elongated and aligned in the direction of fibres on aligned D-PHI/PCNU scaffolds when compared to random D-PHI/PCNU scaffolds and TCPS, where iPSC-CMs were randomly oriented. This is shown by phalloidin staining for actin filaments (**Figure 4.7**). This can be substantiated quantitatively by the higher cell alignment value, lower cell circularity, and higher aspect ratio of iPSC-CMs on aligned D-PHI/PCNU scaffolds when compared to on random D-PHI/PCNU scaffolds and TCPS (**Figure 4.8**). The alignment and elongation of iPSC-CMs was comparable between different surface protein coatings on aligned D-PHI/PCNU scaffolds.
**Figure 4.7:** Immunofluorescence staining of iPSC-CMs for phalloidin (green) on (A) Matrigel coated aligned D-PHI/PCNU scaffolds, (B) gelatin coated aligned D-PHI/PCNU scaffolds, (C) media conditioned aligned D-PHI/PCNU scaffolds, (D) gelatin coated random D-PHI/PCNU scaffolds, and (E) gelatin coated TCPS after 7 days.
**Figure 4.8:** (A) Cell alignment, (B) cell shape, and (C) cell aspect ratio on aligned D-PHI/PCNU scaffolds, random D-PHI/PCNU scaffolds, and TCPS. Box shows mean ± standard error. *p < 0.01 significant from aligned scaffolds, **p < 0.01 significant from aligned scaffolds (n = 6)

There was a significant increase in cell area from 24 h to 7 days for all surfaces **Figure 4.9.** After 24 h, the cell area appeared to be smaller on the scaffold surfaces as compared to on TCPS; however, only the random D-PHI/PCNU scaffolds showed a significant difference when compared to TCPS. There was no significant difference in area between the aligned D-PHI/PCNU scaffolds and TCPS at 7 days, and all cells were significantly more spread at 7 days versus 24 h.
Figure 4.9: Cell area on aligned D-PHI/PCNU scaffolds, random D-PHI/PCNU scaffolds, and TCPS after 24 h and 7 days. Box shows mean ± standard error. *p < 0.01 (n = 6)

4.5 Discussion

This study investigated the iPSC-CM compatibility of a uniform, aligned nanofibrous D-PHI/PCNU electrospun elastic scaffold for potential uses in cardiac tissue regeneration. Previous work has shown that A10 vascular SMCs had high adhesion, viability, and expression of alpha smooth muscle actin contractile marker on D-PHI/PCNU electrospun scaffolds (Chapter 3).

Since CMs are more sensitive to their environment as compared to typical cell lines such as the A10 vascular SMCs, the work here was intended to probe whether the D-PHI/PCNU electrospun scaffold could promote effective attachment and viability for iPSC-CMs, while expressing functional CM markers.

The myocardial ECM is composed of a mixture of collagens, glycoproteins, and proteoglycans that provide mechanical support for cell attachment and influence CM morphology, proliferation, and function [97,196]. ECM proteins govern cell fate through receptor or integrin-mediated transduction [97]. Thus, when culturing iPSC-CMs in vitro, surfaces should try to recapitulate aspects of this native ECM environment in order to generate a physiologically representative surface that can ultimately support iPSC-CMs. D-PHI/PCNU scaffolds provide an elastomeric surface that aims to recapitulate the in vivo environment to encourage cell attachment through a diverse surface chemistry. Previous work has shown that D-PHI/PCNU scaffolds have a high
surface energy (low equilibrium water contact angle of approximately 40 °) and stiffness conducive for general tissue engineering applications (around 50 MPa) (Chapter 3). However, many previous studies have reported that CMs may require additional ECM protein coatings adsorbed to these synthetic surfaces to further enhance CM adhesion [94]. Commonly used ECM protein coatings that promote CM adhesion include Matrigel, collagen, gelatin, laminin, and fibronectin [94]. In particular, gelatin is the most readily accessible, has a very well defined composition, and has low cost [94].

In this study, D-PHI/PCNU scaffolds were coated with different protein surface coatings to determine the effect on iPSC-CM adhesion, viability, alignment, and expression of functional markers. D-PHI/PCNU scaffolds were conditioned for 24 h with CM media to determine whether the adsorption of serum proteins onto the scaffold would be sufficient to enable iPSC-CM adhesion and spreading. In addition, Matrigel and gelatin were used as ECM protein coatings to determine their potential use to further enhance iPSC-CM adhesion.

Matrigel is a common surface coating used for CM attachment and is composed of a heterogeneous and gelatinous protein mixture secreted from mouse sarcoma cells [95,96]. It has been reported to consist of many relevant proteins that are contained in the in vivo extracellular environment. Specifically, it is composed of laminin (~60%), collagen IV (~30%), entactin (~8%), and a mixture of poorly defined growth factors [97]. However, because of its heterogeneity and unknown elements, there are concerns for use in human applications, and thus may never be granted approval for use in humans [97,197]. Also, the unknown chemical composition of the material could result in lot-to-lot variability [97]. As a result, gelatin, which has been approved for use in humans, may be a more suitable alternative for promoting CM adhesion. Gelatin is hydrolyzed collagen and is highly degradable, as well as being reported to be cell compatible [90,98]. Gelatin has been reported to have good antigenicity, while being cost-effective [198]. Given these favorable parameters, gelatin was the chosen protein coating when the aligned fibres were compared to random D-PHI/PCNU scaffolds and TCPS substrates.

In this study, a high iPSC-CM viability (> 95 %) was achieved on all protein coated D-PHI/PCNU scaffolds and the values were comparable to well established iPSC-CMs seeded on
TCPS after 7 days (Figure 4.2). Thus, it can be concluded that the D-PHI/PCNU scaffold chemistry is compatible with iPSC-CMs and not toxic to cells.

Additionally, iPSC-CMs were able to adhere well onto the D-PHI/PCNU scaffolds as shown through the SEM images and the DNA assay (Figure 4.3 and Figure 4.4 respectively). Previous work has shown that electrospun D-PHI/PCNU scaffolds have a surface chemistry that yields a low water contact angle and enhanced surface energy (Chapter 3). This is in part enabled by the favored migration of low molecular weight polar components (urethane oligomers and methacrylic acid) to the surface of the forming fibres during electrospinning. Since D-PHI monomers are lower in molecular weight as compared to PCNU, they will have fewer chain entanglements and interactions with the bulk of the material prior to their polymerization, thereby potentially facilitating their movement to the surface of the scaffold relative to the PCNU chains. The different component chemistries of D-PHI lead to its unique properties consisting of hydrophilic (ionic MAA, polar urethane) and hydrophobic (MMA) functional character. The surface chemistry of a material plays an important role in regulating cell-material interactions by influencing the adsorption of ECM proteins to the material surface [164,199,200]. Previous studies have demonstrated the ability of D-PHI films to modulate the surface protein binding conformation of fibronectin when fibronectin was deposited as a single layer and multi-layer onto D-PHI films [201]. The material chemistry played an important role in protein organization, with small changes in surface hydrophobicity and anionic content affecting protein presentation to cells [201]. In another study, D-PHI was shown to change the conformational state of bound immunoglobulin G, which in turn modulated monocyte-biomaterial interactions [19]. Other studies have shown that there was better cell attachment for rat embryonic CMs onto carboxyl containing surfaces when compared to hydroxyl containing surfaces [202]. In the current work, there are abundant carboxylic acid groups introduced on the material surface from the D-PHI chemistry, which could potentially help with iPSC-CM attachment.

Although the DNA assay did not show significant differences in adhesion for the different surface protein coatings, the iPSC-CMs seemed to adhere better on the Matrigel and gelatin coated D-PHI/PCNU surfaces as compared to the media conditioned surfaces. In one study that used a microarray to screen for human embryonic stem cell derived CM (ESC-CM) adhesion to various methacrylate and methacrylamide monomers with different side chain chemistries, it was
determined that polymers pre-conditioned with fetal bovine serum supported enhanced ESC-CM adhesion when compared to polymers without serum [203]. On serum pre-conditioned arrays, it was determined that the surface was dominated by multiple types of adsorbed proteins [204]. The surface chemistry of polymers affects protein adsorption, which in turn affects cell adhesion [205]. In the absence of serum, only 7 out of 116 polymers tested supported ESC-CM adhesion at a level comparable to a gelatin control [203]. However, in these serum-free conditions, the two best polymers tested had ESC-CM cell sizes that were considerably lower (274 μm² and 110 μm²) when compared to ESC-CM cultured on a gelatin control (1560 μm²) [203]. In general, it is desirable to have CMs with a larger cell area, which is usually indicative of more mature CMs that are more likely to have well-organized structural proteins required for functional cell contraction [206]. This highlights the importance of serum protein adsorption on CM adhesion to biomaterial surfaces. Although conditioning some polymers with serum helped to enhance CM adhesion to a level comparable to a gelatin control, the adsorption of serum proteins to other polymer surfaces was not sufficient to achieve the same level of CM adhesion as the gelatin control [203]. In the current study, adhesion of iPSC-CMs to media conditioned D-PHI/PCNU is lower than Matrigel and gelatin coated surfaces, which may imply that additional ECM surface proteins are required to enhance iPSC-CM adhesion on top of an adsorbed medium protein layer.

