Angiogenesis in Patches and Injectable Biomaterials for Cardiac Repair

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

Treatment of cardiac diseases involves transplantation of donor hearts, since the damaged heart has limited self-regeneration potential. An alternative treatment option has emerged as engineered cardiac tissues, grown in vitro by cultivation of cardiac cells on biomaterials, have comparable properties to native myocardium and can be implanted for cardiac repair. Major current limitations are a viable cell source and adequate vascularization to support cell survival. In this thesis, two proangiogenic biomaterials, a scaffold and a hydrogel, were developed to achieve vascularization in vitro and in vivo for cardiac repair. Scaffold patches are suitable for repairing congestive heart failure or congenital malformations, while injectable biomaterials allow minimally-invasive treatment post-myocardial infarction (MI). In the first aim, a collagen scaffold with covalently immobilized vascular endothelial growth factor (VEGF) was developed, and improved cell mobilization, survival and proliferation when used for free wall repair in adult rats. This increased angiogenesis, which aided in retaining the biomaterial size to allow tissue growth. In the second aim, a collagen-chitosan hydrogel with encapsulated thymosin β4 (Tβ4) was developed to 1) recruit cells from the heart epicardium for repair post-MI in vivo, and 2) guide capillary outgrowths from arteries and veins to form oriented capillary structure for in vitro cardiac tissue engineering. Results showed that the encapsulation of Tβ4 into collagen-chitosan...
hydrogels led to cell outgrowths from rat or mouse cardiac explants in vitro. A portion of the recruited cells were CD31-positive endothelial cells (ECs) that formed tubes. The hydrogel was injected in vivo to increase vascularization and number of cardiomyocytes within the infarct area post-MI, which improved left ventricular wall thickness. Tβ4-hydrogel also promoted the outgrowth of capillaries from vascular explants that followed the direction of the hydrogel-coated grooves of a micropatterned polydimethylsiloxane (PDMS) substrate. These capillary outgrowths eventually formed a vascular bed for engineering vascularized cardiac tissues. This thesis presents two bioinstructive biomaterials with sustained and localized delivery of angiogenic molecules to be used for in situ cardiac repair based on improved vascularization. The use of cell-free bioactive materials overcomes limitations of cell isolation and expansion as required for cell therapies or implantation of engineered tissues.
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Susanna and my sister Mary, who support and believe in me unconditionally, regardless of what career path I take.
Declaration of Co-Authorship

The original scientific content of the thesis is comprised of three previously published, peer reviewed articles in internationally recognized journals and a fourth article submitted. These articles were primarily the work of Loraine Chiu. The contributions of co-authors are stated in the thesis, in conformity with the requirements for the degree of Doctor of Philosophy.
Abstracts of Articles Appearing in the Thesis

Biodegradable collagen patch with covalently immobilized VEGF for myocardial repair


Vascularization of engineered tissues *in vitro* and *in vivo* remains a key problem in translation of engineered tissues to clinical practice. Growth factor signalling can be prolonged by covalent tethering, thus we hypothesized that covalent immobilization of vascular endothelial growth factor (VEGF-165) to a porous collagen scaffold will enable rapid vascularization *in vivo*. Covalent immobilization may be preferred over controlled release or cell transfection if the effects are desired within the biomaterial rather than the surrounding tissue. Scaffolds were prepared with 14.5 ± 1.4 ng (Low) or 97.2 ± 8.0 ng (High) immobilized VEGF, or left untreated (control), and used to replace a full right ventricular free wall defect in rat hearts. In addition to rapid vascularization, an effective cardiac patch should exhibit neither thinning nor dilatation upon implantation. *In vitro*, VEGF enhanced the growth of endothelial and bone marrow cells seeded onto scaffolds. *In vivo*, High VEGF patches had greater blood vessel density (*p* < 0.01) than control at Day 7 and 28 due to increased cell recruitment and proliferation (*p* < 0.05 vs. control). At Day 28, VEGF-treated patches were significantly thicker (*p* < 0.05) than control, and thickness correlated positively with neovascularization (*r* = 0.67, *p* = 0.023). Importantly, angiogenesis in VEGF scaffolds contributed to improved cell survival and tissue formation.

**Contributions:** L.L.Y.C.-concept and design, performed all experiments and data analysis, manuscript writing; Y.M.-concept and design, performed surgical work and data analysis, manuscript writing; M.C.-concept and design, data interpretation; R.D.W., M.R., R.K.L.-concept, data interpretation, final approval of manuscript
Controlled release of thymosin β4 using collagen-chitosan composite hydrogels promotes epicardial cell migration and angiogenesis


Rapid vascularization at the infarcted site is crucial for cardiac repair following myocardial infarction. Thymosin β4 (Tβ4), a 43-amino acid peptide, is both angiogenic and cardioprotective. Tβ4 in soluble form was previously shown to promote cell migration from quiescent adult cardiac explants. Here we developed a collagen–chitosan hydrogel for the encapsulation of Tβ4, which allowed its controlled release over 28 days to elicit localized and prolonged effects. Contrastingly, Tβ4 was fully released over 3 days when encapsulated in collagen-only hydrogels due to charge repulsion and lack of interconnected pores as shown by SEM. The charge of encapsulated molecules affected their release from collagen–chitosan hydrogels. While the release of neutral polyalanine was size-controlled diffusion, that of negatively-charged Tβ4 and positively-charged polylysine was affected by electrostatic interactions of peptides with collagen/chitosan molecules. Hydrogels with encapsulated Tβ4 significantly increased cell migration and outgrowth of CD31-positive capillaries from mouse and rat epicardial explants in vitro, compared to Tβ4-free and soluble controls. Potential advantage of Tβ4 over commonly-used angiogenic growth factors is that it can induce recruitment and differentiation of both endothelial and smooth muscle cells necessary for vascular stability. Importantly, Tβ4-encapsulated collagen–chitosan hydrogels promoted angiogenesis in vivo upon subcutaneous injection, compared to collagen-only hydrogels.

Contributions: L.L.Y.C.-concept and design, performed all experiments and data analysis, manuscript writing; M.R.-concept, data interpretation, final approval of manuscript
Controlled release of thymosin β4 from injected collagen-chitosan hydrogels promotes angiogenesis and prevents tissue loss after myocardial infarction in rats


Acute myocardial infarction (MI) often leads to fibrosis and severe left ventricular (LV) wall thinning. Enhancing vascularization within the infarct can reduce cell death and maintain thick LV wall essential for proper cardiac function. Thymosin β4 (Tβ4) supports cardiomyocyte survival post-MI by inducing vascularization and upregulating Akt activity. Here we injected collagen-chitosan hydrogel with controlled release of Tβ4 into the infarct after performing LAD ligation in rats. The Tβ4-encapsulated hydrogel (Thymosin) significantly reduced tissue loss post-MI (13±4%), compared to 58±3% and 30±8% tissue loss for no treatment (MI Only) and Tβ4-free hydrogel (Control). There were significantly more Factor VIII-positive blood vessels with diameter >50μm in Thymosin group compared to both MI Only and Control (P<0.0001), showing Tβ4-induced vascularization. Wall thickness was positively correlated with the mature blood vessel density (r=0.9319, P<0.0001). Thus, controlled release of Tβ4 within the infarct enhances angiogenesis and presence of cardiomyocytes necessary for cardiac repair.

Contributions: L.L.Y.C.-concept and design, performed all experiments and data analysis, manuscript writing; L.A.R-concept and design, assistance in surgical work and preparation of samples for histology; A.M.-performed surgical work; M.R.-concept, data interpretation, final approval of manuscript
Perfusable branching microvascular bed generated in vitro through control of substrate topography and presentation of angiogenic factors

Loraine L.Y. Chiu, Yan Liang, Milica Radisic. Submitted to Proceedings of the National Academy of Sciences.

Vascularization is critical for survival of engineered tissues in vitro and in vivo. In vivo, angiogenesis involves proliferation and sprouting of a sub-set of endothelial cells followed by anastomosis, which is accomplished via connection of extended cellular processes and subsequent lumen propagation through vacuole fusion. Here, we engineered an endothelial cell network in vitro, to be used as a vascular bed to generate vascularized cardiac tissue, following biomimetic principles. An organized capillary network anchored by an artery and a vein at the ends was generated by inducing directed capillary sprouting from vascular explants on micropatterned substrates coated with thymosin β4 (Tβ4)-encapsulated hydrogel. Tβ4 peptide was selected based on its angiogenic and cardioprotective potential. By Day 14, there was increased capillary outgrowth on substrates with Tβ4-encapsulated hydrogel compared to substrates coated with bare collagen-chitosan hydrogel, which also correlated with Tβ4-induced increases in the concentration of autocrine vascular endothelial growth factor (VEGF). The capillary outgrowths connected between the parent explants by Day 21, a process which could be shortened to 14 days by application of soluble VEGF and hepatocyte growth factor (HGF). Immunostaining, confocal microscopy and transmission electron microscopy indicated the presence of tubules with lumens formed by endothelial cells that expressed CD31, VE-cadherin and von Willebrand factor. Vascularized cardiac tissues engineered using the resulting vascular network showed improved functional properties, cell striations and cell-cell junctions compared to tissues without prevascularization. This strategy is not only beneficial for allowing perfusion in tissue engineering applications, but it may also provide means for connecting the host circulation to the vascularized engineered tissue in vivo.

Contributions: L.L.Y.C.-concept and design, performed all experiments and data analysis, manuscript writing; Y.L.-VEGF ELISA, image analysis for branch density, length and width; M.R.-concept, data interpretation, manuscript writing and approval
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Chapter 1

1 Introduction

1.1 Overview

Cardiovascular diseases are the main cause of death in the world. Cardiac tissue engineering may provide a novel approach to treat heart disease, by replacing, repairing or regenerating the damaged myocardium using tissue- and cell-based strategies. A major limitation in cardiac tissue engineering is the need for functional vasculature to support transport of oxygen and nutrients to the highly metabolic cardiac cells. Biomaterials such as three-dimensional scaffolds and hydrogels are essential for providing a protective environment for cell growth during in vitro cardiac tissue engineering and in vivo cardiac repair. Promoting angiogenesis (growth of vasculature) within these biomaterials is required for improving mass transport and in turn cell survival during cardiac regeneration. In this thesis, the delivery of angiogenic molecules such as vascular endothelial growth factor (VEGF) and thymosin β4 (Tβ4) through their incorporation into scaffolds and hydrogels was explored for vascularization in vitro and in vivo.

Both scaffolds and hydrogels were studied in this thesis. Scaffolds are used for repair of congenital heart diseases such as septal defects in children, while hydrogels are used for minimally invasive repair of myocardial infarction in adults. Modification of scaffolds and hydrogels with the incorporation of angiogenic molecules renders the biomaterials bioactive and capable of vascularization without the incorporation of cells, thus eliminating the need to find a viable cell source. In addition, the incorporation of angiogenic molecules into biomaterials protects the molecules from degradation and washout from the injection site, thus improving their efficacy by maintaining their sustained and localized presence. Controlled delivery of angiogenic molecules is also important for lowering the required dose as compared to systemic administration, potentially eliminating side effects.

Here, VEGF was selected to be covalently immobilized to scaffolds for repair of ventricular wall defects since it is a widely studied and potent angiogenic factor. Moreover, its bioactivity does not depend on cell internalization, thus allowing its covalent immobilization in this thesis. Covalent immobilization ensures that the effect of VEGF is localized within the biomaterial that replaces the wall defect, in turn promoting the infiltration of cells and blood vessels into the
biomaterial such that formation and integration of new cardiac tissue occurs at the site of implantation. On the other hand, Tβ4 was chosen for treatment of myocardial infarction due to its ability to promote myocardial and vascular repair simultaneously. The encapsulation of Tβ4 into hydrogels gives a controlled release of the molecule, thus allowing its cell internalization as required for its bioactivity. As well, the controlled release of Tβ4 ensures that the entire infarct area surrounding the injection sites can undergo repair, while maintaining localized bioactivity.

1.2 Hypothesis

The hypothesis of the thesis is that cardiac tissue engineering and cardiac repair can be facilitated by rapid vascularization using bioactive scaffolds with immobilized VEGF or injectable hydrogels with encapsulated Tβ4.

1.3 Specific aims

The overall objective of this study is to promote angiogenesis in patches and injectable biomaterials for tissue engineering and cardiac repair. The specific aims of the work include:

1. The development of scaffolds with covalently immobilized VEGF to improve angiogenesis for cardiac repair.

   1.1 The proliferation and organization of endothelial cells using scaffolds with covalently immobilized VEGF in vitro (Chapter 3).
   1.2 In vivo cardiac repair using scaffolds with immobilized VEGF (Chapter 3).

2. The release of Tβ4 from hydrogels for cell recruitment and vascularization.

   2.1 The recruitment of cells from cardiac explants using collagen-chitosan hydrogels with encapsulated Tβ4 in vitro (Chapter 4).

   2.2 The guidance of capillary outgrowths from an artery or vein into the grooves of a micropatterned PDMS substrate, in order to obtain an oriented perfusable capillary structure in vitro (Chapter 5). The grooves are filled with the developed Tβ4-encapsulated hydrogel from Aim 2.1.
2.3 *In vivo* release of Tβ4 from collagen-chitosan hydrogels for post-MI treatment (Chapter 6).

1.4 Scope of the thesis

The body of work reported here describes the development of biomaterials for the delivery of angiogenic biomolecules for cardiac repair. The two delivery systems investigated here include 1) a scaffold patch with covalently immobilized angiogenic growth factor, VEGF, and 2) an injectable hydrogel with the controlled release of angiogenic peptide, Tβ4. These systems are described in:

- **Chapter 3.** The evaluation of collagen scaffolds with covalently immobilized VEGF for vascularization and cardiac repair.

- **Chapter 4.** Development of a collagen-chitosan hydrogel for the sustained and localized release of Tβ4, an angiogenic and cardioprotective peptide.

- **Chapter 5.** The combined use of topographical cues and controlled release of Tβ4 for the engineering of oriented capillary structures.

- **Chapter 6.** The evaluation of collagen-chitosan hydrogels with encapsulated Tβ4 for cardiac repair.
Chapter 2

2 Literature Review

2.1 Motivation for cardiac tissue engineering

Cardiac tissue engineering is motivated by the need to provide functional tissue replacements for the damaged heart. Cardiovascular diseases are one of the leading causes of death in the world. The high mortality due to cardiovascular diseases is in part due to the limited ability of the damaged cardiac tissue to regenerate itself. After myocardial infarction (MI), the loss of cardiac tissue impairs the function of the left ventricle and leads to the formation of non-contractile scar tissue. Treatment for cardiovascular diseases remains hindered by the shortage of donor organs.

The engineered tissue replacements should match the morphology and function of the native myocardium. They should be mechanically stable with Young’s modulus of 22-50kPa [1], ~1cm in thickness, and capable of propagation of electrical impulse. In order to support proper cardiac function, cardiomyocytes within the tissue construct should be elongated and aligned at high cell density of ~10^8 cells/cm^3, and organized within a vascular network with intercapillary distance of ~20μm.

While engineered cardiac patches have been evaluated in vivo in animal models [2-4], their safety and efficacy in humans are not fully investigated. However, engineered cardiac tissues can also serve as high-fidelity models for drug testing, studies of cardiac development, and studies of cardiovascular diseases. As a first step, mouse engineered cardiac tissue was used as an in vitro model system to study the effectiveness of transplanting different cell types for cardiac repair [5]. In this study, it was found that mouse embryonic stem cell (ESC)-derived
Flk1+/PDGFRα+ cardiac progenitors integrated with the host engineered cardiac tissue and improved its function, while mouse ESC-derived cardiomyocytes showed limited potential to functionally integrate with the host tissue.

The main considerations in cardiac tissue engineering include: 1) selection of an appropriate cell source, 2) design of a bioinstructive biomaterial to provide a three-dimensional environment for cell infiltration, growth, differentiation and organization, and 3) incorporation of biomimetic cues such as electromechanical and topographical cues to establish physiological conditions in which the cardiac tissue constructs can be cultivated. In this thesis, we focused on the role of biomaterials in cardiac tissue engineering, particularly for vascularization of the engineered tissues.

2.2 Biomaterials for cardiac tissue engineering

The role of biomaterials in cardiac tissue engineering is to 1) support cell function, survival, proliferation and differentiation, 2) allow infiltration of cells and blood vessels, 3) provide mechanical support during tissue regeneration and degrade after the regenerated tissue performs its proper function. Biomaterials should also have appropriate tensile strength such that the engineered cardiac tissue can achieve simultaneous contraction with the native cardiac tissue. For example, the adult rat left ventricular myocardium is anisotropic, with Young’s modulus of ~160kPa in the circumferential direction and ~85kPa in the longitudinal direction [6]. Engelmayr et al. [6] previously designed an anisotropic scaffold based on poly(glycerol sebacate) (PGS) to match the circumferential and longitudinal Young’s moduli of the adult rat right ventricular myocardium (~55kPa and ~20kPa respectively).

Many natural and synthetic biomaterials have been studied for cardiac tissue engineering. Natural biomaterials include collagen [7-8], gelatin [9], alginate [10-11], Matrigel [8], fibrin [12] and laminin [13]. Synthetic biomaterials include polyglycolic acid [14-18], poly-L-lactide [19], polyurethane [20] and PGS [6].