As per the discussion above, both Matrigel and gelatin are popular options for surface coatings intended for CM adhesion [81,95,119,192,207–209]. The adhesion of iPSC-CMs to Matrigel and gelatin coated surfaces were comparable while other studies have reported differentiated levels of CM adhesion for surfaces coated with these proteins. Choi et al. showed that HL-1 CMs adhered better on fibronectin and laminin (main components of Matrigel) surfaces when compared to collagen and gelatin surfaces [192]. They hypothesized that the differences in cell adhesion could be due to size differences in the protein nanostructures as they observed that larger structure proteins had higher adhesion levels [192]. In another study, PU films (composed of lysine diisocyanate, polycaprolactone, and phenylalanine chain extender) were coated with gelatin, laminin, or collagen IV [209]. CMs were seeded onto the PU films for 30 days and it was determined that cell attachment was higher on gelatin, laminin, and collagen IV coated films compared to gelatin coated TCPS and uncoated PU films after 30 days [209]. This further
demonstrates the consideration of matching the ECM protein coating to the base scaffold materials in order to optimize iPSC-CM adhesion.

In terms of CM phenotype, iPSC-CMs expressed high levels of cTnT and MLC on the protein coated aligned and random D-PHI/PCNU scaffolds after 7 days (Figure 4.5). No significant difference could be detected for the ratio of MLC to cTnT positive cells on any of the protein surface coatings for D-PHI/PCNU scaffolds. There was a higher proportion of MLC positive cells relative to cTnT positive cells on D-PHI/PCNU scaffolds when compared to TCPS. Since cTnT is expressed in all CMs, it can be used as a marker for the total CM population. On the other hand, MLC is only expressed in ventricular CMs [210]. The iPSC-CMs used in these experiments consist of a mixed population of atrial, nodal, and ventricular CMs [194]. A higher MLC to cTnT ratio suggests that there is a larger proportion of CMs that differentiate towards a ventricular CM phenotype on the scaffolds when compared to on TCPS. In addition, since there was no difference in MLC to cTnT ratio between the aligned and random D-PHI/PCNU scaffolds, this difference in CM phenotype can likely be attributed to surface chemistry and its inherent elastomeric character, rather than a surface topography effect.

Previous studies have shown that Matrigel can help promote iPSC-CM adhesion and maturation [211][198]. When iPSC-CMs were delivered in Matrigel to a rat myocardial infarction model, iPSC-CMs were more mature when compared to iPSC-CMs that were delivered alone or in a hyaluronan hydrogel [211]. iPSC-CMs were larger in size, had better organized sarcomere structures, had longer sarcomeres, and had greater expression of cardiac troponin-I (a marker of matured CMs) when delivered with Matrigel [211]. Similarly, gelatin coated surfaces have been shown to maintain cardiac phenotype and can also help enhance cardiac differentiation [207]. Ogasawara et al. showed that iPSC-CMs cultured on gelatin-coated dishes displayed a higher expression of cTnT and longer beating periods when compared to collagen coated dishes and TCPS [211]. When differentiating a carcinoma stem cell line to CMs, cTnT expression on gelatin coated dishes was five times the levels of those seen for cells that were on collagen coated dishes or TCPS after 11 days [211]. Although collagen and gelatin are similar in amino-acid composition, there are some key structural differences that may lead to this difference in CM phenotype and function. Gelatin has a wide molecular weight distribution and can be easily hydrolyzed by proteases [207]. Also, gelatin has a higher dynamic storage and binds fibronectin
with a higher affinity than collagen, resulting in a higher production of fibrils when compared to collagen, which could lead to enhanced cell adhesion [207].

The iPSC-CMs used for the current study’s experiments were greater than 95 % positive for cTnT while the other cell types mainly consisted of fibroblasts, which were obtained through the differentiation procedure [98]. Since CM proliferation is typically extremely low, while fibroblasts are able to proliferate, it was important to determine whether the D-PHI/PCNU scaffolds could help support iPSC-CMs while maintaining a relatively low proportion of fibroblasts. The ratio of vim to cTnT positive cells was lower on TCPS when compared to the D-PHI/PCNU scaffolds, thus indicating a lower proportion of fibroblasts relative to CMs on TCPS compared to the scaffolds (Figure 4.6). This could indicate that the scaffold is enhancing fibroblast proliferation relative to when culturing on TCPS. If D-PHI/PCNU scaffolds are able to support high iPSC-CM purities in conjunction with allowing fibroblasts to co-exist, this could be a beneficial characteristic of the D-PHI/PCNU scaffolds when extending this work to represent physiological tissues as fibroblasts are naturally present [125,128].

In the human heart, fibroblasts outnumber CMs by 3 to 5 times and are the largest cell population by number [128]. A dense network of cardiac fibroblasts surrounds CM clusters of two to four cells in the ventricular myocardium and act as bridges between myocardial tissue layers [125]. Cardiac fibroblasts are important in maintaining normal cardiac function and cardiac remodelling during MI and hypertension [125]. Fibroblasts are responsible for synthesizing and depositing ECM and cell-to-cell communication with CMs, monocytes, endothelial cells, and other fibroblasts [125]. The interaction of fibroblasts with CMs affects cardiac development, myocardial structure, cell signalling, electrophysiological properties of CMs, and secretion of growth factors in the healthy and diseased heart [125]. Thus, the D-PHI/PCNU scaffold could be used as a potential platform for co-culturing CMs and fibroblasts to enhance CM function and maturity.

When iPSC-CMs were cultured on the aligned D-PHI/PCNU scaffolds, they were more elongated and aligned in the direction of fibres as compared to random D-PHI/PCNU scaffolds and TCPS where the iPSC-CMs were randomly oriented (Figure 4.7 and Figure 4.8). The surface protein coating on the D-PHI/PCNU scaffolds did not seem to have an impact on cell
alignment and indicates that it is the nanotopography of the fibres which is most influential on the cellular alignment. A higher cell aspect ratio on the aligned D-PHI/PCNU scaffolds indicates a more elongated cell morphology as there is a larger difference between the dimensions of the major and minor axes. This cellular elongation is further supported by a lower cell circularity which indicates that the cells are less circular and more spread out. The elongated and aligned morphology of iPSC-CMs on aligned D-PHI/PCNU scaffolds is more representative of the native morphology of CMs in vivo and indicates that the nanotopography of the electrospun scaffold is having a positive effect on cell morphology.

The cardiac ECM is composed of proteins that form a fibrous, anisotropic network to generate aligned CMs [92]. Muscle fibres are aligned within each layer of the myocardium to produce an anisotropic environment and the layers vary from approximately -70° in the epicardium to +80° in the endocardium [122]. This anisotropic organization helps with CM contractions and electromechanical coupling as the action potential is directed to propagate in a specific direction, thus helping with CM maturity [92]. In order to recapitulate the nanotopography of the myocardium, many groups have attempted to generate surfaces with similar nanopatterning to the native myocardium. One group studied the effect of different sized nanogroove widths (350-2000 nm) on the structural development of iPSC-CMs in vitro and found that iPSC-CMs had the highest amount of alignment, longest sarcomere length, and highest surface area when cultured on 800 nm nanogrooves [95]. Another group assessed the effect of 450 nm grooves with a 100 or 350 nm depth on neonatal rat CM organization and found that the nanotopography affected the organization of the actin cytoskeleton and focal adhesion complexes of cells, thus affecting the morphology, orientation, and contractile function of CMs [123]. The cells became elongated and aligned in the direction of the grooves and the alignment of CMs increased with groove depth [123]. The nanotopography of the D-PHI/PCNU scaffolds attempts to mimic the native nanotopography of the myocardium and helps to generate aligned iPSC-CMs. The aligned nature of the iPSC-CMs on the D-PHI/PCNU scaffolds could lead to enhanced iPSC-CM maturity and an improvement in electrophysiological response over time.

After 24 h, the iPSC-CMs had a significantly smaller cell area on random D-PHI/PCNU scaffolds compared to TCPS (Figure 4.9). The cell area on aligned D-PHI/PCNU scaffolds was smaller than that on TCPS, but the difference was not statistically significant. In general, CM
spreading is a function of matrix rigidity, where CMs are able to sense the stiffness of their environment using myosin contractions [212]. Pandey et al. showed that neonatal rat CMs had a faster spreading rate on stiffer matrices [212]. Since TCPS is much stiffer compared to D-PHI/PCNU scaffolds (~1 GPa compared to ~50 MPa respectively), this could explain the faster initial rate of iPSC-CM spreading on TCPS compared to D-PHI/PCNU scaffolds. Although the iPSC-CMs initially spread out faster on TCPS compared to D-PHI/PCNU scaffolds, there was no difference in cell area on all surfaces after 7 days. This indicates that the iPSC-CMs were able to effectively spread out to the same extent on the D-PHI/PCNU scaffolds as seen on TCPS surfaces after 7 days, despite the slower initial spreading rate.

The iPSC-CMs had a significantly smaller cell area after 24 h (300 to 700 μm²) compared to 7 days (1200 to 1400 μm²) for all surfaces (Figure 4.9). In general, iPSC-CMs more closely resemble the properties of fetal CMs that have a much smaller surface area (1000-1300 μm²) compared to adult CMs (10,000-14,000 μm²) [92,100,101]. This highlights the immature phenotype of iPSC-CMs and emphasizes the importance of developing substrates that can induce CM maturity. Since D-PHI/PCNU scaffolds are able to induce iPSC-CM alignment, they may potentially also be used to enhance iPSC-CM maturity through increasing iPSC-CM surface area during longer term cultures.

Overall, the stiffness of the ECM influences CM contractility, calcium handling, and cytoskeletal structure. The human myocardium has an elastic modulus of approximately 0.02 to 0.5 MPa [24]. In order to improve CM maturity, many groups have attempted to generate scaffolds with stiffness similar to that of the native myocardium. Although TCPS may be a suitable culture substrate for CMs in the short term, some groups have shown that due to the stiffness of TCPS surfaces, when iPSC-CMs were grown on TCPS for five weeks, there were signs of excessive mechanical stress and loss of sarcomere integrity [213]. Thus, to ensure that the D-PHI/PCNU scaffolds are more conducive to promoting CM maturity, more work has to be done to decrease the stiffness of D-PHI/PCNU scaffolds to a value more similar to that of the native myocardium. This could be accomplished by co-spinning the D-PHI/PCNU scaffolds with a softer material, like gelatin, to decrease scaffold stiffness. Incorporating gelatin into the bulk of the scaffold could help with improving scaffold mechanical properties while also enhancing CM attachment, as shown in this work.
4.6 Conclusion

This study investigated the compatibility of iPSC-CMs with an aligned electrospun nanofibrous D-PHI/PCNU scaffold for potential uses in cardiac tissue regeneration. The D-PHI/PCNU scaffold was coated with different surface protein coatings to enhance iPSC-CM adhesion. The scaffold was compatible with iPSC-CMs and supported high cell viability and adhesion on all surface protein coatings. There was a high expression of CM functional markers, cTnT and MLC, on D-PHI/PCNU scaffolds after 7 days. The ratio of MLC to cTnT positive cells was higher on aligned and random D-PHI/PCNU scaffolds compared to on TCPS, indicating a higher proportion of ventricular CMs on the scaffolds. Although there was a higher ratio of vim to cTnT positive cells, which corresponds to a higher proportion of fibroblasts, on the D-PHI/PCNU scaffolds compared to on TCPS, this could be beneficial when extending this work to use D-PHI/PCNU as a co-culture platform for CMs and fibroblasts. In addition, the aligned D-PHI/PCNU scaffolds were able to induce iPSC-CM alignment and elongation along the direction of fibres, which was not seen on random D-PHI/PCNU scaffolds and on TCPS. This elongated and aligned morphology of iPSC-CMs is more representative of the native morphology of CMs in their in vivo environment, and could help with enhanced maturity and electrophysiological response. Out of the surface protein coatings used on D-PHI/PCNU scaffolds, gelatin is the most favourable in terms of achieving a balance between adhesion, expression of functional markers, and inherent properties of the coating. Thus, gelatin can be used for future studies as a coating for D-PHI/PCNU scaffolds. Overall, the D-PHI/PCNU scaffold is highly compatible with iPSC-CMs, helps maintain a CM phenotype, and generates aligned iPSC-CMs that are more representative of their in vivo morphology. Thus, D-PHI/PCNU scaffolds should be further explored as a potential platform for cardiac tissue regeneration and repair after myocardium damage to treat heart failure.

4.7 Limitations

There are several limitations with the current work which should be considered and further examined for future work. The D-PHI/PCNU scaffold is stiffer than the native human myocardium so further work would be required to decrease the elastic modulus to generate a more representative scaffold for CM culture. This could be accomplished by incorporating softer
materials into the bulk of the D-PHI/PCNU scaffold (eg. gelatin) by co-spinning with D-PHI/PCNU. In addition, although Matrigel helped to promote iPSC-CM adhesion as a surface protein coating on the D-PHI/PCNU scaffolds, there are problems with heterogeneity and unknown elements, which would make it difficult for use in clinical applications. Furthermore, the iPSC-CMs used in these experiments displayed an immature phenotype that was more representative of fetal CMs rather than adult CMs. More mature CMs would be required for eventual clinical applications so various methods could be explored to enhance CM maturity (eg. co-culturing with fibroblasts, mechanical and electrical stimulation). Moreover, the immunofluorescence analysis performed in these experiments was semi-quantitative and limited the extent of analysis that could be performed. In the future, more quantitative analysis could be performed to study CM phenotype in more detail. For example, flow cytometry could be used to stain for populations of cells with specific cell markers to obtain a quantitative distribution of cell populations that are expressing different functional markers. Methods for removing CMs from the D-PHI/PCNU scaffolds would need to be developed to minimize cell loss.

4.8 Acknowledgements

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5 Chapter 5: Conclusions

5.1 Summary

The overall objective of this thesis was to investigate whether degradable polar hydrophobic ionic polyurethane (D-PHI) could be electrospun to generate a nanofibrous scaffold to support cardiomyocyte (CM) culture for applications in cardiac tissue regeneration. This main objective was broken down into two sub-objectives which were the synthesis and characterization of the electrospun D-PHI composite scaffold (Chapter 3), and the determination of induced pluripotent stem cell derived CM (iPSC-CM) compatibility on the scaffold (Chapter 4).

In Chapter 3, the synthesis and characterization of the electrospun D-PHI composite scaffold was described in the context of broad tissue engineering applications. First, D-PHI was incorporated with a degradable linear polycarbonate polyurethane (PCNU) and electrospun with in situ UV cross-linking to generate aligned nanofibrous scaffolds. Subsequently, the D-PHI/PCNU scaffold fibre morphology, cross-linking efficiency, surface energy, mechanical properties, \textit{in vivo} degradation and integration, and \textit{in vitro} cell compatibility were characterized. The D-PHI/PCNU scaffolds were found to have a high cross-linking efficiency. When compared to pure PCNU scaffolds, the composite scaffolds had a higher surface energy and lower stiffness. \textit{In vivo}, the D-PHI/PCNU scaffold degraded relatively slowly, but was well integrated with the surrounding tissue over time. \textit{In vitro}, the D-PHI/PCNU scaffold was able to support high levels of A10 vascular smooth muscle cell (SMC) viability and adhesion, with high expression of contractile marker alpha smooth muscle actin, after a 7-day culture period. These characterization results demonstrated that the D-PHI/PCNU scaffold could be used to mimic the native extracellular matrix (ECM) in order to generate a tissue engineered scaffold that supports functional tissue regeneration. (Chapter 3)

Given the successful generation of an electrospun nanofibrous D-PHI/PCNU scaffold with appropriate properties for tissue engineering applications, Chapter 4 aimed at investigating the CM compatibility of the scaffold for cardiac tissue engineering applications. To accomplish this, iPSC-CMs were cultured on D-PHI/PCNU scaffolds with different protein coatings (Matrigel, gelatin, and medium proteins) in order to determine the effect of the scaffold and the protein coatings on cell viability, adhesion, expression of functional markers, and cell alignment. D-
PHI/PCNU scaffolds were found to be able to support high iPSC-CM viability and adhesion on all surface protein coatings, while expressing high levels of cardiac troponin-T (cTnT) and myosin light chain 2 (MLC) functional markers after a 7-day culture period. There was a higher ratio of MLC to cTnT positive cells on the polyurethane scaffolds when compared to on TCPS, indicating a higher proportion of ventricular-like CMs. In addition, the iPSC-CMs were more aligned and elongated on protein coated aligned D-PHI/PCNU scaffolds when compared to gelatin coated random D-PHI/PCNU scaffolds or on gelatin coated TCPS. This indicated that the nanotopography of the scaffold could induce a morphological change in the iPSC-CMs that was more representative of in vivo CM morphology. These results demonstrated that the D-PHI/PCNU scaffold was compatible with iPSC-CMs and could be used for functional cardiac tissue regeneration. (Chapter 4)