Biomaterials can be tailored to any size or shape, and their mechanical properties can be controlled. McDevitt et al. [13] microcontact printed laminin on a biodegradable polyurethane scaffold to control the spatial organization of cardiomyocytes. Zong et al. [19] seeded cardiomyocytes on fine textured matrices that were fabricated by electrospinning of poly-L-
lactide and polyglycolide. These matrices were mechanically stretched to attain oriented fibres. As a result, the seeded cells oriented and elongated along the fibre direction, in turn improving sarcomere development and contractile function. Zimmermann et al. [8] encapsulated neonatal rat cardiomyocytes in collagen I/Matrigel into circular molds and applied unidirectional stretch to the tissue construct to engineer a heart tissue with functional and morphological properties of differentiated muscle. To decrease immunogenicity of cultivation methods for eventual clinical application, these engineered heart tissues were cultivated in serum-free and Matrigel-free conditions [21].

Different biomaterial structures have been created for cardiac tissue engineering. Engelmayr et al. [6] created an accordion-like PGS scaffold to guide orientation of cardiomyocytes and achieve an anisotropic structure similar to the native myocardium. The honeycomb scaffold consisted of overlapping 200μm x 200μm squares at a 45° angle. Cultivation of fibroblasts and cardiomyocytes in the scaffold led to aligned cells and contractile cardiac grafts with mechanical properties similar to the native rat right ventricle. To facilitate transport of oxygen and nutrients within the cardiac construct, porous collagen scaffolds have been used for the engineering of functional contractile cardiac patches [22]. Another strategy to improve mass transport was to mimic blood flow through a capillary structure by seeding cells in a scaffold with an array of channels and cultivate the cardiac construct under perfusion with culture medium supplemented with perfluorocarbon, which is a synthetic oxygen carrier [23]. Cardiac constructs showed higher cell density and viability, mature cell morphology and improved contractile properties.

2.2.1 Scaffolds

There are many forms of heart disease. While the most common form is myocardial infarction in adults, congenital heart diseases are present at birth. The most common type of congenital heart disease is the ventricular septal defects. Septal defects are defects in the interatrial or interventricular septum, allowing blood to flow from the left side of the heart to the right side and reducing the efficiency of the heart. In these cases, scaffolds with a three-dimensional structure and appropriate mechanical properties would be necessary to surgically repair the full-thickness defects, by either direct implantation or implantation of engineered cardiac tissues grown on these scaffolds.
Both naturally-derived and polymeric scaffolds have been used to engineer cardiac tissues (reviewed in [24-25]). Naturally-derived scaffolds have shown biocompatibility and biodegradability. Fetal rat cardiac cells were cultured in porous alginate scaffolds for 4 days \textit{in vitro} and then implanted into the rat peritoneal cavity for 1 week to vascularize the cardiac patch [26]. The patch was then transplanted into the heart for cardiac repair. The attachment of adhesion peptide $G_4RGDY$ and heparin-binding peptide $G_4SPPRRARVTY$ to alginate scaffolds improved the formation of a functional cardiac tissue compared to unmodified scaffolds [27]. The scaffolds with both peptides showed an isotropic myofiber arrangement of cultivated cardiac cells, with an increase in Connexin-43 expression. In a recent study, collagen scaffolds were shown to support differentiation of mouse embryoid bodies into beating cardiomyocytes [28].

On the other hand, polymeric scaffolds can be easily modified to control structure and mechanical properties. A porous polycaprolactone scaffold was designed with an automated algorithm and fabricated using selective laser sintering [29]. The compressive stiffness of the scaffold could be predicted based on the porosity to design a scaffold with appropriate mechanical properties for cardiac tissue engineering. The scaffold supported the \textit{in vitro} growth of C2C12 myoblast cells for 21 days. Multi-layered PGS scaffolds were also fabricated by laser microablation of PGS membranes to create sheets with accordion-like honeycomb shaped pores and a subsequent oxygen plasma treatment of multiple stacked sheets [30]. Cardiac cells that were cultured on these scaffolds responded to electrical field stimulation after 7 days. Micromolding and microablation technologies were combined to fabricate biodegradable PGS scaffolds with well defined surface patterns and pores that ultimately guided the orientation of muscle cells [31]. The stiffness of PGS elastomer scaffold could be tailored by controlling the extent of polymer cross-links [32]. It was found that the contractility of engineered cardiac tissues using PGS scaffolds had a positive correlation with low compressive stiffness. A biodegradable porous scaffold composed of 50% poly-L-lactic acid and 50% polylactic glycolic acid was seeded with human ESC-derived cardiomyocytes, endothelial cells and embryonic fibroblasts [4]. The cardiac constructs were cultivated for 2 weeks and then grafted onto the left ventricle for 2 additional weeks, showing formation of both donor and host vasculature within the tissue constructs and functional integration of donor vessels with the host coronary vasculature.
2.2.1.1 Bioinstructive scaffolds for vascularization

An essential component of cardiac tissue engineering is the vascularization of the tissue (vascularization strategies further discussed in Section 2.3). Recent research has been focused on making proangiogenic scaffolds for cardiac tissue engineering, by 1) controlling structure of the scaffolds and 2) incorporating angiogenic molecules. Madden et al. [33] used microtemplating to make bimodal scaffold with parallel channels for the organization of cardiomyocytes and interconnected pores to enhance angiogenesis. These scaffolds supported the survival and proliferation of human ESC-derived cardiomyocytes in vitro. When cell-free scaffolds were implanted in the nude rat myocardium, maximal vascularization was achieved with scaffolds with 30-40μm pore diameter.

In addition, angiogenic molecules such as VEGF, angiopoietin-1 (Ang-1), and basic fibroblast growth factor (bFGF) have been incorporated into scaffolds for cardiac tissue engineering to improve angiogenesis and in turn cardiac repair. VEGF and Ang-1 were previously covalently immobilized onto porous collagen scaffolds using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry. These modified scaffolds increased the proliferation of seeded H5V endothelial cells [34] and primary rat aortic endothelial cells [35] as compared to cells grown on unmodified scaffolds with or without supplementation of soluble growth factors. These cells also formed capillary-like tubes on modified scaffolds. Scaffolds with co-immobilized VEGF and Ang-1 enhanced angiogenesis in the chicken chorioallantoic membrane assay compared to scaffolds with immobilized VEGF or Ang-1 alone [34], due to the synergistic effects of combined growth factors. Covalent immobilization of angiogenic growth factors onto scaffolds can localize and sustain the bioactivity of the biomolecules by preventing their washout from the site of interest and their internalization by cells. This ultimately improves the efficacy of the bioactive molecules, and is a suitable delivery method of biomolecules that do not require internalization for their bioactivity.

A novel approach to prevascularize cardiac patches involved the implantation of patch onto the omentum. Dvir et al. [36] incorporated prosurvival and angiogenic factors, insulin-like growth factor-1 (IGF-1), stromal-cell derived factor (SDF-1) and VEGF, into macroporous alginate scaffolds. The alginate scaffolds included alginate-sulfate, which allows high affinity binding of the factors to the matrix. These scaffolds were then seeded with neonatal rat cardiac cells,
cultured for 48 hours and then implanted onto rat omentum for 7 days to induce patch vascularization. There was a clear infiltration of host blood vessels into the cardiac patches with biomolecules, while patches without the factors remained acellular at the centre. When the vascularized cardiac patch was subsequently grafted onto the infarcted rat heart tissue, it fully integrated into the host myocardium after 4 weeks and improved cardiac function.

2.2.1.2 Decellularized hearts as scaffolds

Whole heart decellularization provides an alternative method of generating a naturally derived scaffold with proper cardiac architecture. The decellularized heart provides an ideal scaffold for cardiac tissue engineering as the chamber geometry, vascular architecture, heart extracellular matrix and mechanical properties are all preserved, thus exposing seeded cells to the same environment as the native heart. Moreover, the generation of an autologous bioartificial heart using decellularized whole heart as a scaffold would overcome immunorejection of allograft heart transplantation. Ott et al. [37] decellularized cadaveric rat hearts by coronary perfusion with detergents, preserving its extracellular matrix, vascular structure and chamber geometry. The decellularized heart acts as a scaffold for whole heart engineering by reseeding it with cardiac cells and cultivating the construct in vitro. In a recent study, Godier-Furnemont et al. [38] seeded human mesenchymal progenitor cells (MPCs) in fibrin hydrogel onto decellularized sheets of human myocardium. The decellularization process preserved most elements of the extracellular matrix, as well as the underlying mechanical properties. The MPCs were conditioned with a low concentration of transforming growth factor β (TGF-β) to promote an arteriogenic profile of gene expression. After implantation of these constructs into the infarct bed in rats, significantly enhanced vascular network formation was observed. Echocardiography demonstrated the recovery of baseline levels of left ventricular systolic dimensions and contractility.

Human hearts were decellularized by coronary perfusion with sodium dodecylsulfate for 4-5 days, and maintained three-dimensional structure, chamber geometry, valve competency, fiber orientation, extracellular matrix components and microvascular structure after decellularization [39]. The decellularized human heart supported the attachment, alignment and survival of human mesenchymal bone marrow derived stem cells cultivated on the left ventricle in vitro. Adult porcine hearts were also decellularized using pulsatile retrograde aortic perfusion, and
retained mechanical integrity and extracellular matrix components such as collagen, elastin and glycosaminoglycans [40]. The decellularized heart supported the organization of sarcomeres in the cultivated chicken cardiomyocytes in vitro. In a separate study, porcine hearts were also decellularized using the modified Langendorff perfusion decellularization model with an ionic detergent-based protocol [41]. Akhyari et al. [42] developed a software-controlled automatic coronary perfusion method to decellularize whole rat hearts while keeping the preset perfusion pressure constant. The goal of an automatic protocol was to achieve reproducible scaffolds with the same extracellular matrix components, which may be important for the fate of cardiomyocytes and cardiac progenitors. For example, elastin and collagen IV were found removed by previous protocols. In addition, varying degrees of acellularity were achieved as indicated by the remaining DNA content within the decellularized matrices. Thus, the standardization of an automatic protocol may be necessary to fully exploit the potential of decellularized whole hearts as scaffolds for cardiac tissue engineering.

Recently, Ng et al. [43] studied the differentiation potential of human embryonic stem cells (hESCs) and human mesendodermal cells (hMECs) derived from embryonic stem cells in decellularized hearts. These cells showed upregulated expression of cardiac markers after 2 weeks of static culture, showing that the properties of the decellularized heart could direct differentiation of stem cells and progenitors into cardiac cells. Moreover, there was higher expression of myosin light chain (Myl2 and Myl7) in differentiated hMECs and higher expression of myosin heavy chain (Myh6) in differentiated hESCs, showing the presence of different cardiomyocyte subtypes upon differentiation. When subcutaneously implanted in SCID mice, cells that expressed cardiac markers remained in the cardiac tissue but the tissue showed no contractility. Further investigations into how pluripotent cells or lineage restricted progenitors repopulate the decellularized heart and differentiate in a site-specific manner into different populations of the native heart would be essential to creating whole hearts for transplantation.

2.2.2 Injectable hydrogels

Hydrogels are crosslinked networks of hydrophilic polymers and have high water content. They are formed through self-assembly, non-covalent interactions with ionic species, covalent crosslinking via chemical reaction, or gelation via thermal transitions. The advantages of using
hydrogels include: 1) the ability for the biomaterial to be molded into the desired shape or to fill the geometry of the injected space, 2) the need of a non-invasive method of delivery such as catheter delivery. Hydrogels have been used extensively for cardiac tissue engineering and cell transplantation for cardiac repair (reviewed in [44]). On the other hand, hydrogels themselves can be injected into the heart for cardiac repair based on tissue bulking and bioactive properties (reviewed in [45]). Hydrogels have also been used as in situ delivery systems of bioactive molecules in the heart (reviewed in [45]). The delivery of biomolecules within hydrogels into the heart promotes in situ cardiac repair and eliminates the need to find an appropriate cell source for transplantation.

After MI, the extracellular matrix breaks down, leading to geometric changes in both the infarcted and healthy myocardium [45]. Left ventricular dilation after MI results in thinning of the myocardium and in turn increased stress on the myocardial wall. Injectable hydrogels can be applied non-invasively, with or without cells, to limit the expansion of the left ventricle. Wall et al. [46] used a finite element model to simulate the effects of injecting non-contractile materials into the myocardium, and showed that the bulking of the myocardium was sufficient to decrease stress in the myocardial wall post-MI based on their attenuation of geometric changes.

Both natural and synthetic hydrogels have been used for cardiac tissue engineering. Fibrin glue has been developed for the delivery of cells in cardiac repair [47]. Fibrin has binding domains for growth factors and receptors. Moreover, it provides mechanical support for the myocardium. The crosslinked 3D fibrin hydrogel is formed upon injection with a dual-barreled syringe containing fibrinogen and fibrinolysis inhibitor aprotinin in one barrel and thrombin, factor XIIIa and CaCl₂ in the other barrel [47]. The crosslinking mechanism is similar to the normal clotting events in vivo. After infarction in rats, the injection of skeletal myoblasts alone or fibrin glue with or without skeletal myoblasts helped to preserve the infarct wall thickness and in turn cardiac function after 5 weeks [47]. Besides bulking effects, fibrin also has bioactivity that affects left ventricular remodeling, as shown by its ability to improve arteriole density in the infarct area when used to deliver skeletal myoblasts as compared to skeletal myoblasts delivered in bovine serum albumin (BSA) [48]. On the other hand, alginate is a bioinert natural material that requires modification with bioactive peptides to be rendered bioactive. The injection of non-modified alginate hydrogel and alginate modified with RGD conjugation 5 weeks post-MI led to improved cardiac function at 10 weeks post-MI, as compared to control involving the injection
of 0.5% BSA in phosphate buffered saline (PBS) [49]. However, RGD modification further increased angiogenic response. Hyaluronic acid (HA) is a polysaccharide that has biological roles in angiogenesis, cell migration and scar reduction [45]. HA hydrogel was fabricated by mixing acrylated HA with poly(ethylene glycol) (PEG) crosslinker to be crosslinked, and the treatment of rat MI with the injection of this mixture led to significantly decreased infarct size, increased ejection fraction and increased vascularization at 4 weeks post-injection [50]. This improvement was attributed to the biological role of HA, since there was a complete degradation of the HA hydrogel.

Extracellular matrix components that were isolated from the healthy myocardium were also used to treat MI. A myocardial matrix was produced by the decellularization of porcine myocardial tissue [51]. This matrix gelled at 37°C and had collagen and glycosaminoglycan content. The myocardial matrix attracted the homing of endothelial and smooth muscle cells in vitro and in vivo. As a result, the matrix increased arteriole formation when injected into the rat myocardium. The solubilized form of decellularized porcine and human pericardium were investigated as injectable hydrogels for cardiac repair [52]. The gels preserved the extracellular matrix components of the native pericardium and induced neovascularization in vivo. In addition, c-kit+ cells were present in the site of injection. Thus, the use of decellularized cardiac matrix as injectable hydrogels can provide cardiac-specific cues to promote endogenous homing and in turn cardiac-specific tissue formation.

While natural materials are biocompatible and provide necessary cues for cell-biomaterial interactions, synthetic materials are more tunable in their mechanical properties, degradation rates and gelation time. Synthetic injectable hydrogels for cardiac tissue engineering are reviewed in [44-45, 53]. In this thesis, the biomaterials used were collagen-based since collagen is the major extracellular matrix component in the heart [54].

2.2.2.1 Collagen and chitosan

Collagen I is a natural extracellular matrix protein that is widely used for cell culture and tissue engineering. Collagen molecules form microfibrils which in turn form fibrils to assemble into the collagen fiber. As a structural fibrous protein, collagen provides the tensile strength of the native extracellular matrix [55]. The gelation of collagen matrices can occur at 37°C upon injection as a liquid in vivo. Collagen hydrogels injected into rats 1 week post-MI significantly
increased scar thickness, stroke volume and left ventricular ejection fraction as compared with the saline treatment [56]. In a rat model of left coronary artery occlusion followed by reperfusion, the injection of collagen I hydrogel yielded higher levels of capillary formation compared to saline treatment at 5 weeks post-treatment [57]. The level of angiogenesis for collagen injection was similar to the injection of fibrin or Matrigel. However, collagen treatment further increased infiltration of myofibroblasts into the infarct region.

Chitosan is a linear polysaccharide that is biocompatible and biodegradable. Biocompatibility in the context of vascularization is defined as the ability of the biomaterial to elicit a desirable level of inflammatory response that is necessary to promote angiogenesis, but low enough to avoid destruction of blood vessels. Chitosan hydrogels can be formed in several ways. By mixing commercially produced chitosan with a solution of glycerol phosphate and glyoxal solution, chitosan gels can be formed with a thermoresponsive gelation at 37°C [58]. This chitosan hydrogel was used for transplantation of mouse embryonic stem cells to treat myocardial infarction, and showed improved cell retention and 4-week graft size compared to PBS control. Chitosan was also rendered photocrosslinkable by a chemical modification involving the conjugation of azidobenzoic acid to the chitosan backbone [59-60]. The modified chitosan could be crosslinked by UV application, but did not support cell proliferation. However, peptides such as RGD have been incorporated into the hydrogel to support cell survival and viability [59]. Mouse VEGF165 could be encapsulated into the photocrosslinkable chitosan containing RGD peptide, and retained for 24 days [59]. Furthermore, the photocrosslinkable chitosan was conjugated with QHREDGS, an angiopoietin-1-derived peptide that mediates cardiomyocyte attachment and survival via integrin binding [60]. Neonatal rat cardiomyocytes cultured on the modified chitosan hydrogel showed improved attachment and viability after 5 days, as compared to glass substrate, photocrosslinkable chitosan, or photocrosslinkable chitosan with RGD. Upon subcutaneous implantation, the cardiac constructs engineered using photocrosslinkable chitosan with QHREDGS showed enhanced cardiomyocyte elongation, assembly of contractile apparatus, and cell survival compared to cardiac constructs engineered using chitosan modified with RGDS peptide.