5.2 Scientific Contributions

This thesis explored the use of a novel D-PHI polymer for cardiac tissue engineering applications. Previously, D-PHI flat films were prepared for vascular tissue engineering applications using a heat curing process and with porogens [18,21]. With the new capability described in this thesis to electrospin D-PHI to generate a nanofibrous scaffold, there are diverse opportunities to use D-PHI scaffolds for different tissue engineering applications. The nanofibrous structure of the D-PHI scaffolds introduces a new surface chemistry not previously seen on the D-PHI flat films. Compared to pure PCNU scaffolds, D-PHI/PCNU scaffolds have a higher surface energy, which often translates to enhanced protein adsorption and facilitated cell attachment. Furthermore, D-PHI/PCNU scaffolds have a lower stiffness compared to pure PCNU scaffolds, which could be helpful in softer tissue engineering applications, like cardiac tissue engineering. In addition, the nanofibrous nature of the scaffold helps to increase the surface area to volume ratio which could help with tissue biosynthesis, cell growth, and ultimately functional tissue regeneration [23]. (Chapter 3) The method employed to generate the D-PHI/PCNU scaffold was unique as the D-PHI monomers were cross-linked in-flight during the electrospinning process. This study demonstrated for the first time the ability to synthesize a cross-linked co-polymeric urethane elastomer, generated in-flight, while fibres were forming. This method of in-flight cross-linking during the electrospinning process had the potential of generating incomplete vinyl group polymerization, which are toxic. Therefore, it was important
to evaluate the vinyl monomer conversion and cell compatibility. The results showed high cross-linking efficiency and cell compatibility with A10 vascular SMCs. (Chapter 3)

The *in vivo* studies demonstrated that the electrospun D-PHI/PCNU scaffolds were well integrated into the tissue and did not elicit an adverse immune response after a 90-day implantation period. This showed that the combined D-PHI/PCNU chemistry and synthesis procedure yielded scaffolds that were compatible *in vivo*. However, biodegradation was slower than anticipated and this may be something to investigate in the future. (Chapter 3)

Furthermore, this work was the first to demonstrate the use of a D-PHI scaffold for culturing iPSC-CMs and showed the compatibility of the scaffold with these cells. The effect of three different surface protein coatings on the D-PHI/PCNU scaffolds was investigated. The results showed that the D-PHI/PCNU scaffold was able to support high cell viability, adhesion, and expression of functional markers, regardless of the surface protein coating used. It was also shown that the nanotopography of the aligned D-PHI/PCNU scaffold could induce alignment and elongation of the iPSC-CMs, which was not seen on random D-PHI/PCNU scaffolds and TCPS. The alignment of CMs was more representative of their native morphology *in vivo* and could help enhance maturity. (Chapter 4)

Overall, the generation of an electrospun nanofibrous D-PHI composite scaffold opens up the door for use in different tissue engineering applications with a variety of cell types. Both vascular SMCs and iPSC-CMs were found to be highly compatible with the D-PHI/PCNU scaffolds and similar results could be expected from other cell types. D-PHI possesses an immunomodulatory character and is conducive to cell adhesion, which is important for functional tissue engineering. Combined with the electrospinning technique, electrospun D-PHI composite scaffolds provide a powerful platform with a unique chemistry that could be used for a diverse range of tissue engineering applications.

### 5.3 Limitations

The current composition of the D-PHI/PCNU scaffold is limited in terms of its mechanical properties and degradation rates. These properties could be varied by changing the chemical composition as explained in more detail in the subsequent section. In addition, the iPSC-CMs
used in these experiments display a relatively immature phenotype, so more work could be done to enhance CM maturity when cultured on the D-PHI/PCNU scaffolds as described below. Finally, more quantitative analysis could be performed to study the integration and degradation of the D-PHI/PCNU scaffold in vivo and CM phenotype when cultured on the D-PHI/PCNU scaffolds.

5.4 Recommendations

1. **Varying D-PHI/PCNU scaffold composition:** The composition of the electrospun D-PHI/PCNU scaffold could be varied to generate a substrate that is more conducive for enhancing CM maturity. For example, the component ratios of D-PHI, molecular weight of PCNU, and ratio of D-PHI to PCNU could be varied. These changes will affect solution composition, viscosity, and fibre diameter. Additionally, ECM proteins (gelatin, fibronectin, collagen) could be incorporated into the polymer solution and co-spun during the electrospinning process \[16,79\]. Since gelatin has been shown to help with CM attachment as a surface protein coating, it could have a similar effect if it is incorporated into the bulk of the electrospun scaffold during electrospinning. Also, adding gelatin to the D-PHI/PCNU electrospun scaffold could help decrease the elastic modulus of the D-PHI/PCNU scaffolds to a stiffness more comparable to the native myocardium (0.02 to 0.5 MPa), which has been shown to help with CM maturity \[24,214,215\]. Others in the lab have shown that pure gelatin electrospun scaffolds have an elastic modulus of approximately 0.1 MPa and that 80:20 gelatin/PCNU electrospun scaffolds have a modulus of approximately 0.3 MPa, which is more similar to the myocardium stiffness. Furthermore, adding gelatin to the scaffolds could enable the ability to tune the degradation rate of the scaffold as gelatin degrades relatively quickly compared to D-PHI/PCNU \[216\].

2. **Functional electrical testing of CMs:** This thesis has shown that iPSC-CMs have high viability, adhesion, and expression of functional markers on the electrospun D-PHI/PCNU scaffold. In addition, the nanotopography of the D-PHI/PCNU scaffold was able to induce iPSC-CM alignment and elongation in the direction of fibres. Given these results, the next experiments to conduct are functional electrical tests of the CMs on the D-PHI/PCNU scaffolds to ensure that the CMs have appropriate electrophysiological properties. This work could be conducted in collaboration with Ren-Ke Li’s lab, similar to previous work they have published on their
polypyrrole-chitosan conductive biomaterial [217,218]. The fluorescent Fluo-4 AM calcium indicator could be used as an indirect indicator of action potential propagation to visualize the cytoplasmic calcium transient propagation. The calcium transients could be recorded using a camera and the speed of calcium transient propagation and synchronicity of beating could be evaluated. In addition, it would be interesting to determine if the direction of calcium transient propagation is in the same direction as fibres and whether this propagation is uni-directional. In order to determine whether CMs have good cell-cell connections to enable necessary cell communication and electrical coupling, immunostaining could be used to stain for gap junctions (eg. connexin 43 (Cx43)).

3. Effect of D-PHI/PCNU scaffold on CM maturity: The effect of the D-PHI/PCNU scaffold on CM maturity could be investigated through longer term culture of CMs on the scaffold. Currently, experiments have only grown CMs on the D-PHI/PCNU scaffolds for up to 7 days. Thus, it would be interesting to extend the culture period of CMs on the D-PHI/PCNU scaffolds to determine whether the scaffold could help enhance CM maturity. Many groups have shown that longer culture periods tend to help with CM maturity [92].

4. Co-culture of CMs with fibroblasts and endothelial cells: In vivo, CMs interact closely with other cells in the heart, including fibroblasts and endothelial cells (ECs). Non-CM cells have been shown to support the growth, proliferation, survival, differentiation, and maturation of CMs [26,124,126,127]. Thus, the co-culture of CMs with fibroblasts and ECs is important for recapitulating the interaction that CMs have with these cells in vivo [26,124,126,127]. When cardiac fibroblasts were co-cultured with CMs on chitosan nanofibres, the CMs had a more polarized morphology during long term culture as compared to a CM monoculture [78]. Another group showed that the co-culture of pluripotent stem cell derived CMs with cardiac fibroblasts in an electrically stimulated collagen/Matrigel construct increased CM maturity with an optimal cardiac fibroblast to CM ratio of 3:1 [119]. EC co-cultures with CMs have similarly shown enhanced results with ECs having the ability to create capillary-like networks that help with CM reorganization that was not seen in CM monocultures [127]. Also, the ECs promoted synchronized contraction of CMs and Cx43 expression [127]. Initially, fibroblasts could be co-cultured with CMs at a physiologically relevant ratio and then ECs could be further added to this co-culture system to promote CM maturity.
5. **Immunomodulatory nature of D-PHI/PCNU scaffolds:** The immunomodulatory nature of the electrospun D-PHI/PCNU scaffolds could be further investigated, similar to work that has previously been published on D-PHI flat films [19]. The activation of macrophages on electrospun D-PHI/PCNU scaffolds could be compared to PCNU scaffolds to determine if D-PHI is able to reduce the pro-inflammatory character of macrophages, similar to what was seen for D-PHI films [19].

6. **Mechanical and electrical stimulation of CMs:** Mechanical and electrical stimulation are vital for CM maturity [92]. In the heart, CMs constantly experience mechanical stimuli from stretching experienced during heart filling, hemodynamic loads from blood flow, and cytoskeletal contractions during heart ejection [92]. Thus, it will be important to simulate these mechanical stimulations on the D-PHI/PCNU scaffold with the help of bioreactors in order to enhance CM maturity. Many groups have used bioreactor systems to recapitulate the physiological mechanical stimuli in the heart and have shown improvements in CM maturity [92,109,114,115]. Similarly, electrical stimuli directly impact the electrophysiological function of CMs. Exogenous electrical stimuli could be used to mimic in vivo electrical impulses by directing electrical currents through CMs to improve CM electrophysiological maturity [116,117]. A combination of both mechanical and electrical stimuli could further enhance CM function and maturity [118,119].

7. **Generation of multi-layered scaffold:** The cardiac patch will need to have a clinically relevant thickness in order to generate functional tissue. The alignment of CMs on each layer could be varied to mimic the anisotropy of the heart. This could be accomplished by stacking multiple layers of cell seeded D-PHI/PCNU scaffolds to generate a thicker construct. The polymer compositions of each of the layers could be varied to modulate the degradation rates and cellular interaction. For example, a multi-layered scaffold composed of an aligned conductive nanofibre yarn network was able to mimic the myocardium anisotropy with aligned and elongated CMs on each layer [219]. Another study demonstrated the ability to generate a 5 mm thick, multi-layered patch composed of albumin electrospun fibre scaffolds [220]. The patch layers were grown separately and then glued together with an ECM-based glue prior to transplantation [220]. With the generation of a thicker tissue construct, oxygen and nutrient perfusion into the inner cell layers will need to be investigated.
8. *In vivo* testing of cell-seeded D-PHI/PCNU scaffold in myocardial infarction model:
Ultimately, the iPSC-CM seeded D-PHI/PCNU scaffold will need to be tested in an animal myocardial infarction model to determine whether the scaffold could help with tissue repair and regeneration. Likely, the first animal experiments would be in a small animal model (eg. rats) and involve a single layer cell-seeded scaffold. Methods to deliver the scaffold and secure it in place will also need to be investigated.
References


[38] A.P. Cotrim, B.J. Baum, Gene therapy: some history, applications, problems, and


[63] S.H. Kim, S.H. Kim, S. Nair, E. Moore, Reactive electrospinning of cross-linked poly(2-


[111] C. Mummery, D. Ward-van Oostwaard, P. Doevendans, R. Spijker, S. van den Brink, R.


A. Marsano, R. Maidhof, L.Q. Wan, Y. Wang, J. Gao, N. Tandon, G. Vunjak-Novakovic, Scaffold stiffness affects the contractile function of three-dimensional engineered cardiac


[190] I.C. Parrag, P.W. Zandstra, K.A. Woodhouse, Fiber alignment and coculture with


Appendices

Appendix A: Chapter 3 supplementary information

A1 Scaffold implantation site

Figure A 1: Subcutaneous implantation site of scaffold showing (A) scaffold implanted between muscle and skin layer of rat 30 days post-implant and (B) explanted section of skin with scaffold and blood vessel 7 days post-implant.

A2 Scoring system scale

Table A 1: Semi-quantitative histological scoring system. Higher scores are more indicative of better integration of the scaffold with surrounding tissue. Six 100 μm scaffold sections were analyzed per image with three sections in areas with low scaffold density and three sections in areas with high scaffold density if possible. Areas up to 100 μm away from the scaffold in a perpendicular direction were analyzed.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular infiltration</strong></td>
<td>0 cells</td>
<td>1-4 cells</td>
<td>5-9 cells</td>
<td>10 or more cells</td>
</tr>
<tr>
<td><strong>Vascularity</strong></td>
<td>0</td>
<td>1-4</td>
<td>5-9</td>
<td>10 or more</td>
</tr>
<tr>
<td><strong>Encapsulation</strong></td>
<td>Dense tissue encapsulation</td>
<td>Moderate encapsulation</td>
<td>Slight encapsulation</td>
<td>No encapsulation</td>
</tr>
</tbody>
</table>
A3 Representative histology figures at each scoring scale

**Cellular infiltration**

0

1

2

3

**Vascularity**

0

1

2

3

**Encapsulation**

No samples in this category

---

**Figure A 2:** Representative H&E stained histology images for each category of the scoring scale. S: scaffold, I: infiltrating cell, B: blood vessel
A4 Scaffolds with different scaffold densities

Figure A 3: Scaffolds with (A) low scaffold density and (B) high scaffold density.

A5 Individual scoring system results

Figure A 4: Histological scoring system (range is 0 to 9) results for PCNU and D-PHI/PCNU scaffolds after 7, 30, 60, 90 days separated between areas with high scaffold density and low scaffold density. Box shows mean ± standard error, whiskers show range of data. (n = 11-12)
Figure A 5: (A) Immunofluorescence staining of SMCs for SMA (green) on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS after seeding, 24 h, and 7 days. (B) SMA mean fluorescence intensity normalized to cell number on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS. Two images were taken per scaffold, two scaffolds were used for each experiment, and the experiment was repeated three times. Box shows mean ± standard error. *p < 0.05 for PCNU scaffolds after seeding compared to after 24 h and 7 days, after 24 h compared to after 7 days. *p < 0.05 for D-PHI/PCNU scaffolds after seeding compared to after 7 days, after 24 h compared to after 7 days. *p < 0.05 for TCPS after seeding compared to after 24 h and 7 days.
A7 DNA content of SMCs on scaffolds and TCPS

**Figure A 6:** DNA content on D-PHI/PCNU scaffolds, PCNU scaffolds, and TCPS after 24 h and 7 days. The TCPS DNA data was obtained using a different DNA assay protocol, as described previously [221], due to limitations with performing the same protocol that was used for the scaffolds. The data was adjusted to samples without cells to account for differences between the two DNA protocols. Box shows mean ± standard error. (n = 9)
Appendix B: Method development

B1 Method development for D-PHI/PCNU electrospun scaffold optimization

An appropriate setup was designed to hold the UV lamp in the electrospinning machine enclosure and protect it from polymer overspray during electrospinning. The UV lamp holder includes a removable, transparent front glass panel to protect the UV lamp from polymer overspray, two side panels to protect the lamp, and a base with variable height. The base of the lamp setup consists of a square board with two posts to enable stacking of additional boards to increase the height of the UV lamp if necessary. All parts are made of wood (except for the front panel) to prevent static charge build-up. A L-bracket is used to secure the UV lamp to the base of the lamp setup.

The electrospinning process depends on several parameters: solution composition, electrospinning settings, UV lamp setup, and environmental conditions. Solution composition parameters include the ratio of D-PHI to PCNU, the ratio of the three components of D-PHI (a divinyl oligomer (DVO), methacrylic acid (MAA), and methyl methacrylate (MMA)), the weight percentage of PCNU in the solvent, the molecular weight of PCNU, and solution viscosity. The electrospinning settings that can be changed are the voltage difference, flow rate of polymer solution, tip-to-collector distance between the syringe tip and the collecting mandrel, and the mandrel speed. In terms of the UV lamp setup, the distance between the UV lamp and the syringe tip and the distance between the UV lamp and collecting mandrel can be varied. Lastly, environmental conditions involve the surrounding room temperature and humidity.