Several properties of the collagen-chitosan blend make it suitable for tissue engineering applications. While most polymer blends are immiscible with each other, mixtures of collagen with other synthetic and natural polymers, such as chitosan, exhibit miscibility due to specific
interactions between the polymeric components [61]. In the natural extracellular matrix, proteoglycans provide mechanical stability and compressive strength by intertwining with the fibrous structure of collagen [55]. The components associated with collagen in native tissue, including elastin and proteoglycans, modulate collagen fibrillogenesis, fill space and bind water [55]. Specific interactions within collagen-chitosan blends include hydrogen bonding and ionic bonding [61-62]. The OH groups of hydroxyproline in collagen are involved in hydrogen bonding between chains. Other side groups interact with individual molecules to form fibrils. These side groups, as well as the end groups COOH and NH$_2$, are capable of forming hydrogen bonds with the OH and NH$_2$ groups in chitosan. Chitosan has large numbers of OH groups and thus the long chitosan chain can wind around the collagen triple helix to form a complex involving the entanglement of the two macromolecules. In addition, collagen and chitosan can interact through ionic bonding. These molecules can form polyanion-polycation complexes through polyelectrolytic interactions, since the polysaccharide is cationic and collagen has an anionic COOH group. The formation of this electrostatic complex competes with the formation of a complex with collagen triple helices for the gelation of collagen [62]. A third phenomenon was observed in collagen-chitosan blends, as wide-angle X-ray diffraction data indicated that the miscibility of the molecules turned the collagen helix structure to a more disordered structure [61]. Moreover, a simple biphasic mixture could not explain the higher viscosity of the collagen-chitosan blends than both components in isolation. The presence of a third component, gelatin, was speculated since it is the denatured form of collagen and has a higher intrinsic viscosity than collagen. FTIR measurements showed that a triphasic system involving the interactions of molecules is present, since collagen was not simply denatured to gelatin and existing separately from the chitosan [61].

The addition of chitosan to a previously developed collagen matrix improved the stability and angiogenic potential of the hydrogel [63]. The collagen-chitosan hydrogel increased vascular endothelial-cadherin expression compared to collagen-only matrix and promoted the formation of vascular-like structures by endothelial cells in vitro. Subcutaneously implanted collagen-chitosan matrices recruited angiogenic cells and stimulated vascular growth. In a separate study, the addition of chitosan was found to influence the crosslinking of collagen fibers, in turn reinforcing the structure [55].
2.2.2.2 Delivery of angiogenic factors in hydrogels

An important strategy to preserve the myocardium post-MI is to stimulate formation of neovasculature to restore blood flow to the ischemic tissue. Recent approaches include the localized release of angiogenic growth factors, such as fibroblast growth factor (FGF) [64-65], VEGF [66-67] and platelet derived growth factor (PDGF) [66], using hydrogels. As with the controlled delivery of biomolecules with scaffolds (Section 2.2.1.1), the bioactivity of growth factors and peptides incorporated into hydrogels can be localized and sustained since the biomolecules are protected from washout and cell internalization.

Fibroblast growth factor is an angiogenic molecule with a short half-life *in vivo*, as demonstrated by the lack of detection of radiolabeled FGF 24 hours after administration to the myocardium [64]. The use of gelatin hydrogel microspheres allowed the sustained delivery of absorbed FGF at effective concentrations in the peri-infarct area of the rat myocardium, based on the degradation of gelatin. This in turn increased vascular density and improved left ventricular systolic and diastolic function. FGF was also mixed with chitosan solution and was then encapsulated in the thermoresponsive chitosan hydrogel upon injection into the myocardium [65]. Sustained delivery improved cardiac function compared to injection of soluble FGF in a rat chronic MI model.

To form more stable blood vessels supported by smooth muscle cells, more recent approaches used dual delivery of angiogenic growth factors with hydrogels. VEGF and PDGF were co-delivered in an alginate hydrogel, with a sequential release in which VEGF was released more quickly than PDGF [66]. The controlled release was based on the degradation of the hydrogel, and the release of both factors was sustained over 30 days *in vitro*. As such, VEGF can first stimulate endothelial vessel formation and PDGF can then recruit smooth muscle cells to support the immature vessels. Sequential delivery induced mature vessels and improved cardiac function 4 weeks post-injection compared to the delivery of individual factors alone. The delivery of VEGF, a potent angiogenic factor, is further discussed in Section 2.4.

2.2.3 Scaffold-free cell sheet engineering

Alternatively, Shimizu et al. [68] developed a novel technology to engineer contractile cardiac grafts by layering monolayers of cell sheets without the use of matrix materials. Surfaces coated
with a temperature responsive polymer that is non-adhesive below 32°C, poly-N-isopropylacrylamide, were used to culture neonatal cardiomyocytes. By lowering the temperature, cell sheets detached spontaneously, while preserving cell junctions and adhesive proteins, and could be stacked together [68-69]. Another matrix-free self organization approach involves spontaneous wrapping of confluent cardiomyocyte monolayers around a poly(glycolic acid) suture [70].

2.3 Vascularization of engineered cardiac tissues

Cardiac tissue engineering and regenerative medicine is often complicated by the need for vascularization to provide oxygen and nutrients within highly metabolic cardiac tissues [71-72]. To generate tissue engineered cardiac patches, cardiomyocytes were seeded on scaffolds and cultivated in bioreactors. However, oxygen concentration is greatly reduced near centre of cardiac constructs due to the high oxygen consumption rates by cardiomyocytes, leaving a 100μm thick outer layer of viable cells with a nearly cell-free interior [14, 73-74]. Different bioreactors were used to improve the transport of oxygen for cultivation of cardiac constructs in vitro, including perfusion bioreactors [14, 73] and rotating bioreactors [75].

Although perfusion bioreactors can support the growth and viability of cardiac tissues with physiological thickness and high cell density in vitro, rapid vascularization and connection to the host vasculature is essential upon in vivo implantation. In the native myocardium, capillaries of ~7μm in diameter are spaced at distances of ~20μm, with each myofiber located between two capillaries [76]. Recent approaches for cardiac tissue engineering recognize the need for in vitro vasculogenesis and cardiac protection by the incorporation of endothelial cell and progenitors, and the fabrication and use of bioinstructive biomaterials incorporating proangiogenic bioactive molecules such as VEGF [34, 67, 77-78], bFGF [79] and small peptides like RGD [49].

Coronary vessel formation involves vasculogenesis, angiogenesis and arteriogenesis [80]. Vasculogenesis is the formation of vascular structures by the differentiation of endothelial
precursors. Angiogenesis is the formation of new vessels by the proliferation and migration of endothelial cells, and occurs through intussusception (the division of the original vessel in two) or vascular sprouting. Arteriogenesis is the remodelling of the microvessels to form mature arteries by the migration of pericytes and vascular smooth muscle cells.

The main strategies for vascularization of engineered cardiac tissues include 1) incorporation of endothelial cells and networks, 2) fabrication of proangiogenic scaffolds based on geometry and matrix components, 3) incorporation of growth factors and peptides, and 4) seeding of cells in subcutaneously implanted chambers around an arteriovenous loop. While some of the vascularization strategies described here have not been assessed in cardiac tissue engineering, they have important relevance as they can be potentially applied to create vascularized cardiac tissues.

2.3.1 Incorporation of endothelial cells and networks

The co-culture of fibroblasts and endothelial cells (ECs) with CMs can improve vascularization of engineered cardiac tissues [4, 81]. Lesman et al. engineered vascularized cardiac tissue constructs by a tri-culture of CMs with fibroblasts and endothelial cells, and transplanted them to rat hearts [4]. There was increased formation of donor and host-derived vasculature in tri-culture constructs compared to scaffolds with CMs alone. Donor-derived vessels were functionally integrated with host coronary vasculature, as demonstrated by their incorporation of intraventricularly-injected fluorescent microspheres. Naito et al. used the cell population from the native rat heart, which contained both CMs and non-myocytes, to engineer the cardiac tissue with completely defined culture conditions (i.e. serum and Matrigel free) [21]. It was also found that the pre-culture of fibroblasts and ECs followed by the seeding of CMs improved the functionality of microscale cardiac organoids, as compared to simultaneous tri-culture [81-82]. In a separate study, ECs were sandwiched between stacked cell sheets to form pre-vascular networks [83], which then connected to the host blood vessels upon implantation in an MI model to support further vascularization of the cell sheets [84-85].

2.3.2 Proangiogenic scaffolds

Vascularization can be further enhanced using appropriate geometries [33, 86-87] and extracellular matrix components [88-89] in biomaterials and scaffolds. Microtemplating was
used to fabricate tissue engineering scaffolds consisting of interconnected pores of 30-40μm in diameter that promoted angiogenesis post-MI in rats [33]. Raghavan et al. [86] cultured ECs within 50-200μm wide and 50-100μm high collagen-filled channels. Cells organized into tubes with lumens within 1-2 days. Branched tube formation was also achieved [86]. Bettinger et al. [87] showed that nanofabricated substrates of 1.2μm in period and 0.6μm in depth improved alignment, elongation and migration of endothelial progenitor cells (EPCs), thus enhancing capillary tube formation. Besides micropatterning and controlling porosity, Deng et al. [88] showed increased vascular endothelial-cadherin expression and formation of vascular-like structures by ECs on collagen-chitosan matrix compared to collagen alone.

### 2.3.3 Incorporation of proangiogenic biomolecules

Proangiogenic growth factors can also be used for vascularization of engineered cardiac tissues. They can be delivered in various forms, including soluble factors, encapsulated in microparticles, and physically or covalently immobilized into biomaterials for tissue engineering. Saif et al. [90] fabricated poly(lactic-co-glycolic acid)-based microparticles to release VEGF, hepatocyte growth factor and Ang-1 upon injection in murine hindlimb ischemia models. The release of multiple factors improved vasculogenic progenitor cell therapy by promoting homing and incorporation of intravenously-administered progenitors, in turn increasing microvessel density and vascular smooth muscle cells necessary for vessel stabilization. Similarly, alginate microparticles were integrated into collagen/fibronectin gels to transplant ECs with co-delivery of VEGF and monocyte chemotactic protein-1 (MCP-1) [91]. VEGF improved survival of transplanted ECs and vessel formation, while MCP-1 induced mural cell recruitment and vascular stability. Incorporation of biomolecules is essential for cell-based therapeutic vascularization to overcome apoptosis and lack of recruitment of host cells.

Small molecules such as Tβ4 [92] or ascorbic acid [89] can also be utilized to enhance angiogenesis. Smart et al. [92] showed that the peptide Tβ4 facilitates neovascularization by resident progenitors and stabilizes vascular plexus by collateral vessel growth, thus sustaining the myocardium after ischemic damage. Martinez et al. supplemented myocardial grafts with ascorbic acid, which increased number of blood vessels, donor cells and ECs and improved cardiomyoblast survival at 6 days post-implantation in renal pouches of rats [89]. Other biomolecules were used to induce growth factor production and in turn angiogenesis [92-93].
For example, erythropoietin-induced VEGF expression in CMs was shown to stimulate myocardial endothelial proliferation and increase homing of EPCs to the myocardium in rats with heart failure [93].

Physical and covalent immobilization of growth factors can localize growth factor activity and prolong receptor/ligand signaling to promote rapid vascularization. Zhang et al. [94] produced a fusion protein, CBD-VEGF, consisting of VEGF and a collagen-binding domain (CBD). Once injected into rats, CBD-VEGF was bound to the collagen-rich cardiac extracellular matrix while retaining growth factor activity, in turn increasing capillary vessel density in infarcted hearts and reducing scar size as compared to PBS and VEGF controls. Previously, we covalently immobilized VEGF and angiopoietin-1 onto collagen scaffolds to increase the proliferation of H5V endothelial cells [34] and primary rat aortic endothelial cells [35] compared to scaffolds with no growth factor or soluble factors, thus leading to tube formation. Co-immobilization of VEGF and angiopoietin-1 further led to enhanced angiogenesis in a chicken chorioallantoic membrane assay compared to immobilized VEGF or angiopoietin-1 alone [34]. Dvir et al. prevascularized a cardiac patch, which consisted of cultured cardiac cells on an alginate scaffold capable of sustained release of several incorporated prosurvival and angiogenic factors (i.e. insulin-like growth factor-1, stromal cell derived factor 1, VEGF), by heterotopic transplantation onto the omentum for 7 days [36]. The vascularized cardiac patch was explanted from the omentum and subsequently transplanted onto infarcted rat hearts post-MI. The patch prevented dilatation by inducing thicker scars, and showed both structural and electrical integration into the host tissue.

2.3.4 In vivo vascularization

Morritt et al. seeded neonatal rat CMs into a plastic chamber that contained Matrigel and an arteriovenous blood vessel loop, and implanted the chamber subcutaneously in the groin to attain spontaneously contracting thick three-dimensional constructs with extensive vascularization at 4 and 10 weeks [71]. By co-implanting adipose-derived stem cells with rat CMs in the same vascularized chamber, larger tissue constructs and vascular volumes were achieved compared to chambers with CMs alone [95]. In a separate study, the arteriovenous loop was created and inserted into the cell seeding chamber at Day 0 and the myoblasts were seeded into the chamber at Day 4 or Day 7 rather than Day 0 [96]. The implantation of myoblasts into the chamber at
Day 7, when capillary growth was well-established, led to improved survival of myoblasts compared to implanting them at Day 0. This suggests the need to seed cells on established vascular beds for tissue regeneration.

### 2.4 Vascular endothelial growth factor

VEGF-A, referred to as VEGF, is an important biomolecule in angiogenesis and has been shown to promote vascularization in the infarcted heart. The deletion of VEGF in mice was found to cause abnormal vascular development [97]. VEGF mRNA expression is upregulated by the hypoxia-induced paracrine or autocrine release of other growth factors such as epidermal growth factor, transforming growth factors, keratinocyte growth factor, insulin-like growth factor-1, fibroblast growth factor and platelet-derived growth factor [98]. Due to its role in angiogenesis, VEGF has been targeted using anti-VEGF antibodies to inhibit tumor growth [99]. Importantly, VEGF has also been studied extensively as a pro-angiogenic agent to treat ischemic disorders [67, 100-102].

VEGF is involved in angiogenesis by promoting endothelial cell survival, migration and proliferation in vitro. VEGF induces growth of endothelial cells through the activation of the Raf-Mek-Erk pathway [98]. VEGF prevents apoptosis induced by serum starvation in vitro, as mediated by the phosphatidylinositol-3 kinase (PI3K)-Akt pathway [103]. VEGF also induces expression of antiapoptotic proteins Bcl-2 and A1 in endothelial cells [104].

In vivo, VEGF induces the formation of new and large vessels by increasing vascular permeability. In adult organisms, blood vessels are composed of quiescent endothelial cells and pericytes. Upon activation of quiescent vessels due to a hypoxic stimulus, pericytes detach from the wall of the blood vessel and endothelial cells lose their junctions to allow for the dilation of vessels. VEGF causes vascular permeability by affecting endothelial cell-cell junctions regulated by adhesion molecules that make up tight, gap and adherens junctions [105]. Specifically, VEGF alters connexin-43 phosphorylation via Flk-1 and Src kinase activation, which blocks gap junctional communication between adjacent endothelial cells. VEGF also alters phosphorylation of zonula occluden-1 (ZO-1) and occludin in a Src-dependent pathway, which disrupts tight junctions. Moreover, adherens junction proteins are tyrosine phosphorylated downstream of Flk-1 due to VEGF-mediated Src kinase activity, thus causing the loosening of cell-cell contacts between endothelial cells [105].
In addition, VEGF gradient during vascular enlargement is regulated by soluble and matrix bound isoforms [106]. This gradient is responsible for the upregulation of delta-like ligand 4 expression in tip cells, activation of NOTCH in stalk cells, and downregulation of VEGF receptors. These events make tip cells more responsive to the stimuli and migrate towards the angiogenic signal, while stalk cells divide, elongate the stalk and establish vessel lumen.

VEGF receptors on endothelial cells are kinase insert domain-containing receptor (KDR or Flk-1 or VEGFR-2) and fms-like tyrosine kinase (Flt-1 or VEGFR-1) [98]. VEGF receptors are mostly restricted to vascular endothelial cells, although they have been found on bone marrow-derived cells [98]. Flk-1 is the main mediator of VEGF-induced proliferation, migration, survival, and angiogenesis [98]. Flk-1 binds VEGF with a $K_d$ of 75-125pM [107]. When VEGF binds to Flk-1 or Flt-1, receptor dimerization and autophosphorylation of the receptor occur. VEGF activation of receptor induces tyrosine phosphorylation of cytoplasmic signaling proteins that allow Flk-1 and Flt-1 to communicate with signaling pathways that promote responses to VEGF [108]. Specifically, Flk-1 undergoes dimerization and tyrosine phosphorylation, in turn signaling cell proliferation, migration and survival. Some proteins that are phosphorylated in response include phospholipase C-γ, PI3K, Ras GTPase-activating protein and the Src family [98]. VEGF was found to be released into the surrounding after binding to its receptor on human colonic vascular endothelial cells, but not human umbilical vein endothelial cells [108].