For the preliminary testing of the lamp, only a few parameters were selected in order to minimize the number of parameters to vary. Other parameters were fixed or monitored to ensure the variation was minimized. The factors to vary were changed one at a time to determine the effect on the generation of fibres. Environmental conditions could not be easily controlled, but they stayed within a reasonable range during experimental testing (21 – 23 °C and 20 – 26 % humidity) and the fluctuations did not affect fibre formation. The factors, their tested ranges, optimal value, and effect on electrospinning are summarized in Table B 1. The tested ranges were based on literature results if available.
<table>
<thead>
<tr>
<th>Factor</th>
<th>Range Tested</th>
<th>Optimal Value/Range</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance between lamp and collecting mandrel</td>
<td>10 – 20 cm</td>
<td>20 cm</td>
<td>A shorter distance results in a higher light intensity but more polymer overspray</td>
</tr>
<tr>
<td>Distance between lamp and syringe tip</td>
<td>5 – 7 cm</td>
<td>5 cm</td>
<td>A shorter distance leads to better curing of the polymer stream as it is contacted by the UV light sooner after leaving the syringe</td>
</tr>
<tr>
<td>Tip-to-collector distance</td>
<td>18 cm</td>
<td>18 cm</td>
<td>Not varied, standard to have ~1 kV/cm</td>
</tr>
<tr>
<td>Voltage difference</td>
<td>18 – 23 kV</td>
<td>19 – 22 kV</td>
<td>Voltage difference can be varied between 19 – 22 kV without affecting fibre formation, higher voltage difference (23 kV) does not result in fibre formation</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.25 – 1.00 mL/h</td>
<td>0.25 – 0.75 mL/h</td>
<td>Faster flow rate (0.75 mL/h) results in thicker fibres or no fibre formation; slower flow rates were not tested due to time constraints</td>
</tr>
<tr>
<td>Mandrel speed</td>
<td>6.43 – 12.07 m/s</td>
<td>7.64 – 10.86 m/s</td>
<td>Slower mandrel speed (6.43 m/s) and faster mandrel speed (12.07 m/s) do not result in fibre formation</td>
</tr>
<tr>
<td>PCNU molecular weight (MW)</td>
<td>100,000, 140,000 polystyrene equivalent weight average molecular weight</td>
<td>140,000</td>
<td>Higher MW facilitates the formation of more uniform fibres with an increased D-PHI to PCNU ratio</td>
</tr>
<tr>
<td>Ratio of components of D-PHI</td>
<td>1:5:15 DVO:MAA:MAA</td>
<td>1:5:15</td>
<td>Not varied as this was the standard ratio used in previous experiments with D-PHI for vascular tissue engineering</td>
</tr>
<tr>
<td>Ratio of D-PHI to PCNU</td>
<td>50:50 – 65:35 D-PHI/PCNU</td>
<td>Depends on interaction with cells, likely higher proportion of D-PHI will be beneficial</td>
<td>Higher proportion of D-PHI results in thicker fibres</td>
</tr>
<tr>
<td>Weight percentage of PCNU in HFIP</td>
<td>15 – 20 wt. %</td>
<td>15-16 wt. %</td>
<td>Affects solution viscosity</td>
</tr>
<tr>
<td>Viscosity</td>
<td>60 – 120 s</td>
<td>90 – 120 s</td>
<td>Lower viscosity (~80 s) prevents the formation of fibres; higher viscosity (~140 s) can result in solution curing before reaching the mandrel</td>
</tr>
</tbody>
</table>
A factorial design was conducted for the 65:35 D-PHI/PCNU ratio to determine which factors have a significant effect on the morphology of the nanofibres. The most important factors from the preliminary testing were chosen as the factors of interest for the factorial design: viscosity, voltage difference, flow rate, and mandrel speed (Table B 2). Two zero reference points were conducted. The factors that were kept constant were the distance between the lamp and collecting mandrel (20 cm), distance between the lamp and syringe tip (5 cm), tip-to-collector distance (18 cm), voltage difference (19 kV), PCNU molecular weight (140,000), ratio of components of D-PHI (1:5:15), and weight percentage of PCNU (~15.5 wt. %). The order of trials was randomized to minimize the effect of environmental factors and other random factors that could not be controlled. All runs were replicated twice.

Table B 2: Parameters for factorial design experiment

<table>
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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Viscosity</td>
<td>95 ± 5 s</td>
<td>105 ± 5 s</td>
<td>115 ± 5 s</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.25 mL/h</td>
<td>0.5 mL/h</td>
<td>0.75 mL/h</td>
</tr>
<tr>
<td>Mandrel speed</td>
<td>7.64 m /s</td>
<td>9.25 m/s</td>
<td>10.86 m/s</td>
</tr>
</tbody>
</table>

Pure PCNU fibres have an average fibre diameter of 275 ± 100 nm and alignment of 0.65 (perfect alignment = 1, absolute randomness = 0) and were shown to be compatible with annulus fibrosus cells and gingival fibroblasts, so this was used as a benchmark for the D-PHI/PCNU nanofibres (Figure B 1A). Also, Carson et al. demonstrated that human iPSC-CMs had improved organization and structural development (increased cell area, cell alignment, and sarcomere length) on 800 nm nanogrooves [95]. Thus, the initial goal for electrospinning was to generate fibres with an approximate diameter of 800 nm (or smaller) and alignment of approximately 0.65, while having the maximal ratio of D-PHI/PCNU.

For a 50:50 D-PHI/PCNU solution with a PCNU polystyrene equivalent weight average molecular weight of 100,000, the most uniform and aligned fibres were generated using a 20 wt. % PCNU solution, 112 ± 14 s viscosity, 0.25 mL/h flow rate, and mandrel speed of 9.25 m/s. The voltage difference could be varied between 19 kV to 22 kV without affecting fibre formation. The average fibre diameter was 410 ± 350 nm and the alignment was 0.60 (n = 5) (Figure B 1B). There were some thicker fibres interspersed among the thinner fibres in the D-
PHI/PCNU scaffolds, leading to a slightly larger average fibre diameter and larger standard deviation. In addition, some merging of the fibres was observed due to the presence of D-PHI.

Upon using the same conditions for a 65:35 D-PHI/PCNU solution, fibre formation was not as robust due to the higher percentage of D-PHI in the solution. The average fibre diameter was 851 ± 758 nm and alignment was 0.61 (n = 2) (Figure B 1C). With the higher ratio of D-PHI/PCNU, the standard deviation in fibre diameter was a lot larger due to the presence of thick fibres interspersed among the thinner fibres.

Since the higher ratio of D-PHI relative to PCNU resulted in the formation of more non-uniform fibres, a PCNU with a higher polystyrene equivalent weight average molecular weight (140,000) was used. Using the higher PCNU molecular weight and a 65:35 D-PHI/PCNU solution, consistent fibres were obtained with a flow rate of 0.25 mL/h and 0.5 mL/h and voltage difference of 19 kV. The results are summarized in Table B 3 and representative fibres are shown in Figure B 1D. Using a higher molecular weight PCNU, smaller diameter fibres with a smaller standard deviation could be achieved with a faster flow rate. The fibre alignment was also comparable to that obtained with pure PCNU fibres. This optimal set of conditions with a 0.5 mL/h flow rate was used as the baseline condition for the factorial design testing.

From the factorial design, it was determined that the flow rate and viscosity had a borderline significant effect on the fibre diameter (p = 0.0560 and p = 0.0975 respectively), flow rate and the interaction between the viscosity and flow rate had a significant effect on the standard deviation of the fibre diameter (p = 0.0182 and p = 0.0342), and there were no significant effects for the fibre alignment. From the results, it was determined that to obtain the thinnest fibres (~530 nm), a viscosity of 115 s and flow rate of 0.25 mL/h should be used. In addition, a 95 s viscosity and a 0.25 mL/h flow rate would generate fibres with the smallest standard deviation in fibre diameter (~220 nm). Thus, to obtain a compromise between obtaining thinner diameter fibres with a small standard deviation, it would be best to use an intermediate viscosity of 105 s and a flow rate of 0.25 mL/h to generate fibres with a diameter of approximately 630 ± 235 nm. Since the mandrel speed does not affect fibre diameter, an intermediate speed of 9.25 m/s could be used.
Figure B 1: SEM images of electrospun fibres (A) pure PCNU fibres (B) 50:50 D-PHI/PCNU fibres (with 100,000 molecular weight PCNU) (C) 65:35 D-PHI/PCNU fibres (with 100,000 molecular weight PCNU) (D) 65:35 D-PHI/PCNU fibres (with 140,000 molecular weight PCNU)

Table B 3: Summary of electrospinning results using a 140,000 polystyrene equivalent weight average molecular weight PCNU

<table>
<thead>
<tr>
<th>Flow rate</th>
<th>Average diameter</th>
<th>Alignment</th>
<th>Viscosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 mL/h (n = 4)</td>
<td>710 ± 450 nm</td>
<td>0.69</td>
<td>97 ± 8 s</td>
</tr>
<tr>
<td>0.5 mL/h (n = 2)</td>
<td>526 ± 335 nm</td>
<td>0.67</td>
<td>104 ± 2 s</td>
</tr>
</tbody>
</table>

B2 Method development with embryonic stem cell derived cardiomyocytes

ESC-CMs were obtained through a collaboration with the Centre for Commercialization of Regenerative Medicine, a not-for-profit company that is optimizing and scaling up the ESC-CM differentiation protocol. CMs were differentiated from the ESI-17 cell line (ESI-Bio Stem Cell Solutions) in suspension culture following an established 20 day differentiation protocol and subsequently cryopreserved [186]. ESC-CMs were characterized by flow cytometry for cardiac troponin-T (cTnT) and myosin light chain 2 (MLC) and all cell vials used were greater than 80%
positive for cTnT. Problems with reproducibility of results were encountered when working with the ESC-CMs. Some of these issues are discussed below with suggestions on how to mitigate the issues.

**B2.1 Freezing density of ESC-CMs**

Initial exploratory experiments with the ESC-CMs were on a relatively small scale. Also, since the ESC-CMs were thawed and plated directly on D-PHI/PCNU scaffolds for experiments without a culture period in between (CMs do not proliferate and cannot be re-plated by removing cells from surfaces easily without losing a lot of cells), it was desirable to have ESC-CMs in small volume cryovials to avoid wasting cells. Thus, 1 million cells per 1 mL cryovial were requested from CCRM. Normally, CCRM freezes ESC-CMs in cryovials ranging in size from 5 million to 1 billion ESC-CMs; however, they had never frozen cells at volumes less than 5 million cells per vial prior to this collaboration. When these smaller vials of ESC-CMs were thawed and seeded onto surfaces using the same protocol used by CCRM, very poor cell viability (~40 %) and adhesion was experienced. It was determined that this low cell viability and adhesion was due to the low cell freezing density used as it seemed like the ESC-CMs had a preference to be frozen at a higher density. Other groups have reported similar issues, such as one group reporting the need to freeze a minimum of 2 million iPSC-CMs per 1 mL vial [90]. After encountering this issue, 5 million cells per cryovial samples were used.

**B2.2 Poor CM viability out of thaw**

Even after using the larger cell vials, there was variable cell viability out of thaw (ranging from 40% to 80%). The 5 million cells found in the vial consisted of both live and dead cells, so the number of live cells was a lot smaller than the total cell population. Coupled with this low cell viability out of thaw, the ESC-CMs also had variable cell adhesion rates (CCRM indicated that some batches of ESC-CMs could have adhesion rates as low as 40%). Thus, even if the cells were viable out of thaw, a large proportion of the cells may be unable to attach to the surface and would be washed away during media changes. Many of the cells were rounded and did not spread out on the culture surface. In addition, since there were a lot of dead, rounded cells in the ESC-CM culture, they interfered with the viable ESC-CMs forming the necessary cell-cell connections required to form a healthy, confluent, beating monolayer. In most of the ESC-CM
experiments, beating was only observed in small clusters of cells, instead of throughout the culture well as desired. Only one set of experiments was successful in generating a confluent, beating monolayer of ESC-CMs, but these results could not be reproduced, even with identical protocol and culture conditions. In general, this shows that there was high variability between each of the frozen ESC-CM vials, thus preventing reproducibility of results.

**B2.3 Problems with scaling down culture systems**

Another problem experienced with the ESC-CM culture was problems with scaling down the culture size. Normally, CCRM cultures the ESC-CMs in 6 well plates and the smallest well size they have used was 12 well plates. However, the D-PHI/PCNU scaffold holders are similar in size to a 96 well plate and thus, the cell seeding density, Matrigel coating, and media used had to be appropriately scaled down proportional to the well surface area. In a 6 well plate, each well normally contains 1 million ESC-CMs, 1 mL of Matrigel, and 1 mL of media. This approximately scales down to 50,000 ESC-CMs, 50 μL Matrigel, and 50 μL of media per well of a 96 well plate. However, it was determined that the 50,000 cell seeding density was too low to achieve a confluent monolayer (due to the low cell viability out of thaw and adhesion). By increasing the cell seeding density to 150,000 ESC-CMs per well, the higher cell density could help mask some of the effects of poor viability and adhesion. In addition, it was determined that 100 μL of Matrigel solution was better for cell attachment compared to 50 μL of Matrigel. In terms of the culture media, 50 μL of media was often insufficient for ensuring sufficient media coverage, and thus 100 μL of media was used instead. This problem with scaling down the culture well size has been reported by a few other groups, but the exact reason for the configuration dependent culture is not known.

**B2.4 Effect of various CM culture media**

For the ESC-CM culture experiments, DMEM/F12 based culture media was used throughout the experiment; however, for experiments performed by CCRM, they often switch to an iCell CM maintenance media (CDI) after the first day of culture as this media can help preferentially select for CMs over other cell types (eg. fibroblasts that proliferate quickly). This selection media is rougher on the ESC-CMs and could compromise their viability. Thus, since there were many
issues experienced with basic ESC-CM viability and adhesion, the gentler DMEM/F12 based media was used for the entire culture period.

**B2.5 Proportion of cTnT positive cells**

When differentiating ESCs to CMs, variable purities of ESC-CMs were achieved. Most of the other cells consist of fibroblasts and other cell populations. The CM vials used for experiments were greater than 80% positive for cTnT. However, due to the low viability and adhesion experienced, a greater proportion of the more sensitive CMs may not have survived the thawing and adhesion process, while fibroblasts could survive a lot easier. Thus, an 80% cTnT positive cell population may not be sufficient for generating a sufficiently pure population of ESC-CMs for experiments.

**B2.6 Comparison of ESC-CMs with commercial iPSC-CMs**

In comparison, the commercial CDI iPSC-CMs produced more reproducible results. Each vial came with a guaranteed 5 million live cells and viability was always greater than 80%. In addition, the iPSC-CMs adhered a lot faster compared to the ESC-CMs. The media could be changed 4 hours after seeding the iPSC-CMs, since they were sufficiently adhered, but the ESC-CM media could only be changed 24 hours after seeding cells and there was still poor adhesion of the ESC-CMs. The iPSC-CMs also had greater cTnT purity (greater than 95%) compared to the ESC-CMs, thus producing a purer CM population.

One problem that was encountered for the iPSC-CMs was when the iPSC-CMs were grown on gelatin coated glass surfaces. After 5 days of culture, the iPSC-CMs began to detach and lift off the glass surface. This was due to the interaction of the CM ECM on the gelatin coated glass substrate. To mitigate this issue, the iPSC-CMs can either be grown on gelatin coated TCPS or fibronectin coated glass as a control.
Appendix C: Protocols

C1 Preparing D-PHI/PCNU solutions for electrospinning

1) Weigh out DVO into a scintillation vial
   a) Weigh empty bottle
   b) Weigh DVO in bottle, being careful not to get any DVO on the rim or sides of the bottle
   c) Place bottle with DVO in vacuum oven and vacuum for 30 min at room temperature
   d) Reweigh DVO to determine remaining mass of DVO

2) Calculate required amounts of MMA, MMA, and photoinitiator from Excel spreadsheet

3) Add MMA to bottle dropwise (okay if mass exceeded by 0.01 g)

4) Add MAA to bottle dropwise (okay if mass exceeded by 0.005 g).