2.4.1 Controlled delivery of VEGF

VEGF has a short half-life in vivo and thus many studies have investigated the use of controlled delivery systems for therapeutic angiogenesis with VEGF. Delivery systems of VEGF have been developed using particles, scaffolds and injectable hydrogels.

VEGF-loaded microparticles and nanoparticles were used to sustain the bioactivity of VEGF [101-102, 109-111]. Poly(lactic-co-glycolic acid) (PLGA) microparticles loaded with VEGF was evaluated in a rat model of ischemia-reperfusion by an intramyocardial administration in the border zone around the infarct [101]. The microparticles provided sustained release of VEGF, and an increase in angiogenesis and arteriogenesis resulted in animals with VEGF microparticles compared to VEGF or microparticles alone. This ultimately led to a greater left ventricular wall thickness in the group injected with VEGF microparticles. More complex systems involved incorporating VEGF-loaded particles into scaffolds and hydrogels. VEGF was included in
heparin-functionalized nanoparticles, which were then incorporated in fibrin gel [102].
Sustained release of VEGF was achieved over 30 days, whereas VEGF incorporated in fibrin gel only was released within the first 3 days. The incorporation of VEGF nanoparticles into fibrin increased angiogenesis in a rabbit ischemic hind limb model, compared to VEGF-loaded fibrin.

VEGF was also physically and chemically incorporated in scaffolds and hydrogels [67, 100, 112-114]. Wu et al. [67] developed a temperature-sensitive aliphatic polyester hydrogel conjugated with VEGF, which gelled at 37°C upon injection and completely degraded after 42 days. VEGF-conjugated hydrogels showed improvements in blood vessel density, scar area and cardiac function 4 weeks post-injection, as compared to injection of soluble VEGF or hydrogel with unconjugated VEGF. In a separate study, a collagen-binding domain was fused to human VEGF to enhance the binding of VEGF to collagen [100]. Then, collagen scaffolds were loaded with the modified VEGF and implanted in the rabbit MI model. The collagen scaffolds with modified VEGF promoted more cell infiltration after 4 weeks and more vascularization after 12 weeks as compared to scaffolds with unmodified VEGF, thus showing the need for sustained bioactivity of the growth factor.

2.5 Thymosin β4

Tβ4 is a small 43-amino acid polypeptide that has various biological roles, including actin binding and sequestration, induction of angiogenesis and cardioprotection. Tβ4 was identified as an angiogenic factor by an in vitro screening of early genes (< 4 hours) induced during cultivation of human umbilical vein endothelial cells (HUVECs) on Matrigel [115]. Tβ4 promotes vessel formation and collateral growth during development [116]. Moreover, exogenous Tβ4 enhanced tube formation of HUVECs in vitro and vascular sprouting in the coronary artery ring angiogenesis assay [117]. Its role in vascular development motivated research by several groups to investigate its biological potential in the adult organisms, as discussed here.

2.5.1 Role of Tβ4 in cardioprotection and neovasculogenesis

Tβ4 was reported to be important for cardioprotection and cardiac repair. When injected intraperitoneally or intracardially, Tβ4 improved cardiac repair after coronary artery ligation, with significantly higher fractional shortening and ejection fraction at 4 weeks after MI [118]. In
addition, the scar size was reduced with Tβ4 treatment, due to early protection of the myocardium through Tβ4-mediated cardiomyocyte survival.

A possible mechanism of Tβ4-induced cardioprotection is the stimulation of coronary neovascularisation. Mouse embryos expressed Tβ4 in the cardiovascular system, including the left ventricle, outer curvature of the right ventricle and cardiac outflow tract [118]. Tβ4 was found to play a role in restoring the pluripotency of the otherwise quiescent adult epicardium [116]. Tβ4 treatment induced outgrowth from epicardial explants from hearts of 8 to 12-week-old mice, while untreated explants showed minimal outgrowth. Tβ4 directly promotes epicardial cell migration through its actin binding, filament assembly and lamellipodia formation. The outgrowth cell population was positive for epicardin, an epicardial-specific transcription factor, and could be differentiated into fibroblasts, smooth muscle cells and endothelial cells. This is a significant finding, since the supply of endothelial and smooth muscle vascular precursors required for vascular regeneration was previously attributed to the bone marrow and peripheral circulation. The potential to use Tβ4 to release quiescent epicardial cells as a source of vascular progenitors suggests the ability to deliver endothelial and smooth muscle cells to the sites of interest following cardiac injury for vascular repair.

A recent study by Smart et al. [119] demonstrated that a pretreatment of the adult heart with Tβ4 was important for activating Wilm’s tumour 1-positive (WT-1+) epicardial cells that contributed to both vasculogenesis and cardiomyogenesis after MI. However, if the peptide was applied at the time of MI or after MI, there was no effect of Tβ4 on epicardial cell plasticity [120]. Pretreatment with Tβ4 prior to MI is not feasible in clinical settings. In addition, systemic application must be carefully evaluated, since Tβ4 can cause growth of cancerous lesions in the gut [121]. Thus, formulation of a controlled delivery system that can sustain the effect of Tβ4 even at low doses is required.

In a study by Qian et al. [122], local delivery of Gata4, Mef2c and Tbx5 (GMT) into the boundary between the infarct and border zones converted resident cardiac fibroblasts into cardiomyocyte-like cells in vivo after cardiac injury. The co-delivery of Tβ4 with GMT further improved cardiac function in vivo since it activated the proliferation of cells and in turn increased the delivery of GMT to more cells. In larger animals, controlled delivery, by placing the Tβ4-
containing gel onto the epicardium, may be beneficial as it will require less peptide and the activity of the peptide can be localized to the epicardium as required.

2.5.2 Mechanisms of action

While Tβ4 has many functions, its mechanism of action has not been completely defined. It was previously shown that Tβ4 binds to cells and becomes internalized [117]. Radioiodinated Tβ4 was found to bind to the cell surface of HUVECs. In addition, incubation of exogenous Tβ4 increased intracellular staining of Tβ4. It was thought that the increased intracellular Tβ4 could be due to the uptake of Tβ4 by the cells or the cell response to exogenous Tβ4 to increase transcription and translation of Tβ4 mRNA. The internalization of Tβ4 was necessary for antiapoptotic activity in human corneal epithelial cells [123]. It was not until recently that an extracellular signaling pathway was identified for Tβ4, in which Tβ4 increases the ATP levels on cell surface by binding to β subunit of ATP synthase [124]. In the same study, ATP-responsive P2X4 receptor was found required for Tβ4 to induce migration of HUVECs.

A study performed to determine the portion of Tβ4 responsible for its angiogenic activity showed that the actin binding domain of Tβ4, LKTET, is essential for endothelial cell adhesion and angiogenesis [125]. The addition of exogenous actin blocked adhesion of HUVECs and vessel sprouting in the aortic ring assay as mediated by Tβ4, showing that actin is the cell surface ligand for Tβ4. Various synthetic peptides and naturally occurring proteolytic fragments of Tβ4 were used in the cell migration and vessel sprouting assays, and it was determined that the peptides containing the carboxyl-terminal portion or all of the actin binding domain highly promoted cell migration and vessel sprouting. A small 7-amino acid synthetic peptide containing amino acids 17-23 from Tβ4, LKKTETQ, promoted migration and sprouting, and blocked adhesion to Tβ4. Collectively, Tβ4 is thought to initiate angiogenesis through the binding of its central actin binding domain to the surface actin of endothelial cells. However, it was shown in a separate study that the addition of synthetic peptide with actin binding sequence LKKTETQEKK did not compete with Tβ4 to bind to the cell surface of HUVECs [117]. This suggests that the binding of Tβ4 does not occur through the actin binding domain.

It has also been shown that Tβ4 stimulated angiogenesis through induction of VEGF, a potent angiogenic growth factor. Overexpression of Tβ4 was found to upregulate VEGF in mouse melanoma cells [126]. In addition, the knockdown of Tβ4 downregulated VEGF in mouse hearts
Tβ4 previously upregulated the levels of hypoxia-inducible factor (HIF)-1α in mouse melanoma B16-F10 cells. Further, Jo et al. [127] found that Tβ4 induced the transcriptional expression of VEGF through the stabilization of HIF-1α protein.

In addition, the proangiogenic tetrapeptide N-acetyl-ser-lys-proline (AcSDKP) was found as a bioactive fragment that is formed by cleavage of Tβ4 at its N terminus between Pro 4 and Asp 5 by prolyl oligopeptidase [116, 128]. AcSDKP levels were found significantly reduced after Tβ4 knockdown [116]. AcSDKP is proangiogenic, stimulating the proliferation and migration of endothelial cells and the formation of capillary-like structures on Matrigel by these cells [129-130]. AcSDKP induced angiogenesis in vivo in the rat Matrigel plug angiogenesis assay [129], chick chorioallantoic membrane assay [129], rat corneal micropocket assay [130] and rat myocardial infarction model [130]. In the adult mouse, there was upregulation of both Tβ4 and AcSDKP after ischemia [116]. AcSDKP did not have actin binding function and thus could not promote outgrowth from epicardial explant as Tβ4 did. AcSDKP-treated cells differentiated into Flk-positive endothelial cells but not smooth muscle cells, suggesting that AcSDKP only supports endothelial cell differentiation. Thus, the vasculogenic effect of Tβ4 may be attributed to this fragment. AcSDKP may also have immunomodulatory properties, as AcSDKP-induced vascularization after hind limb ischemia was due to its induction of MCP-1 [131].

Besides improving cardiac repair through inducing vascularization, Tβ4 also acts through the activation of Akt. Akt is a serine/threonine kinase that plays a role in cell survival and angiogenesis [132]. It can be activated by phosphorylation at Ser 473 by an enzyme such as integrin-linked kinase (ILK) [133]. Tβ4 was shown to form a functional complex with particularly interesting Cys-His protein (PINCH) and ILK [118], in turn activating Akt for cell survival. There was an increase in the level of ILK protein and phosphorylated Akt-S473 in the mouse hearts with Tβ4 treatment after coronary ligation compared to PBS treatment [118]. Thus, the activation of ILK, which in turn stimulates Akt activation, is one possible mechanism by which Tβ4 enhances the survival of cardiomyocytes. However, it was later found the Tβ4 knockdown in mice led to high levels of phosphorylated Akt-S473 [116]. This suggests that the activation of Akt may either be a secondary response to Tβ4 treatment or it may be a compensatory mechanism for the lack of Tβ4.
2.5.3 In vitro application and in vivo delivery of Tβ4

The potential for Tβ4 as a therapeutic for treatment of cardiac diseases has been established, yet a full understanding of the mechanism and scope of its potential use is only now being explored. As the internalization of Tβ4 may be required in order to have an effect, there is a slight limitation on the modes in which it can be administered both in vitro and in vivo. Logically, in vitro studies have been focused on using soluble Tβ4 in cell media to study vasculogenesis, cell migration and differentiation, and most in vivo studies have done the same by using Tβ4 in soluble form (reviewed in [134]).

Excitement over the discovery of the angiogenic and cardioprotective effects of Tβ4 has led to animal studies and human clinical trials involving the systemic administration of Tβ4 as a treatment for acute MI [116, 118, 135]. Bock-Marquette et al. [118] treated acute MI through the injection of Tβ4 in mice. In this study, the peptide was delivered systemically through intraperitoneal injections of Tβ4 in PBS every 3 days, or locally within the cardiac infarct through intracardial injections of Tβ4 in a collagen gel once only. Another delivery regime used was a combination of initial intracardial injection followed by intraperitoneal injections every 3 days for 4 weeks. Systemic, intracardial, or a combination of both systemic and intracardial injections of Tβ4 in the mouse acute MI model showed that treatment with Tβ4 led to a greater than 60% improvement in fractional shortening and more than 100% improvement in ejection fraction compared to PBS injection controls [118]. Furthermore, cardiac morphology was significantly improved in Tβ4 treated animals compared to controls. However, there was no difference between systemic or local delivery of Tβ4 in this case.

The phase I human clinical trial involved the determination of the safety and tolerability of single and multiple ascending intravenous injections of soluble Tβ4 in healthy volunteers [135]. Successful completion of the study has paved the way for approval to move to phase II trials, giving soluble intravenous Tβ4 to acute MI patients (ClinicalTrials.gov Identifier: NCT01311518). While animal studies showed no difference in improvement between systemic or local delivery and human trials confirmed the safety of systemic administration with large dose Tβ4 injections, groups are still interested in local delivery of Tβ4 to prevent washout of the peptide and improve efficacy of the peptide at the cardiac infarct.
The development of controlled delivery systems for Tβ4 is motivated by the need to prolong and localize the bioactivity of peptide. Injectable hydrogels capable of sustained, localized delivery of bioactive molecules were studied in many different applications [136-137]. Only a few groups are looking at the development of such hydrogels in direct conjunction with Tβ4 specifically, however. Collaboration between the Langer and Hubbell groups (from Massachusetts Institute of Technology in the US and Ecole Polytechnique Federale de Lausanne in Switzerland, respectively) has led to the development of a PEG-based hydrogel modified with matrix metalloproteinase peptides (MMPs) thereby yielding a cell mediated proteolytic degradable and remodellable hydrogel matrix for cell and molecule delivery [138]. Their system was capable of physically retaining Tβ4 within the matrix and gave controlled release of 90-100% in 160-200 hours depending on the MMP (MMP-2 or MMP-9) used in the hydrogel. The gelling of the hydrogel occurred in approximately 30 minutes at physiological conditions. Results from encapsulating HUVECs within the developed system showed increased survival of cells in hydrogels containing Tβ4, as well as increased upregulation of the genes endothelial-cadherin and angiopoietin-2. Furthermore, Tβ4 improved HUVEC attachment to the hydrogel and induced vascular-like networks to be formed within the matrix [139].

*In vivo*, injection of the developed hydrogel in a rat MI model showed 80% of encapsulated Tβ4 released by day 3 after injection and 95% released by 6 weeks [139]. The gel degraded to about 25% of the injected amount by day 28 and was undetectable at 6 weeks. Injection of the gel (MMP+PEG) significantly reduced infarct size as compared to a PBS control showing that the gel alone can enhance cardiac function. However, addition of Tβ4 with human embryonic stem cell-derived vascular cells further improved host cardiomyocyte functionality in the infarct zone. A modest improvement in cardiac function could be seen with the combined treatment after 6 weeks, with an increase in ejection fraction of ~12% with Tβ4 and hESC-derived cell treatment compared to PBS injection control. There was also more vasculature in the treatment group and transplanted vascular cells were found to form de novo vascular structures. They hypothesized that the gel substituted for the degrading endogenous matrix, providing temporary support and pro-survival factors, while hESC-derived vascular cells contributed to formation of capillary-like vessels, stabilization of host vessels, secretion of paracrine factors, or induction of paracrine factor secretion from native rat cells.
2.6 Methods of quantifying vascularization

The level of vascularization in this thesis was mainly determined by the following measures: 1) endothelial cell proliferation, 2) endothelial cell migration and 3) tube formation for in vitro studies, and 1) blood vessel density and 2) blood vessel diameter for in vivo studies. These parameters were selected as indicators of vascularization due to their importance in the process of angiogenesis in vivo.

Angiogenesis is the formation of new blood vessels from pre-existing ones [140]. During angiogenesis, pericytes detach from the pre-existing blood vessel, and the vascular basement membrane degrades and reforms in response to growth factors and matrix metalloproteinases. These growth factors are either released from the basement membrane or secreted by tumor cells, immune cells and fibroblasts. This triggers the proliferation and migration of vascular endothelial cells. These endothelial cells align into cords, which branch, form lumens and anastomose. The formation of a new vascular basement membrane then follows. The combination of vascular endothelial cells, pericytes and vascular basement membrane forms a new blood vessel.

Since the proliferation and migration of endothelial cells are required for the formation of a new blood vessel, they are major indicators of angiogenesis. In this thesis, in vitro cell proliferation is evaluated using 1) XTT assay to measure the number of metabolically active cells and 2) lactate assay to measure the level of metabolic activity. Cell migration is evaluated by measuring 1) density of migrated cells and 2) displacement of cells. In vitro tube formation assay is widely used to measure the ability of endothelial cells to differentiate and reorganize into capillary-like tubes [141]. Tube formation is quantified by measuring 1) the number or density of tubes and 2) the average length of tubes [142].