5) Add photoinitiator using the microbalance by volume (calculated from mass and density of photoinitiator)

6) Parafilm bottle and wrap in aluminum foil to protect from light

7) Allow D-PHI solution to mix on shaker until monomers completely dissolved (takes a few hours)

8) Weigh out required mass of PCNU into a scintillation vial

9) Add HFIP to PCNU to achieve the desired weight percentage of solution

10) Parafilm bottle and wrap in aluminum foil to protect from light

11) Allow PCNU solution to mix on shaker until completely dissolved (takes a few hours)

12) Add D-PHI solution to PCNU bottle to achieve desired ratio of D-PHI/PCNU (by total mass of D-PHI monomers and mass of PCNU without solvent)
13) Parafilm bottle and wrap in aluminum foil to protect from light

14) Allow D-PHI/PCNU solution to mix on shaker until a homogeneous solution obtained (takes a few hours)

**C2 Protocol for seeding cells on electrospun scaffolds**

1) Vacuum dry scaffolds at room temperature for 24-48 h

2) Autoclave scaffold holders and two tweezers (flat tip tweezers are better)
   a) Cut off lid of 500 μL microfuge tube
   b) Trim cap to fit into 48 well plate
   c) Cut pointed tip off microfuge tube (at end of frosting)
   d) Ensure holder can fit into well in 48 well plate

3) Put scaffold on holders in biosafety cabinet
   a) Make fresh 70 % ethanol with MilliQ water
   b) Cut aluminum foil with scaffold into small rectangular pieces slightly larger than the surface area of the holder
   c) Submerge scaffolds attached to aluminum foil in 70 % ethanol for 2-3 min in sterile petri dish
   d) Scaffold should come off aluminum foil easily by gently pulling them off with tweezers
   e) Put cap of microfuge tube into 70 % ethanol in petri dish
   f) Use both flat tweezers to gently pull scaffold on top of cap of microfuge tube and ensure membrane is flat and smooth
   g) Remove cap with scaffold from 70 % ethanol and place on a sterile petri dish to dry for 2-3 min
h) Carefully cap the tube evenly on all sides to keep scaffold flat in holder

i) Check to ensure that there are no tears or wrinkles in scaffold

j) Place holder with scaffold in 48 well plate

4) Add 70% ethanol to fill each holder for ~24h

5) Parafilm 48 well plate to prevent ethanol from evaporating

6) Wrap with Al foil

7) After ~24h, remove ethanol from scaffolds and dry scaffold in biosafety cabinet with the blower and sash left open

a) Aspirate ethanol

b) Remove the lid of the 48 well plate and cover well plate with aluminum foil

c) Allow the scaffolds to fully dry (takes approximately 12 h)

8) After scaffolds are dry, add PBS (+/+) to the scaffolds for 24 h to remove any residual monomers or contaminants

9) Remove PBS from scaffolds and allow scaffolds to dry in biosafety cabinet

10) Store scaffolds in a sterile environment or use immediately for cell culture

11) Seed cells on scaffold at an appropriate seeding density (surface area of scaffold exposed ~28.2 mm²)

a) For A10 smooth muscle cells, pre-condition scaffolds with 50 μL media per scaffold at 37°C, 5% CO₂ for at least 1 h

b) For cardiomyocytes, coat scaffolds with the appropriate extracellular matrix coating prior to seeding cells

c) Seed cells on scaffolds
12) Incubate cells at 37°C, 5% CO₂

Note: Depending on the cell type, it may help to seed cells with a smaller volume of media and add more media after cells have sufficiently attached.

**C3 Live dead staining protocol**

1) Aspirate media

2) Wash cells with PBS (+/+) for a few minutes

3) Incubate cells in live/dead stain for 30 min in the dark at room temperature
   a) 200 μL/well
   b) 2uM calcein
   c) 4uM ethidium homodimer

4) Rinse with PBS (+/+) for a few minutes

5) Counter-stain cells with Hoechst if required

6) Keep scaffolds in PBS (+/+) before imaging

7) Remove tube of holder and place scaffolds on caps face-down on a coverslip with PBS

**C4 SEM preparation protocol**

1) Aspirate media

2) Wash cells with PBS (-/-) for a few minutes

3) Fix cells with 3% glutaraldehyde in PBS (-/-) (200 μL per scaffold) for at least 2 hours or up to 24h (leave at 4°C overnight).

4) Dehydrate in increasing concentrations of ethanol (diluted in MilliQ water)
   a) 30 %, 50 %, 70 %, 90 %, 100 % (at least 30 min each)
5) Dehydrate in increasing concentrations of hexamethyldisilazane (HMDS) (diluted in 100 % ethanol)
   a) 30 %, 50 %, 70 %, 90 %, 100 % (15 min each)

6) Add 200 μL 100 % HMDS to scaffolds and leave lid of well plate open overnight in the fume hood for HMDS to evaporate slowly

7) The next day after samples are dry, sputter coat samples or move samples to desiccator until ready for sputter coating

8) Sputter coat samples by carefully removing tube of holder and placing cap of holder on SEM stub affixed with carbon tape

**C5 Immunostaining protocol for scaffolds**

1) Wash cells with PBS

2) Fix cells with 4 % paraformaldehyde for 10 min at room temperature (200 μL/scaffold)

3) Wash three times with ice cold PBS

4) Antigen retrieval
   a) Make 0.1 % w/v Trypsin-CaCl$_2$ solutions in DI water (pre-warmed to 37 °C)
   b) Add 200 μL/scaffold and incubate for 30 min at 37 °C

5) Wash twice with PBS

6) Permeabilization (required if target protein intracellular)
   a) Incubate scaffolds with 0.2 % Triton X-100 in PBS for 10 min (200 μL/scaffold)
   b) Wash cells once with PBS for 5 min

7) Rinsing step
a) Wash scaffolds 3 x 5 min with 0.05 % Tween-20 in PBS at room temperature

8) First blocking

a) Prepare 3 w/v% bovine serum albumin (BSA) solution in PBS

b) Pre-warm BSA solution at 37 °C for 10 min

c) Incubate scaffolds in BSA solution for 20 min at 37 °C (200 μL/scaffold)

9) Rinse once with PBS for 1 min

10) Adding primary antibody

a) Prepare 0.3 v/v% Triton X-100 solution in PBS (keep on ice)

b) Dilute primary antibody in Triton X-100 solution as appropriate

c) Add 150 μL primary antibody solution per scaffold

d) Parafilm well plate with scaffolds and cover with aluminum foil

e) Place scaffolds in fridge overnight

11) Rinsing step

a) Wash scaffolds 3 x 5 min with 0.05 % Tween-20 in PBS at room temperature

12) Second blocking

a) Use the same animal serum that the secondary antibody was raised in (eg. for goat anti-mouse secondary antibody, use 10 % goat serum)

b) Ensure serum is heat inactivated (65 °C for 30 min)

c) Make 10 % serum in PBS (keep on ice)

d) Add 200 μL serum solution per scaffold
e) Incubate for 30 min at room temperature

13) Adding secondary antibody
   a) Keep room dark and lights off
   b) Dilute secondary antibody in 10% animal serum as appropriate
   c) Add 150 μL secondary antibody solution per scaffold
   d) Incubate for 30 min in the dark at room temperature

14) Rinsing step
   a) Wash scaffolds 3 x 5 min with 0.05% Tween-20 in PBS at room temperature

15) Nucleus staining
   a) Dilute Hoechst in PBS at 1:1000
   b) Add 150 μL Hoechst solution per scaffold
   c) Incubate for 5 min in the dark at room temperature

16) Rinsing step
   a) Wash scaffold 5 x 1 min with PBS at room temperature

17) Add PBS to scaffolds and store in fridge until imaging

C6 FFT fibre and cell alignment analysis protocol
1) Open image file in ImageJ
2) Make a rectangular box within image (avoid scale bar)
3) Process → FFT → FFT
4) Image → Transform → Rotate 90 Degrees Left
5) Draw a perfect circle in FFT image (ensure width and height of circle are equal)

6) Plugins → Oval Profile (must be installed first)

7) Choose parameters: Number of Points: 360, Analysis mode: Radial Sums, uncheck Show Hotspots

8) Press List to get Plot Values

9) Save values

10) Copy values to Excel

11) Find the lowest value and subtract if from every value

12) Calculate the alignment order parameter (S) using the formula:

$$S = \frac{\sum_{\theta=1}^{180} F_\theta [3 \sin^2(\theta + \alpha) - 1]}{2 \sum_{\theta=1}^{180} F_\theta}$$

where $F_\theta$ is the alignment value at angle $\theta$ and $\alpha = 90^\circ - \theta_p$ is the image deviation of FFT alignment peak angle $\theta_p$ from $90^\circ$. For perfect alignment, $S = 1$, for absolute randomness, $S = 0$.

**Figure C 1**: Protocol for FFT fibre and cell alignment analysis
C7 Cell viability analysis protocol

1) Counting live cells
   a) Open live cell channel image in ImageJ
   b) Perform appropriate thresholding of the image (Image → Adjust → Threshold)
   c) Apply the Watershed function to separate cells that are touching (Process → Binary → Watershed)
   d) Count cells (Analyze → Analyze Particles, set size range and circularity as appropriate)

2) Counting dead cells
   a) Open dead cell channel image in ImageJ
   b) Perform appropriate thresholding of the image (Image → Adjust → Threshold)
   c) Count cells (Analyze → Analyze Particles, set size range and circularity as appropriate)

3) Calculate cell viability using the formula:

\[
\text{% cell viability} = \frac{\text{live cells}}{\text{live + dead cells}} \times 100\%
\]

Figure C 2: Protocol for live/dead cell viability analysis