In vivo, blood vessel density and diameter are evaluated from Factor VIII and smooth muscle actin-stained histology images. Factor VIII is an endothelial marker while smooth muscle actin is a pericyte marker. As such, Factor VIII staining indicates all blood vessels since they are all made of endothelial cells. The increase in the number or density of Factor VIII-positive blood vessels is an indication of vascularization occurring. Smooth muscle actin staining indicates only mature blood vessels supported by pericytes. The increase in the number or density of smooth muscle actin-positive blood vessels is an indication of a shift to vessel maturation since
blood vessels that are not supported by pericytes undergo vessel regression [143]. An increase in blood vessel diameter also indicates vessel maturation. Larger vessels are often stabilized by smooth muscle actin-positive cells, while small vessels are predominantly not stabilized and will regress [144]. In addition, the vessel diameter is a major determinant to control flow resistance. A larger diameter significantly decreases vascular resistance.
3 Biodegradable Collagen Patch with Covalently Immobilized VEGF for Myocardial Repair

3.1 Introduction

Cardiac patches are required to surgically restore normal size and shape to the dilated ventricles of patients with end stage congestive heart failure [145] or to correct congenital cardiac defects [146]. Synthetic patches do not heal or grow as a child matures, but biodegradable scaffolds may produce a compliant tissue that heals without scarring [147]. However, cell-engineered patches created from biodegradable biomaterials require extensive angiogenesis (sprouting of new capillaries) to transport oxygen and nutrients and remove waste, in order to support the engraftment of implanted and/or recruited progenitor cells that contribute to tissue formation as the patch degrades. The optimal method to induce and maintain vascularity within a biomaterial patch has yet to be identified.

Cardiac tissue patches created from decellularized matrices that provide a physiological substrate architecture have been evaluated extensively with positive results [37, 148-149]. Combining biomaterials with cells (e.g., muscle cells, endothelial cells, or fibroblasts) yields a 3-dimensional tissue [72, 147]. However, the survival of the tissue patch in vivo will be dependent on the vascularization process. In vitro, engineered tissues can be grown in a perfusion bioreactor to overcome diffusional limitations of oxygen transport in order to support cell survival [150-151]. In addition, channels can be created within tissue engineering scaffolds to mimic vasculature and to facilitate mass transport. Previous studies involving this approach included microfabrication of capillary-sized conduits in PGS-coated silicone wafers [152], and culturing cardiomyocytes and fibroblasts on channelled scaffolds perfused with perfluorocarbon supplemented culture medium [22]. These in vitro techniques are limited in that the pre-formed channels or vasculature cannot connect fast enough to the host’s circulation upon in vivo implantation. In another approach,

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Morritt et al. [71] engineered vascularized cardiac tissues by seeding cells in a polycarbonate chamber that contained an arteriovenous loop, and then implanting the chamber into a rat groin. Madden et al. [33] recently demonstrated that the scaffold pore size can be tuned (30-40μm) to enhance angiogenesis upon implantation of the cardiac patch.

Angiogenic cytokines can be used to induce vascularization within a cardiac patch [153]. For example, vascular endothelial growth factor (VEGF) - which promotes endothelial cell proliferation, cell migration, tube formation, and functional blood vessel formation [98, 154] – can be delivered to damaged cardiac tissue by soaking a biomaterial in VEGF solution before surgical ventricular repair. However, handling during surgery and the ventricular pressure will ultimately cause the soluble VEGF to leak and limit the duration of its biological effects.

Controlled release [66, 155-156] and physical immobilization of growth factors [157] were also previously evaluated in promoting cell proliferation and angiogenesis in vivo. Steffens et al. [157] physically immobilized VEGF onto heparinized collagen matrices. The VEGF released from the matrices led to improved endothelial cell proliferation and angiogenesis in the chorioallantoic membrane. Similarly, increased angiogenesis around the implantation site in the back subcutis of mice was achieved using glutaraldehyde crosslinked collagen hydrogel loaded with VEGF, compared to soluble VEGF and VEGF-free hydrogels [156]. VEGF was also released from an alginate hydrogel delivery system that responded to compressional loading, which in turn induced blood vessel formation [155]. More recently, a scaffold consisting of alginate/alginate sulphate was used to enhance cardiac patch vascularization and viability by incorporating a mixture of prosurvival and angiogenic factors (SDF, IGF-1 and VEGF) by affinity binding to the scaffold [36].

We hypothesized that biomaterial vascularization can be enhanced in vivo during cardiac repair by use of covalently immobilized growth factors. Growth factor signalling can be prolonged by covalently tethering the factor directly to the surface of the biomaterial [158]. We previously found that covalently immobilized VEGF boosted the in vitro proliferation of seeded endothelial cells within a collagen scaffold more than soluble VEGF added to the culture medium [34]. Covalent immobilization offers an advantage over physical entrapment or controlled release techniques since it enhances the stability of the growth factor, and ensures that the effects are localized within the biomaterial itself, rather than the surrounding tissue.
Here, our aim was to increase the angiogenic potential of a biodegradable scaffold for use as a patch in cardiac repair. We accomplished this by covalently immobilizing different concentrations of VEGF directly to a three-dimensional collagen scaffold in order to maximize local growth factor availability. We assessed the angiogenic properties of our biomaterial in vitro and in vivo. We demonstrated the dose-dependent effects of VEGF on the seeded endothelial and bone marrow cells (BMCs) in vitro, and evaluated healing, vascularization, cell recruitment and survival within scaffolds with immobilized VEGF implanted to replace a full thickness defect of the right ventricular (RV) free wall in rats. Patches of this bioactive biomaterial may be well-suited for cardiac repair.

### 3.2 Materials and methods

#### 3.2.1 Preparation of collagen scaffolds

Recombinant VEGF-165 (Cell Sciences, CRV014B) was immobilized onto Ultrafoam collagen sponge scaffolds (2cm x 2cm x 2mm; Davol, 1050050) using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry. Briefly, the scaffolds were immersed in a filtered solution of EDC (Sigma, E7750) and Sulfo-NHS (N-hydroxysulfosuccinimide; Pierce Chemicals, 24510) in phosphate-buffered saline (PBS; Gibco, 10010-023) for 20 minutes. The concentrations of EDC and Sulfo-NHS used in the activation step for different experimental groups are summarized in Table 3-1. Next, the scaffolds were immersed in a solution of VEGF [0.5μg/mL (Low VEGF) or 2.0μg/mL (High VEGF)] in PBS for 2 hours (Table 3-1). Collagen scaffolds immersed in PBS only (both steps) were used as controls. Finally, the scaffolds were washed in PBS.

<table>
<thead>
<tr>
<th>Name of Experimental Group</th>
<th>EDC Activation Step (First Step)</th>
<th>Immobilization Step (Second Step)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDC (mg/mL PBS)</td>
<td>Sulfo-NHS (mg/mL PBS)</td>
</tr>
<tr>
<td>Control</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Low VEGF</td>
<td>4.8</td>
<td>12</td>
</tr>
<tr>
<td>High VEGF</td>
<td>24</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 3-1. Concentrations of reagents used in VEGF immobilization.
3.2.2 Scanning electron microscopy (SEM)

The collagen scaffolds were imaged using environmental scanning electron microscopy (Hitachi S-3400N) as previously described [34]. First, the scaffolds were washed twice with distilled water to remove PBS solution and then placed into the specimen chamber. Next, filter paper was used to remove excess water from the sample. Finally, the chamber was closed, and the chamber temperature decreased to -20°C. Samples were imaged under variable pressure mode at 70Pa and 15kV. Images were captured at 100X.

3.2.3 Quantification of immobilized and released amounts of VEGF

VEGF was quantified using an ELISA kit (Biovision, K4364-1000) according to the manufacturer’s instructions. The amount of immobilized VEGF per scaffold was measured indirectly. First, an ELISA assay was performed to measure the non-immobilized VEGF remaining in the immobilization and washing solutions. Next, the amount of non-immobilized VEGF was subtracted from the starting amount as previously described [34]. To determine the amount of VEGF released from the scaffolds over 28 days in vitro, scaffolds (n=3/group) were prepared and incubated in 200µL PBS at 37°C. PBS was collected from each incubated sample on days 3, 8, 14, 21, and 28, and stored at -20°C. VEGF levels in the PBS (released VEGF) were measured using ELISA. The total amount of VEGF released from the scaffold was calculated by summing the amounts released at all time points.

3.2.4 In vitro cell cultivation

The in vitro activity of immobilized VEGF on cell growth and lactate production was measured. Fresh scaffolds (n=4-6/group; 7mm diameter, 2mm thickness) were seeded with H5V endothelial cells or cultured BMCs (50,000 cells/scaffold) [34, 159] and maintained for 3 days as previously described [34]. Alternatively, scaffolds were incubated in 200µL PBS at 37°C for 28 days (Aged scaffolds), and then seeded with endothelial cells and cultured. H5V endothelial cells were isolated from murine embryonic heart endothelium and grown in H5V culture medium comprising Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma, D5796) with 4.5g/L glucose, 4mM L-glutamine, 10% fetal bovine serum (FBS, Gibco, 16000-044), 100units/mL penicillin, and 100µg/mL streptomycin (Gibco/Invitrogen, 15140122). BMCs were isolated from green fluorescent protein (GFP)-positive Wistar rats and grown in BMC culture medium comprising Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco, 12440-053) with 10% FBS. Before
seeding, collagen scaffolds were washed in PBS, incubated in culture medium for 30 minutes, and dried on autoclaved Kimwipes. The scaffolds were transferred to a clean, 24-well plate. The desired number of cells was centrifuged into a pellet and resuspended in a volume of cell type-specific culture medium corresponding to 10µL per collagen scaffold. H5Vs or BMCs were seeded onto the scaffolds (50,000 cells/scaffold) in 10µL culture medium. The scaffolds were incubated for 40 minutes at 37°C to allow cell attachment. After incubation, 1mL fresh culture medium was added to each well, and the samples were cultured for 3 days.

The number of cells in the collagen scaffolds after the 3 day *in vitro* culture was determined using an XTT (sodium 3’-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay (Roche Diagnostics, 1465015) as previously described [34]. Briefly, collagen samples were incubated with 100µL culture medium and 50µL XTT labelling solution at 37°C with 5% CO₂ for 2 hours. Using this technique, live cells should cleave the yellow tetrazolium salt XTT to form an orange formazan dye. The absorbance of the supernatant (100µL) was measured at 450nm using a plate reader (Apollo LB911, Berthold Technologies). Calibration curves were created by performing XTT assays on standard scaffold samples seeded with known numbers of cells. The number of cells in each sample was determined based on the absorbance value, using the calibration curve.

After seeded scaffolds were cultured for 3 days *in vitro*, culture medium was collected from each well and diluted (1:50) in distilled water. Lactate concentrations were measured in the medium samples using a lactate assay kit (Biomedical Research Service Centre, A-108L) according to the manufacturer’s instructions. Absorbance was measured using a plate reader at 492nm. Since the intensity of the coloured formazan product is proportional to the concentration of lactate, lactate concentrations were determined based on the absorbance values, using calibration curves from the series of lactate standards.

3.2.5 Experimental animals for in vivo studies

Animal procedures were approved by the Animal Care Committee of the University Health Network. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Sprague Dawley rats (200-225g) and Wistar rats (250-275g) underwent RV free wall replacement. Implanted scaffold morphology, histology, and cell proliferation were measured in Sprague Dawley recipients; circulating cell recruitment
to the implanted scaffold and cell survival within the scaffold were evaluated in Wistar recipients. BMCs expressing green fluorescent protein (GFP) were isolated from female transgenic Wistar rats [Wistar-TgN(CAG-GFP) 184ys] (carrying an enhanced GFP transgene driven by chicken β-actin promoter and CMV enhancer) and used for cell recruitment and survival experiments.

### 3.2.6 RV free wall resection and replacement with collagen scaffold

This procedure was performed as we previously described [160]. Briefly, rats were ventilated and anesthetised with 1.5-2.5% isoflurane. In each rat, the heart was exposed through a median sternotomy. A 5-6 mm diameter section of the RV free wall was isolated with a purse-string suture to create a “neck” of the bulging RV free wall. A 16-gauge angiocatheter was passed over the stitch to create a tourniquet and prevent bleeding when a full thickness circular segment of the RV free wall was removed. A collagen scaffold patch (5 x 5 x 2 mm) was laid on top of the resected ventricular wall and then sutured to the margin of the defect using a purse-string stitch (with 7-0 polypropylene monofilament material and an over-and-over running suture technique) to close the RV free wall defect. The tourniquet was released and the purse-string stitch was removed. The chest wall was closed with 5-0 silk.

### 3.2.7 In vivo cell recruitment

To evaluate cell recruitment into the patch, BMCs (1x10^6 in 50µL of serum-free medium) isolated from GFP transgenic Wistar rats were injected intravenously into syngeneic Wistar rats at 1 day after patch implantation (n=3-4/group). Five days after cardiac repair, rats were sacrificed and scaffolds were removed and fixed in 10% formalin for 48 hours. Fixed samples were cut into 5µm thick sections. GFP-positive BMCs that were recruited into the patch were identified using immunohistochemical staining. Four sections were analyzed per group. GFP-positive cells were counted in 5 randomly selected high power (20×) fields within the patch area.

### 3.2.8 In vivo cell survival

The effect of immobilized VEGF on cell survival within the patch was determined by identifying GFP-positive BMCs (1x10^6 initially seeded) that survived after they were seeded into the scaffold (directly injected into the patch at the time of surgical repair). Patches were collected and fixed at 3 days (n=4/group) or 28 days (n=4/group) after cardiac repair. GFP-positive BMCs
were identified using immunohistochemical staining. Four sections were analyzed per group and time point. The area of the patch that contained GFP-positive cells was determined in 5 randomly selected high power (20×) fields per patch (ImageJ software, NIH software).

3.2.9 In vivo cell proliferation

The thymidine analogue 5-bromo-2’-deoxyuridine (BrdU; 50 mg/kg) was injected intraperitoneally into rats (n=5/group) for 3 consecutive days after patch implantation (BrdU is taken up by nuclei in S-phase). After 3 days, rats were sacrificed and patches were collected. BrdU-positive cells in the patches were identified using immunohistochemical staining as previously described [161], and counted in 5 randomly selected high power (40×) fields within the patch area. Five sections were analyzed per group.

3.2.10 Scaffold morphology and histology

Rats were euthanized by anesthetic overdose at 7 days (n=6/group) or 28 days (n=4/group) after patch implantation. Hearts were perfused with KCl supplemented with heparin. The RV free wall of each heart was photographed. Patch and RV free wall areas were measured using ImageJ software. Patch areas were calculated for all groups as previously described [160].

Next, the heart tissue was fixed in 10% formalin-PBS solution and cut into 5μm thick sections. Sections were stained with Masson’s trichrome (to identify extracellular matrix and collagen), anti-CD31 antibody (1:400, Invitrogen; to identify blood vessels), or with a monoclonal anti-α-smooth muscle actin (α-SMA) antibody (1:3000, Sigma-Aldrich Corp, St. Louis, MO; to identify muscle tissue). Patch thickness was quantified using computerized planimetry (ImageJ software) as described previously [160]. Vascular density was estimated by counting CD31-positive blood vessels in 5 randomly selected high power (40×) fields within the patch area of sections stained with anti-CD31 antibody. In sections stained with anti-α-SMA antibody, ImageJ software was used to measure the areas occupied by α-SMA-positive pixels. Blood vessel formations were identified and digitally removed. The remaining α-SMA-positive pixels were quantified to yield the area (expressed as a percentage of total possible pixels) of non-vascular, α-SMA-positive tissue. For patch thickness, vessel density and α-SMA-positive tissue measurements, 4-6 sections were analyzed per group and per time point. All quantifications were carried out by a blinded observer using a Nikon Eclipse TE200 microscope.
3.2.11 Statistical analyses

Data were expressed as mean ± standard error. Analyses were performed using SPSS software (v.12.0), with the critical α-level set at p<0.05. Comparisons among multiple groups were made using analyses of variance (ANOVA). When F values were significant, differences between the groups were specified with Tukey’s multiple comparison post-tests or least significant difference (LSD) multiple range post-tests.

3.3 Results

3.3.1 Characterization of the collagen scaffold

SEM imaging demonstrated that the chemical process used to conjugate VEGF to the collagen scaffold did not significantly change the physical characteristics of the biomaterial (Figure 3-1A-C). Pore structure and size were similar in control and VEGF-treated scaffolds, though control scaffolds in the wet state exhibited slightly collapsed pores (Figure 3-1A).

Low and High VEGF-treated scaffolds contained 14.5±1.4ng and 97.2±8.0ng, respectively, of covalently immobilized VEGF. Therefore, the amount of VEGF immobilized increased as the VEGF concentration in the immobilization solution increased (p<0.01; Figure 3-1D). Over the course of a 28 day incubation in PBS, most VEGF release from the scaffolds occurred between days 0 and 3. Total VEGF release over 28 days was 0.005±0.005ng from the Low VEGF and 0.073±0.005ng from the High VEGF scaffolds (p<0.01; Figure 3-1E, F). The amount of VEGF released was therefore insignificant compared to the initial amount immobilized onto the collagen sponges, indicating that the growth factor was stable after covalent immobilization to the scaffold.

3.3.2 Immobilized VEGF increased seeded cell growth and lactate production in vitro

Fresh scaffolds were seeded with endothelial cells or BMCs (since BMCs could also migrate into an implanted scaffold in vivo) and cultured for 3 days. The number of endothelial cells or BMCs within the scaffold was significantly increased in VEGF-treated scaffolds (p<0.05 vs. controls), with a further increase in the High VEGF group (p<0.05 vs. Low VEGF) (Figure 3-2A, B). This suggests a dose response in seeded cell growth as a function of immobilized VEGF concentration. Lactate production rates were significantly increased (p<0.05) in Low and High
VEGF-treated scaffolds compared to controls whether the biomaterial was seeded with endothelial cells or BMCs (Figure 3-2A, B).

Figure 3-1. *In vitro* properties of porous collagen scaffolds with covalently immobilized VEGF. (A-C) SEM micrographs demonstrating pore size and structure in scaffolds either untreated (control, A), or with 14.5±1.4ng (Low VEGF, B) or 97.2±8.0ng (High VEGF, C) immobilized VEGF. (D) Amount of immobilized VEGF per scaffold (using ELISA). (E) Cumulative (total) amount of VEGF released from scaffolds over 28 days of incubation. (F) Time course illustrating immobilized VEGF release from scaffolds over 28 days.
Figure 3-2. *In vitro* bioactivity of immobilized VEGF.

(A-C) Number of cells (XTT assay) and lactate production rate at 3 days after endothelial cells (A) or bone marrow cells (B) were seeded onto a fresh scaffold, or endothelial cells were seeded onto a scaffold pre-aged for 28 days (aged scaffold, C). Collagen scaffolds were untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF.

To investigate whether VEGF that remained immobilized on the collagen scaffolds over a 28 day incubation period retained its bioactivity, the same endothelial cell growth and lactate production
assays were performed using scaffolds that were first incubated in PBS for 28 days. Similar to the results with fresh scaffolds (Figure 3-2A), the “aged” Low and High VEGF scaffolds maintained significantly higher cell numbers (p<0.05) and lactate production rates (p<0.01) than the aged controls (Figure 3-2C). Therefore, VEGF exhibited sustained bioactivity when covalently immobilized onto collagen scaffolds.

3.3.3 Immobilized VEGF improved right ventricular repair with a collagen scaffold

3.3.3.1 Increased BMC recruitment to the implanted patch

Cell homing into an implanted collagen patch was assessed using GFP-positive BMCs delivered intravenously at 1 day after patch implantation. Immunohistochemical staining revealed that GFP-positive BMCs were recruited to control and VEGF-treated patches by 5 days after patch implantation (Figure 3-3A-C). The number of GFP-positive cells was greater in the High VEGF patches than in the control patches (GFP-positive BMCs per 0.4mm²: control=8.0±1.3, Low VEGF=15.7±4.7, High VEGF=25.0±5.5; p<0.05 for control vs. High VEGF) (Figure 3-3D).

3.3.3.2 Increased cell proliferation within the implanted patch

BrdU injected into rats for 3 consecutive days after patch implantation was incorporated into proliferating cells within control and VEGF-treated patches (Figure 3-3E-G). Significantly more BrdU-positive cells were identified immunohistochemically in the High VEGF patches than in the control patches (BrdU-positive cells per 0.2mm²: control=3.5±0.7, Low VEGF=6.5±1.4, High VEGF=9.9±1.2; p<0.05 for control vs. High VEGF) (Figure 3-3H).

3.3.3.3 Reduced expansion of the implanted patch

The surface areas of the control and VEGF-treated patches were similar before implantation. After implantation, the control patches thinned and dilated, while the High VEGF-treated patches resisted expansion. Specifically, patch surface areas increased 56.4% and 73.5% at 7 and 28 days, respectively, after implantation in the control group, but only 12.1% and 18.2% at those time points in the High VEGF group (Figure 3-4A-F). Surface areas were therefore significantly reduced (p<0.05 vs. control patches at both time points) in patches treated with the higher VEGF concentration (Figure 3-4G).
Figure 3-3. Cell mobilization to and proliferation within the collagen scaffold.

(A-C) Representative images of GFP expression (arrows) at 5 days post-implantation. Collagen patches that are untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF were implanted, followed by systemic injection of GFP-positive bone marrow cells (BMCs) 1 day post-implantation. (D) Numbers of GFP-positive BMCs identified in the patches at Day 5. (E-G) Representative images of BrdU expression (arrows) at 3 days post-implantation. Collagen patches were implanted, followed by 3 consecutive days of intraperitoneal BrdU delivery. (H) Numbers of BrdU-positive cells identified in the patches at Day 3.
Figure 3-4. Remodelling and angiogenesis within the collagen scaffold.

(A-F) Representative images illustrating the extent of the scaffold (dotted outline) at 7 (A-C) or 28 (D-F) days after implantation of collagen patches [untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF]. (G) Patch surface areas before (pre-implantation) and 7 or 28 days after implantation. (H-J) Representative images of Masson’s trichrome staining at 28 days after patch implantation. Arrows indicate thickness of the patch. (K) Patch thickness at 7 or 28 days after implantation. Normal RV=normal right ventricular tissue; red dotted line indicates patch thickness at the time of implantation. (L-N) Representative images of CD31 expression (arrows identify CD31-positive vascular structures) at 28 days after patch implantation. (O) Blood vessel density within the patches at 7 or 28 days after implantation.
3.3.3.4 Increased wall thickness and blood vessel density within the implanted patch

Patch thickness (Figure 3-4H-K) and blood vessel density (Figure 3-4L-O) were assessed using Masson’s trichrome staining and immunohistochemical staining for CD31, respectively. The collagen patches were 2mm thick at the time of implantation. Over the following 28 days, the average patch thickness decreased by 82% in the control group and 64% and 54% in the Low and High VEGF groups, respectively. Most of this decrease occurred within the first 7 days after implantation in the control and Low VEGF patches. At 7 days, wall thicknesses in High VEGF patches (1.92±0.41mm) were largely preserved, and were significantly greater (p<0.05) than those in the other two groups (Figure 3-4K). At 28 days, wall thicknesses in both VEGF-treated patches were significantly greater than those in the control patches (p<0.05 for Low VEGF vs. control; p<0.001 for High VEGF vs. control) (Figure 3-4K). Blood vessel density within the patch was significantly increased in the High VEGF group at 7 days (p<0.001 vs. control) and 28 days (p<0.01 vs. control; p<0.05 vs. Low VEGF) after patch implantation (Figure 3-4O).

![Figure 3-5. Linear relationship between patch thickness and vessel density.](image)

Patch thickness and blood vessel density in individual animals at 28 days after implantation of collagen patches [untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF]. There was a positive correlation between the variables.

Covalently immobilizing VEGF (particularly at the higher concentration) on the implanted collagen scaffold increased both patch thickness and blood vessel density within the biomaterial. Further, a positive correlation was found between these two measures (p=0.023, r=0.67, Figure 3-5).
suggesting that increasing capillary numbers (via VEGF immobilization) will promote increased patch thickness.

### 3.3.3.5 Increased recruitment of myofibroblasts to the implanted patch

Quantification of non-vascular α-SMA-positive pixels (myofibroblast tissue) within the patches (Figure 3-6A-F) indicated that the myofibroblast response was significantly greater in VEGF-treated patches compared to control patches at 7 and 28 days after implantation (p<0.01 for Low or High VEGF vs. control), with greater myofibroblast recruitment in the High VEGF patches compared to the Low VEGF patches (p<0.001) at the 7 day time point (Figure 3-6G).

![Figure 3-6](image_url)

**Figure 3-6. Formation of myofibroblast tissue within the collagen scaffold.**

(A-F) Representative images of α-SMA expression (white arrows identify α-SMA-positive tissue) at 7 (A-C) or 28 (D-F) days after implantation of collagen patches [untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF]. Black arrows identify remaining collagen scaffold fragments. (G) Area (expressed as % of total tissue area) of non-vascular, α-SMA-positive tissue within the patches at 7 or 28 days after implantation.
Figure 3-7. Survival of seeded bone marrow cells within the collagen scaffold in vivo.

(A-F) Representative images of GFP expression (arrows) at 3 (A-C) or 28 (D-F) days after implantation of collagen patches [untreated (control), or with 14.5±1.4ng (Low VEGF) or 97.2±8.0ng (High VEGF) immobilized VEGF]. GFP-positive bone marrow cells (BMCs) were injected directly into the patches before implantation. (G) Area (expressed as % of patch area) that contained GFP-positive BMCs at 3 or 28 days after implantation.

3.3.3.6 Increased survival of seeded BMCs within the implanted patch

In all groups, some of the GFP-positive BMCs seeded into the patches at the time of implantation remained in the patch area (Figure 3-7A-F). At 3 days after implantation, the area of the patch containing GFP-positive cells was significantly greater (p<0.01) in both Low VEGF (11.2±5.1%) and High VEGF (13.5±11.5%) groups than in the control group (1.3±1.5%) (Figure 3-7G). By 28 days, seeded cell number dropped sharply in the Low and High VEGF groups (%
GFP-positive patch areas: High VEGF=4.2±1.7%, Low VEGF=1.5±0.4%, control=1.2±0.7%), but remained significantly greater (p<0.001 vs. Low VEGF and control) in the High VEGF patches (Figure 3-7G).

3.4 Discussion

Engineered cardiac patches hold a great promise in improving functional properties and attenuating pathological remodelling upon myocardial infarction [2]. However, rapid vascularization is required to ensure survival of the implanted tissue graft. It takes at least 3 to 7 days post-implantation for the ingrowth of new blood vessels to occur in vivo [162]. Thus, the viability of engineered tissues with physiologically relevant thickness remains low. We developed an effective method to incorporate an angiogenic growth factor into the 3-dimensional structure of a biodegradable cardiac patch. This is the first report of enhanced angiogenesis after a full thickness RV repair using a collagen scaffold with covalently immobilized VEGF. Successful cardiac repair with a biodegradable patch requires extensive perfusion [163] to promote the survival of recruited or implanted cells and to ensure that the patch material is replaced by viable tissue, rather than fibrotic scar tissue, as it degrades [164-165]. In addition, an effective cardiac patch should maintain its geometry upon implantation as patch thinning and dilatation may ultimately result in pathological remodelling of the heart.

In vitro, the conjugated VEGF was active for at least the 28 day duration of the study (Figure 3-2). In vivo, collagen scaffolds with covalently immobilized VEGF improved tissue formation by promoting cell proliferation within the graft, thus increasing blood vessel density and cellular engraftment and reducing construct thinning in a rat model of full thickness RV free wall repair. Interestingly, we documented a dose response in which High VEGF patches supported greater cell survival and more vascular structures than Low VEGF patches by 28 days after implantation (Figure 3-4L-O, 3-7). Importantly, we did not explore implantation of the patches with non-conjugated (soluble) VEGF because our previous in vitro studies clearly demonstrated that soluble growth factors were inferior to covalently immobilized growth factors for promoting endothelial cell proliferation and infiltration into the scaffold [34, 166].

EDC chemistry was used to couple carboxyl groups in the collagen scaffold to amine groups of VEGF. EDC is a water-soluble, zero-length crosslinking agent that creates stable amide bonds between proteins [167]. To do so, EDC reacts with carboxyl groups of collagen to form an
amine-reactive O-acylisourea intermediate. Then, sulfo-NHS is added to convert the intermediate into an amine-reactive sulfo-NHS ester [167]. The reactive esters then react with amine groups on VEGF to form amide bonds [167]. In our previous studies, we conclusively demonstrated that the utilized method resulted in covalent immobilization of the growth factor to the biomaterial, rather than physical adsorption [34]. The side-reactions may cause the crosslinking of the scaffold to itself, or immobilization of the VEGF molecule in various configurations as well as the crosslinking of the active site as we previously analyzed [168]. Thus, more specific chemistries such as click chemistry will be explored in future studies.

After covalent immobilization, more than 99% of immobilized VEGF remained on the scaffolds over 28 days of incubation in PBS in vitro (Figure 3-1D, E). That the VEGF remained bioactive was illustrated by enhanced cell proliferation in vitro (Figure 3-2) and in vivo (Figure 3-3E-H). Endothelial cell proliferation occurs early in the angiogenic process (before tube formation, pericyte recruitment, or vessel stabilization [169]). Not surprisingly, we found that proliferation of endothelial cells or BMCs was increased in vitro (vs. control) in the VEGF-treated scaffolds. This effect was dose-dependent in fresh scaffolds, but not aged ones (incubated in PBS for 28 days before use) (Figure 3-2). Previously, Shen et al. showed that degradation of these collagen scaffolds was minimal during the first 7 days of incubation, but significant after 12 days [166]. Thus, the early effects of VEGF may be attributed to the immobilized VEGF, while later effects may be partially due to VEGF released during scaffold degradation.

In vivo, regenerative responses were enhanced in cardiac patches that had been modified with the immobilized VEGF. Specifically, an increased number of intravenously administered GFP-positive BMCs were recruited to High VEGF patches compared to control patches (Figure 3-3A-D). BMC mobilization was previously found to promote healing after myocardial infarction [170]. This process is thought to be the initial step of angiogenesis within the collagen patches, and BMC engraftment enhances the recruitment of endothelial progenitor cells [171]. Both postsurgery cell proliferation (evaluated using BrdU labelling) and seeded BMC survival were significantly increased in the High VEGF patches (Figure 3-3E-H, 3-7). These patches also exhibited the highest blood vessel densities (Figure 3-4L-O) - probably due to VEGF-induced recruitment and subsequent proliferation of progenitor cells. Angiogenesis was probably not the result of an increased inflammatory response in the VEGF-treated scaffolds because inflammatory responses (assessed by two blinded observers) were similar among groups (data...
not shown). Future patches might use two immobilized growth factors (e.g., VEGF and angiopoietin-1 [34]) to further promote maturation of the newly-formed blood vessels within.

Over 28 days after patch repair, both the area and the thickness of the implanted scaffold were best preserved in the High VEGF group (Figure 3-4A-K). Further, patch thickness was positively correlated with blood vessel density (Figure 3-5). Increased vessel density enhances the capacity of the biomaterial for oxygen and nutrient transport, preventing thinning and dilatation of the repaired region by contributing to the formation of viable myogenic tissue [172] as the biomaterial degrades. Indeed, improved vascularization in the High VEGF patches was associated with tissue formation and cell survival, as evidenced by increased formation of α-SMA-positive myogenic tissue that was sustained for 28 days after patch repair in this group (Figure 3-6). On the other hand, patch remodelling was most pronounced in the control patches (no VEGF), in association with the lowest rates of injected BMC recruitment and myogenic tissue formation.

An ideal cardiac patch should maintain its thickness and assume the shape of the native cardiac structure it replaces. Here, patch thickness was sustained most effectively when VEGF was covalently immobilized to the collagen scaffold. Importantly, the increased patch thickness did not compromise cell viability (Figure 3-7). In general, thicker cardiac patches should provide greater flexibility and enhanced mechanical support for tissue growth rather than being resorbed during the formation of a fibrotic scar. We found that the thickness of the High VEGF patch was actually similar to that of the normal RV wall at 28 days after implantation (Figure 3-4K), demonstrating the potential of the VEGF-treated patch to maintain both the geometry and integrity of the RV free wall.

Bioactive biomaterials with covalently immobilized angiogenic growth factors may be a useful alternative to current cell-, gene-, or biomaterial-based strategies for cardiac repair. The VEGF-treated collagen scaffold is simpler to engineer and apply than other vascularized cardiac tissues [69, 71-72]. Unlike cell transplantation or gene therapies, patch repair does not require the consideration of cell type or source, and has limited potential for oncogenesis [163, 173]. This approach is not limited by significant cell loss and washout after implantation [174]. Rather, cytokine enhancement can increase the survival of recruited cells and those injected into the biodegradable scaffold at the time of implantation [146]. Future studies will be required to
optimize the configuration of these particular scaffolds, to determine the optimal dose of immobilized VEGF, and to formally compare cell seeded and non-seeded patches. Covalent immobilization localizes the effects of cytokines to the exact location of repair (the biomaterial itself), and ensures growth factor stability in an aqueous environment. The approach we described may eventually provide off-the-shelf, biodegradable biomaterials that permit prolonged cardiac structural repair with a viable elastic tissue rather than a dense scar.

3.5 Conclusions

Collagen scaffolds with covalently immobilized VEGF improved tissue formation by promoting cell proliferation within the graft both in vitro and in vivo, thus leading to increased blood vessel density and reduced construct thinning in a rat model of RV free wall repair. Interestingly, there was a dose response in which High VEGF patches showed an increased presence of positively stained vascular structures compared to Low VEGF patches by Week 4 of the implantation. As such, these modified collagen scaffolds supported vascularization within the graft. Improved and rapid vascularization of implanted grafts can overcome the limited transport capacity of oxygen and nutrients into the growing tissue. Thus, collagen scaffolds with covalently immobilized VEGF have suitable mechanical and biological properties for potential use in repairing heart defects.

3.6 Acknowledgements

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3.7 Commentary

In this chapter, I described the design, fabrication and evaluation of scaffolds with covalently immobilized VEGF. Scaffold patches are suitable for repair of ventricular septal defects, the most common cardiac disease in children. In this disease, a full thickness defect exists in the heart wall, allowing oxygen-rich blood to flow from the left ventricle to the right ventricle. This
increases the volume of blood that is pumped back to the lungs and in turn burdens the heart. Currently, Gore-Tex is the most used material for replacing such defects. However, synthetic materials do not grow with the child. In this study, I used collagen scaffolds to replace synthetic materials as a repair method for septal defects. Moreover, biomaterials need extensive vascularization to support cell survival and tissue growth. Here, VEGF-165 was chosen to be immobilized to the collagen scaffolds since it is the most potent and widely studied angiogenic factor [175]. In particular, VEGF-165 is the predominant VEGF isoform and acts on endothelial cells to promote cell migration, proliferation and angiogenesis upon binding to the receptor VEGFR2 [176]. Its bioactivity does not require cell internalization [177], thus allowing its covalent immobilization onto scaffolds. Immobilization of the growth factor is important since it prevents cell internalization and degradation of the biomolecule, in turn prolonging its presence and bioactivity. In addition, the immobilized VEGF is maintained within the collagen scaffolds, thus localizing the bioactivity at the site of implantation and promoting vascularization and tissue growth at the septal defect.

EDC chemistry was used to immobilize VEGF onto the scaffolds. In this reaction, EDC reacts with the carboxyl groups of collagen to form an amine-reactive O-acylisourea derivative, which is converted into amine-reactive sulfo-NHS esters by the addition of sulfo-NHS [167]. Then, the reactive esters react with the amine groups of VEGF to form stable amide bonds between VEGF and collagen. This process can lower the bioactivity of VEGF by interfering with the receptor binding region of the growth factor if the amino acids from the receptor binding region are involved in the reaction. VEGF is a homodimer of two molecules and contains two binding sites for VEGFR2. Four of 66 amine groups on the outer surface of the VEGF homodimer are located in the VEGFR2 binding clefts. Thus, the probability of interference due to VEGF immobilization was previously calculated to be 6% [166]. Since VEGF bioactivity is affected by the immobilization process, it would be expected that VEGF in the immobilized form on a two-dimensional surface has lowered bioactivity than soluble VEGF. However, in three-dimensional situations, immobilized VEGF has improved bioactivity compared to soluble VEGF due to limitations of soluble VEGF diffusing into the three-dimensional biomaterial. It was previously shown that the bioactivity of VEGF in immobilized form in three-dimensional substrates was better than that in the soluble form [35, 166]. This justifies the use of covalently immobilized VEGF in this study. In addition, soluble and matrix-bound VEGF showed different modes of
action. Soluble VEGF leads to short VEGFR2 phosphorylation, Akt activation and vascular dilation or hyperplasia, while matrix-bound VEGF causes prolonged phosphorylation of VEGFR2, activation of p38 and vascular sprouting [177-178].

No gradient of VEGF was used in this study. As such, cells that were seeded *in vitro* infiltrated into the scaffolds by a combination of gravity and cell migration along the biomaterial, which occur independently of VEGF gradient. *In vivo*, the localized presence of VEGF within the scaffolds acted as a chemoattractant to promote cell recruitment and infiltration into the biomaterial.

Although it was shown here that the developed scaffolds promote endothelial cell proliferation *in vitro* and vascularization in the heart *in vivo*, a limitation of this study is the lack of long term evaluation of these VEGF-immobilized scaffolds. In MI, ventricular remodeling can continue for weeks or months [179]. This may also be the case with the remodeling of the implanted collagen scaffold for repair of full thickness defects. Vascular remodeling may also continue for weeks or months. In future work, it would be of interest to evaluate the VEGF scaffold for a longer period in large animal models so as to show its potential to be used as a commercialized patch for cardiac repair. For this thesis, the evaluation of vascularization at 1 week and 4 weeks is sufficient as these are common termination time points for studies involving the implantation of biomaterials or tissues in the rat heart [36, 180]. The use of the same experimental termination time points as other studies eases a more direct comparison of the VEGF scaffolds developed here with previous angiogenic biomaterials.

Although biomaterials with incorporated growth factors have been extensively shown to induce vascularization necessary for supporting cell survival, this is the first report of the use of VEGF-immobilized scaffolds in the heart. Moreover, the sustained *in vitro* bioactivity of VEGF after aging the scaffolds for 28 days is an important finding of this thesis, since the effect of the modified scaffolds is due solely to immobilized VEGF. By contrast, previously used angiogenic biomaterials involved the release of growth factors. Although VEGF is expected to be released more quickly in the *in vivo* environment due to the degradation of the collagen scaffold, this finding suggests that biomaterials can be created in future studies to further prolong the bioactivity of immobilized VEGF or other growth factors by controlling the biodegradability rate of the base material.
While scaffolds are suitable for repair of full thickness defects, as discussed in this chapter, their implantation is not feasible for other cardiac diseases since it would require resection of the heart and invasive surgeries. For cardiac diseases such as MI, the use of injectable hydrogels is more suitable and will be described in the next chapter.
Chapter 4

4 Controlled Release of Thymosin β4 using Collagen-Chitosan Composite Hydrogels Promotes Epicardial Cell Migration and Angiogenesis

4.1 Introduction

Cardiovascular diseases, including myocardial infarction (MI) and congestive heart failure (CHF), are the leading causes of death in the world, causing around 2.5 million deaths each year in US alone [181]. Heart transplantation is often limited by shortage of donors. Potential novel treatment strategies that are currently under investigation include: 1) implantation of engineered cardiac patches [9], 2) injection of cells [182-183], and 3) injection of bioactive molecules [184]. Implantation of engineered cardiac patches is limited by availability of an appropriate cell source, invasive surgical procedures and effective coupling of engineered tissues to the native tissue [185-187]. Injections of cells or bioactive molecules are less invasive, but are limited by the rapid washout from the injection site. Also, soluble biomolecules generally exhibit short half-life in vivo [59]. Injectable hydrogels could provide a minimally invasive method [188] for the delivery and controlled release of cells or bioactive molecules to the infarct site for cardiac repair.

A key challenge in cardiac repair using both patches and injectable biomaterials is the lack of functional vasculature, which leads to the low survival of engineered cardiac tissues or injected cells [189]. While several angiogenic growth factors such as vascular endothelial growth factor (VEGF) and angiopoietins are well known for regulating the formation of blood vessels, each of them is inadequate individually to sustain both vascular growth and stability [190-192]. In contrast, thymosin β4 (Tβ4) has been shown to be important for vascular stability due to its ability to cause recruitment and differentiation of both endothelial and smooth muscle cells [92].

4 Copyright © 2011 Elsevier. Contents of this chapter have been published in Journal of Controlled Release: Chiu LLY and Radisic M. Controlled release of thymosin β4 using collagen-chitosan composite hydrogels promotes epicardial cell migration and angiogenesis. Journal of Controlled Release. 2011 Nov; 155(3):376-385. Reuse with permission from Elsevier. A link to the published paper can be found at: www.sciencedirect.com/science/article/pii/S0168365911003841
Tβ4 is a new therapeutic agent currently investigated for the treatment of damages caused by coronary obstruction or acute myocardial infarction [135]. Tβ4 is a highly conserved 43-amino acid (MW = 4964 Da), acidic (PI 4.6), G-actin sequestering peptide [118, 135]. Importantly, Tβ4 is an angiogenic factor, a chemoattractant, and a cardioprotective biomolecule [116, 118, 135]. It has also been shown to stimulate VEGF synthesis [193]. The adult epicardium is a potential source of vascular progenitors, which migrate under Tβ4-stimulation and can differentiate into smooth muscle and endothelial cells [116, 135]. Tβ4 has been shown to be essential for cardiac vessel development during vasculogenesis, angiogenesis and arteriogenesis [116, 118, 135]. Previous studies showed that in vivo, Tβ4 improved cardiomyocyte survival by inducing coronary vascularization and upregulating Akt activity [116, 118]. Tβ4 treatment after coronary artery ligation in mice resulted in upregulation of ILK and Akt activity in the heart, enhanced early survival of myocytes, reduced scar volume, improved cardiac function, and attenuated adverse remodelling [118, 135].

Previous in vitro studies showing Tβ4-induced cell migration from cardiac explants were performed using soluble Tβ4 [116, 118]. However, a more suitable delivery method is necessary for in vivo injections into the heart. In previous studies, Tβ4 treatment after myocardial infarction in mice was applied intracardially in collagen gel or intraperitoneally in phosphate buffered saline (PBS) [118]. No significant improvements were seen when Tβ4 was locally injected in collagen gel compared to a systemic injection [118]. This may be due to the fact that localized and sustained release cannot be achieved with unmodified collagen gels [194].

The main objective of this study was to evaluate the applicability of a hydrogel based on collagen I and chitosan for the encapsulation and controlled release of Tβ4 to promote angiogenesis. Tβ4 was encapsulated to elicit highly localized and sustained activity. Collagen and chitosan are both known to be biocompatible. As a component of extracellular matrix, collagen contributes to the tensile strength in tissues. More importantly, the heart wall is composed of collagen-based extracellular matrix [195-196]. Collagen has the ability to promote cell attachment and proliferation [197], and has been used as a scaffold for engineering of blood vessel grafts, artificial skin, ligament and tendon [198]. However, it has a fast biodegradation rate and low mechanical strength [197]. Chitosan is a positively charged, hydrophilic and nontoxic polysaccharide that is used for tissue engineering, drug delivery, wound healing and surgical adhesives [199]. Due to its mechanical stability, it can be used in combination with
collagen to tailor the biological and mechanical properties of hydrogels for specific applications [197]. Recently, chitosan has been shown to increase the compression modulus of previously developed injectable collagen hydrogel, which can potentially improve its ability to stabilize the ventricular wall to help reduce heart dilatation upon MI [200]. The addition of chitosan also enhanced neovascularization *in vitro* and *in vivo* [200]. Here, we hypothesized that a long-term controlled release of Tβ4 can be achieved from composite collagen-chitosan hydrogels. We also demonstrated that the released Tβ4 induced cell migration and tube formation from cardiac explants *in vitro* and improved angiogenesis *in vivo*.

4.2 Materials and methods

4.2.1 Preparation of hydrogels

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<th>Amount of Polyalanine (ng)</th>
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Stock solutions were first prepared as follows: collagen I (BD Biosciences, cat #354236, 4.08mg/mL rat tail collagen I in acetic acid), chitosan (75-90% deacetylation, MW=150-200 kDa, Protasan, NovaMatrix, G113) at different concentrations (ranging from 4.60mg/mL to 5.45mg/mL) in distilled water, and the peptides (702μg/mL), thymosin β4 (Prospec, cat # HOR-
275), poly-L-lysine hydrobromide (Sigma, cat # P0879, MW=1000-5000), poly-DL-alanine (Sigma, cat # P9003, MW=1000-5000) and poly-L-glutamic acid sodium salt (Sigma, cat # P1943, MW=750-5000) in distilled water. The molecular weights are as per the manufacturers’ specifications. The individual stock solutions were then mixed in an appropriate volume ratio in a single step on ice to achieve the final concentrations of 2.5mg/mL collagen I and 1.25mg/mL chitosan and the final amounts of peptides as indicated in Table 4-1. Additionally, 10X PBS (one tenth of the final volume) and 1N sodium hydroxide (0.025 times volume of collagen) were added in the same step to neutralize the solution. For the collagen-only hydrogel with encapsulated Tβ4, water was added in place of chitosan, and the final amount of Tβ4 was 1500ng. The solutions were gently vortexed to mix the components. Then, 50μL of solutions were pipetted into the centre of individual wells of a 24-well plate and gelled at 37°C for 1 hour. Experimental groups included: collagen-chitosan hydrogels without Tβ4 (Control), with soluble Tβ4 in culture medium (Soluable), with 100ng encapsulated Tβ4 (Encap100), with 150ng encapsulated Tβ4 (Encap150), with 750ng encapsulated Tβ4 (Encap750), with 1500ng encapsulated Tβ4 (Encap1500; Gel with Thymosin β4), with 1500ng encapsulated polyllysine (Gel with Polyllysine), with 1500ng encapsulated polyalanine (Gel with Polyalanine), and with 1500ng encapsulated polyglutamic acid (Gel with Polyglutamic Acid).

4.2.2 Characterization of hydrogels

The hydrogels were imaged using environmental scanning electron microscopy (Hitachi S-3400N), as described previously [34]. They were individually placed into the specimen chamber, which was set at -17°C. The samples were imaged under variable pressure mode at 70Pa and 15kV. Images were captured at 200X and 500X.

Storage (G’) and loss (G’’) moduli were measured using a Carri-Med CSL 2500 rheometer (TA Instruments) with a Peltier plate for temperature control and a 6cm diameter 4° cone. Amplitude and frequency sweep tests were performed to confirm that the frequency and strain were within the linear viscoelastic region. Frequency sweep tests are shown in Figure 4-3. Based on the results from these tests, time sweeps were conducted at 1Hz and 1% strain for all hydrogel samples. The Peltier plate of the rheometer was set at 25°C and 2.5mL of hydrogel solution was loaded onto the plate. The temperature was then set to the gelation temperature of 37°C and time ramping was performed for 1500s. The solution was fully gelled at the point of maximum
storage modulus. The plateau storage and loss moduli were determined by averaging the storage or loss moduli from the point of maximum modulus to the end of time ramping. The gelation time was defined as the time at which there was a sudden increase in the viscosity measurement, as described [200]. Briefly, viscosity was plotted against time, and a line was fitted to the linear portion of the data. The x-intercept of the line was calculated as the gelation time as described [200].

To perform degradation studies \textit{in vitro}, the hydrogels were incubated at 37\(^\circ\)C in 500\(\mu\)L PBS. At different time points, hydrogels were removed, weighed and placed into individual 15mL centrifuge tubes with holes that were punctured on the lids with a needle (\(n=3\) for each experimental group). For Day 0, hydrogels were immersed in PBS for 30 minutes. The tubes were placed in liquid nitrogen to snap freeze the samples. The tubes containing the samples were then placed into the lyophilizer for approximately 20 hours to dry under vacuum. Samples were removed from the lyophilizer and weighed. The dry weights at different time points were compared to determine degree of degradation at Day 3, 7, 14, 21 and 28. Swelling ratios, Q, were calculated as a ratio of wet weight of the sample at different time points and its weight before the addition of 500\(\mu\)L PBS at 37\(^\circ\)C, as described in [201]. The volume of PBS added (500\(\mu\)L) was selected based on the volume used for \textit{in vitro} studies to cover the explant samples completely.

To perform degradation studies \textit{in vivo}, the mixture of collagen and chitosan was labelled with biotin using biotin labelling kit (Innova Biosciences, cat #704-0015) and used to prepare the hydrogel solution. Then, 50\(\mu\)L hydrogel was injected subcutaneously into the upper back of adult rats as described in Section 4.2.6. At Day 0 and Day 7, the hydrogel samples were extracted and the biotin content of the samples was quantified (Pierce Biotin Quantification Kit, Thermo Scientific, cat #28005). The quantification is based on the displacement of 2-(4´-Hydroxyazobenzene) Benzoic Acid (HABA) from the HABA: avidin complex by biotin. The HABA:avidin complex absorbs strongly at 500nm and the decrease in absorbance at the detection of biotin is used to calculate the amount of biotin.

4.2.3 Release of encapsulated peptides

The hydrogels with different amounts of encapsulated fluorescently-labelled T\(\beta\)4 (100ng, 150ng, 750ng and 1500ng), as well as poly-L-glutamic acid sodium salt, poly-L-lysine hydrobromide and poly-DL-alanine (1500ng for all), were incubated at 37\(^\circ\)C in 1mL PBS for 28 days (\(n=4-8\))
per experimental group). Continuous shaking was achieved using a horizontal orbital shaker at 70rpm. Tβ4, polyglutamic acid, polylysine and polyalanine were first labelled using an aminomethylcoumarin acetate (AMCA) labelling kit (Lightning-Link, Innova Biosciences) prior to hydrogel preparation. At different time points, the PBS samples were collected and the fluorescence was measured using a plate reader (excitation wavelength of 350nm, emission wavelength of 440nm). The intensity of fluorescence was directly related to the concentration of labelled peptide. A series of standards consisting of different known peptide concentrations was used to determine the amount of labelled peptides released into the PBS.

Infinite sink condition was achieved since peptides from a 50μL collagen-chitosan hydrogel was released into 1mL PBS, thus ensuring that the concentration in the PBS (maximum concentration of ~410ng/mL at Day 3, for polylysine) can only be 4% of that in the hydrogel (minimum concentration of ~10μg/mL at Day 28, for polylysine).

### 4.2.4 Cell recruitment from cardiac explants

Adult rat (Sprague-Dawley) and mouse (5-week old YFP transgenic, 129-Tg(CAGEYFP) 7AC5Nagy/J; Jackson Laboratory) hearts were harvested according to a protocol approved by the University of Toronto Committee on Animal Care. They were cut into pieces of ~3mm x 3mm x 3mm, with each piece containing the epicardium (n=8-12 per experimental group). Each piece was placed onto the centre of a hydrogel (Section 4.2.1). Culture medium, 500μL, was added to each well after 40 minutes of incubation. Tβ4 (1500ng in total in 500μL medium) was supplemented to the culture medium for the soluble Tβ4 group. The culture medium was changed every other day. At Day 7, cell migration from the explants was evaluated by taking brightfield microscopy images of the samples and measuring cell number, cell migration distance, branch number and branch length using ImageJ. Cell number and branch number were manually counted. The distance between two nodes or one node and the end of the tube was counted as one branch. The branch length was measured by drawing a line on top of the branch, and then using ImageJ to measure the length of the line. Cell migration distance was measured by drawing a line perpendicular to the surface, from the edge of the explant to the cell that is furthest from the explant, and then using ImageJ to measure the length of the line.

The culture medium for rat explants consisted of Dulbecco’s modified Eagle’s medium (DMEM, Gibco, cat # D5796) with 10% fetal bovine serum (FBS, Gibco, cat # 16000-044), 10mM 4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES Buffer, Gibco), 100 units/mL penicillin and 100µg/mL streptomycin (Gibco, cat# 15140122). The culture medium for mouse explants consisted of 83% DMEM with 4.5g/L glucose, 4mM L-glutamine, 15% FBS, 100units/mL penicillin, 100µg/mL streptomycin and 1% HEPES buffer.

4.2.5 Immunostaining

Immunostaining was performed on frozen sections. For preparation of frozen sections, the heart explants were removed from the hydrogels at Day 7 (n=3 for each experimental group), and the hydrogel samples were fixed in 10% neutral buffered formalin for 1 hour at room temperature and immersed in a solution of 30% sucrose in PBS overnight. They were then each placed facing down in a separate cryomold (Cryomold Biopsy Disposable Vinyl Specimen Mold 10mm x 10mm x 5mm, Tissue-Tek) that had a thin layer of OCT compound (Embedding Medium for Frozen Tissue Specimens to ensure Optimal Cutting Temperature, Tissue-Tek, 4583) at the bottom. The mold was filled up with OCT to cover the construct and snap frozen in liquid nitrogen. The snap frozen samples were stored at -80°C before being cryosectioned at -22°C using a cryostat (Leica, CM3050S) at a thickness of 10µm.

The frozen sections were air dried at room temperature for 30 minutes. Then, they were fixed in 200µL of 100% acetone (ACS grade) for 20 minutes, until the acetone evaporated. The slides were then blocked in 10% normal horse serum (NHS, Vector Laboratories) in PBS for 10 minutes at room temperature in a humidified chamber. The samples were incubated in 100µL primary antibody (rabbit polyclonal anti-CD31, AbCam; mouse anti-smooth muscle myosin heavy chain, SM-MHC, Millipore) at a dilution factor of 1:50 at 4°C overnight. On the next day, they were incubated in 100µL secondary antibody (Fluorescein or rhodamine-conjugated goat anti-rabbit IgG; FITC or rhodamine-conjugated goat anti-mouse, Jackson ImmunoResearch) with Hoechst dye at a dilution factor of 1:100 (for both antibody and Hoechst) for 1 hour at room temperature. For vimentin (Vim) staining, Cy3-conjugated primary antibody for vimentin (Sigma) was used (dilution factor of 1:50 with Hoechst dye at 1:100, 1 hour at room temperature), thus eliminating the need for separate primary and secondary antibody incubation steps. The slides were mounted with Fluoromount aqueous mounting medium, covered and imaged. Cells were identified as endothelial cells (CD31+/Vim+), smooth muscle cells (SM-
MHC+/Vim-), myofibroblasts (SM-MHC+/Vim+) and fibroblasts (Vim+). Percentages of different cell populations were determined based on cell counts from immunostained images.

4.2.6 Subcutaneous injection

The hydrogel solutions were prepared as described in Section 4.2.1. Collagen hydrogels (100μL) with 1500ng Tβ4 and collagen-chitosan hydrogels (100μL) with 0ng, 100ng or 1500ng Tβ4 were subcutaneously injected at four marked spots on the upper back of adult female Sprague-Dawley rats using 23¾G needles. After 7 days, samples (n=4 per group) were extracted and evaluated for cell infiltration and angiogenesis by staining for hematoxylin and eosin, Masson’s trichrome, Factor VIII and smooth muscle actin (SMA), as well as measuring the hemoglobin concentration within hydrogel samples.

For immunohistochemical staining, the samples were fixed in 10% neutral buffered formalin (Sigma, HT501129) for at least 24 hours at room temperature. Then, the fixed samples were sent to the Pathology Research Program (PRP) histology lab at University Health Network for paraffin embedding, sectioning, as well as hematoxylin and eosin, Masson’s trichrome, Factor VIII and smooth muscle actin (SMA) staining. Images (n=5 for each section sample) were taken with an optical microscope at 400X. The images were analyzed for cell density by determining the percentage of area with positive trichrome staining using ImageJ. The Factor VIII and SMA staining images were also analyzed for angiogenesis by counting the number of blood vessels and measuring the vessel diameters using ImageJ.

To detect the blood vessels connected to the host’s circulation, QuantiChrom hemoglobin assay kit (BioAssay Systems, DIHB-250) was used according to manufacturer’s instructions. Hemoglobin assay works by the principle that hemoglobin is converted into a uniform coloured end product through the Triton/NaOH method. The samples for the assay (n=3 per experimental group) were prepared by digesting the subcutaneously injected hydrogels in a sterile filtered solution of 0.6mg/mL collagenase (254U/mg, Worthington) in Hank’s Buffered Salt Solution (HBSS, Gibco, 14175) at 37°C for at least 24 hours.

4.2.7 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007 and GraphPad Prism 5.0. Differences between experimental groups were analyzed by using one-way ANOVA with post-
hoc Tukey tests or two-way ANOVA with Bonferroni post-tests. P < 0.05 was considered significant. Results were plotted as means with error bars representing standard errors.

4.3 Results

4.3.1 Hydrogel characterization

There was no significant loss in mass of the hydrogel over 28 days in PBS (Figure 4-1A, P = 0.4229). There were also no significant differences in the swelling ratios of the hydrogel in PBS and culture medium over time (Figure 4-1B, 4-1C, P = 0.8961 for PBS, P = 0.4825 for medium). The swelling ratios of the hydrogel in both PBS and medium were low and well below 1.25, which is the point when swelling ratio becomes a significant factor in drug release [202].

The release profiles of Tβ4 at different initial concentrations are shown in Figure 4-1D, E. Encapsulation of Tβ4 in collagen-chitosan hydrogel enabled a sustained release of this bioactive molecule over 28 days in PBS (Figure 4-1D, E). By the end of the 28-day incubation in PBS, 54-92% Tβ4 (87±12%, 92±26%, 81±9% and 54±3% for Encap100, Encap150, Encap750 and Encap1500 respectively) was released, depending on the dose encapsulated. Thus, the percent total Tβ4 load released for Encap1500 group after 28 days was lower than in the other groups.

We hypothesized that the controlled release was due to the negative charge of Tβ4, which interacts with the positively charged chitosan. Thus, we studied the release kinetics of negatively charged Tβ4 and polyglutamic acid, positively charged polylysine, and neutral polyalanine, peptides with similar range of molecular weights, from the collagen-chitosan hydrogel (Figure 4-1G). There is no significant difference between the release kinetics under cell culture conditions (Figure 4-1F, 500μL PBS and static incubation) and infinite sink conditions with continuous shaking (Figure 4-1G, 1mL PBS and continuous shaking at 70rpm). At the loading of 1500ng, 54±3% Tβ4, 55±4% polyglutamic acid, 65±4% polylysine and 57±3% polyalanine were cumulatively released from the hydrogel by Day 28 with no significant differences in the percent final load released (P = 0.07882).
Figure 4-1. Controlled release from collagen-chitosan composite hydrogel was dependent on the size and charge of the encapsulated molecule and independent of degradation and swelling of the hydrogel.

(A) Degradation of hydrogel in PBS over 28 days. (B) Swelling ratio of hydrogel in PBS over 28 days. (C) Swelling ratio of hydrogel in culture medium with 15% FBS over 28 days. (D) Cumulative release profile of encapsulated Tβ4 at different initial concentrations in PBS over 28 days, shown as the amount of the peptide released. Experimental groups include hydrogels with 100ng (Encap100), 150ng (Encap150), 750ng (Encap750), and 1500ng encapsulated Tβ4 (Encap1500). (E) Cumulative release profiles of encapsulated Tβ4 at different initial concentrations in PBS over 28 days, shown as a fraction of initial loading released over time. (F-G) Cumulative release profiles of encapsulated Tβ4, polyglutamic acid, polylysine and polyalanine in PBS over 28 days (1500ng peptide encapsulated for all groups), shown as a fraction of initial loading released over time, with (F) culture conditions that cells experienced and (G) infinite sink conditions with continuous shaking. Tβ4 and polyglutamic acid are negatively charged, polylysine is positively charged and polyalanine is neutral.
Several kinetic models have been proposed previously to describe the release characteristics of biomolecules from a controlled release matrix. The zero-order model equation (Eq. 1), Higuchi’s square-root equation (Eq. 2) and the Ritger-Peppas empirical equation (Eq. 3) are the most commonly used kinetic models [203]. In these equations, $M_t/M_\infty$ is the fraction of molecules released at time $t$, and $K_0$, $K_H$ and $K$ are release rate constants for Equations 1, 2 and 3 respectively, while $n$ in Eq. 3 is the diffusional exponent that indicates the mechanism of release. $M_t$ is the cumulative amount released at time $t$. $M_\infty$ is the cumulative amount released at infinite time, and is also defined as the amount incorporated within the controlled release system at $t = 0$ [204].

\[
\frac{M_t}{M_\infty} = K_0 t \\
\frac{M_t}{M_\infty} = K_H \sqrt{t} \\
\frac{M_t}{M_\infty} = K t^n
\]  

The release kinetic parameters for different peptides encapsulated in collagen-chitosan hydrogels are given in Table 4-2 for zero-order model equation, Higuchi’s square root equation, and Ritger-Peppas empirical equation. There was a slight burst release of 18% to 28% for all peptides over the first 3 days. However, the release was found to be more controlled at the later time points, showing nearly zero-order release kinetics. Specifically, the release profiles were well-fitted with a linear relationship between Day 3 to 28 (Table 4-2, Modified zero-order model, $R^2$ of 0.9800, 0.9944, 0.9930 and 0.9968 for Tβ4, polylysine, polyalanine and polyglutamic acid respectively). The burst release in the first three days can be explained by a small amount of the peptides remaining on the surface of the composite hydrogel upon gelation and are thus easily released into the environment at early time points. Using Ritger-Peppas empirical equation, the $n$ value for polyalanine was 0.488, showing a Fickian release (Table 4-2). On the other hand, the $n$ values for the release of Tβ4, polyglutamic acid and polylysine were 0.466, 0.476 and 0.406 respectively, indicating that the release mechanism was diffusion-controlled but affected by the charge of the peptides. The kinetic parameters were significantly
different for the release of different peptides (P < 0.0001, P < 0.0001, P = 0.0002 for modified zero-order, Higuchi’s square root and Ritger-Peppas empirical models respectively; P < 0.05 indicates rejection of null hypothesis that the same parameters can be used for release of different peptides).

Table 4-2. Release kinetics parameters for the controlled release of encapsulated peptides.

Different peptides were encapsulated individually into collagen-chitosan hydrogels to determine whether the release profile depends on molecular charge. Tβ4 and polyglutamic acid are negatively charged, polylysine is positively charged, and polyalanine is neutral. Parameters for zero-order model, Higuchi’s square root equation, and Ritger-Peppas empirical equation were determined for the entire experiment duration from Day 0 to Day 28, while the parameters for modified zero-order model were determined for only Day 3 to Day 28. K₀, Kₘ, K and K₀’ are the release rate constants for the corresponding equations, R² is the coefficient of determination indicating the goodness of fit of the data to the respective models, n is the diffusional exponent in the Ritger-Peppas empirical equation, and b is the y-intercept indicating the released amount of peptide at Day 0 as predicted by the modified zero-order model.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameters</th>
<th>Thymosin β4 (-)</th>
<th>Polyglutamic acid (-)</th>
<th>Polylysine (+)</th>
<th>Polyalanine (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero-order model</td>
<td>K₀</td>
<td>0.022 ± 0.003</td>
<td>0.022 ± 0.003</td>
<td>0.027 ± 0.004</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>R²</td>
<td>0.4402</td>
<td>0.5745</td>
<td>0.0888</td>
<td>0.5279</td>
</tr>
<tr>
<td>Higuchi’s square root equation</td>
<td>Kₘ</td>
<td>0.102 ± 0.002</td>
<td>0.101 ± 0.003</td>
<td>0.126 ± 0.004</td>
<td>0.107 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>R²</td>
<td>0.9914</td>
<td>0.9597</td>
<td>0.9541</td>
<td>0.9901</td>
</tr>
<tr>
<td>Ritger-Peppas empirical equation</td>
<td>K</td>
<td>0.113 ± 0.007</td>
<td>0.108 ± 0.020</td>
<td>0.165 ± 0.012</td>
<td>0.111 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>0.466 ± 0.023</td>
<td>0.476 ± 0.064</td>
<td>0.406 ± 0.026</td>
<td>0.488 ± 0.033</td>
</tr>
<tr>
<td></td>
<td>R²</td>
<td>0.9950</td>
<td>0.9613</td>
<td>0.9908</td>
<td>0.9905</td>
</tr>
<tr>
<td>Modified zero-order model</td>
<td>K₀’</td>
<td>0.014 ± 0.001</td>
<td>0.014 ± 0.001</td>
<td>0.015 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>0.173 ± 0.019</td>
<td>0.169 ± 0.008</td>
<td>0.245 ± 0.011</td>
<td>0.173 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>R²</td>
<td>0.9800</td>
<td>0.9968</td>
<td>0.9944</td>
<td>0.9930</td>
</tr>
</tbody>
</table>

The ability to show sustained release of both neutral and charged peptides suggests that the controlled release was due to the range of molecular sizes as well as the electrostatic interactions between charged peptides and the hydrogel. Since the peptides were of comparable molecular weight but different charge (negative Tβ4 and polyglutamic acid, positive polylysine, neutral polyalanine), we concluded that the charge had an appreciable influence on the release profile.
PyMOL, a software that allows the visualization of three-dimensional molecular structures, was used to approximate the size of the peptides. PyMOL approximations showed that polyalanine has a length of 14nm, compared to 5nm, 7nm and 11nm for polylysine, polyglutamic acid and Tβ4. Since neutral polyalanine is a longer molecule, its sustained release relied on its size. Although polyglutamic acid and Tβ4 have different molecular sizes, their release profiles were almost identical (Figure 4-1G) since they are both negatively charged and have similar interactions with the hydrogel.

![Figure 4-2](image)

**Figure 4-2.** Scanning electron microscopy images of hydrogels showing smooth surface for collagen-only gel and porous structures for collagen-chitosan gels with or without encapsulated peptides. (A) Collagen-only gel. (B) Collagen-chitosan composite hydrogel. (C) Composite hydrogel with 1500ng encapsulated thymosin β4 (MW=4963.55Da). (D) Composite hydrogel with 1500ng encapsulated polylysine (MW=1000-5000Da). (E) Composite hydrogel with 1500ng encapsulated polyalanine (MW=1000-5000Da).
Interestingly, when Tβ4 was encapsulated in hydrogels composed of collagen only, all of the Tβ4 (1490.99±27.70ng) was released over the first 3 days. This is likely related to the structure of the collagen hydrogel compared to the collagen-chitosan composite hydrogel, as shown in the SEM images (Figure 4-2). The collagen hydrogel (Figure 4-2A) had folds within its structure, but no interconnected pores that were clearly evident in the collagen-chitosan hydrogels with or without encapsulated peptides (Figure 4-2B-E). As a result, Tβ4 in collagen-only gels was not entrapped within pores. Moreover, the negatively charged Tβ4 on the surface of the collagen-only gels would quickly be repelled by the similarly negatively charged collagen molecules to be burst released. This further motivated our work to develop a collagen-chitosan hydrogel system.

Rheological measurements indicated that stable gels were formed in all groups, irrespective of the charge of the encapsulated molecules (Table 4-3, Figure 4-3), as indicated by the lower plateau loss moduli compared to the higher plateau storage moduli (Table 4-3). The storage and loss moduli of the collagen-chitosan hydrogel were unaffected by the encapsulation of charged or uncharged peptides (Table 4-3, one-way ANOVA, P = 0.9935 for storage modulus, P = 0.9164 for loss modulus). The gelation time was also similar for all hydrogels with or without encapsulated peptides (Table 4-3, P = 0.3256). This was expected since the amount of peptides added was small compared to the collagen and chitosan molecules (the amount of peptide was only ~1-2% by weight of collagen or chitosan). Thus, the addition of peptides did not have steric and charge interference with collagen and chitosan during gelation.

**Table 4-3. The presence of charged encapsulated peptides did not affect the rheological properties of collagen-chitosan hydrogels.**

Gelation time, plateau storage modulus and plateau loss modulus of collagen-chitosan hydrogels without modifications or with different encapsulated peptides were determined by the cone and plate rheometer.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Gelation Time (s)</th>
<th>Plateau Storage Modulus, G’ (Pa)</th>
<th>Plateau Loss Modulus, G” (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen-Chitosan Gel</td>
<td>299±28</td>
<td>33.2±2.8</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>Gel with Thymosin β4</td>
<td>233±20</td>
<td>28.7±5.6</td>
<td>3.4±0.7</td>
</tr>
<tr>
<td>Gel with Polyllysine</td>
<td>304±44</td>
<td>26.7±6.4</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>Gel with Polyalanine</td>
<td>247±10</td>
<td>27.0±4.9</td>
<td>2.9±0.4</td>
</tr>
</tbody>
</table>

No significant differences amongst groups.
Figure 4-3. Frequency sweep tests for collagen-chitosan hydrogels

(A) without peptides, or with encapsulated (B) Tβ4, (C) polylysine, or (D) polyalanine. Tests were performed at 1% strain.

4.3.2 Effect of encapsulated thymosin β4 on cardiac explants

Cell migration and infiltration, as well as tube formation, from adult cardiac explants was investigated using Tβ4 encapsulated in the developed collagen-chitosan hydrogels. In mouse explants, there was significantly higher cell density in Encap150, Encap750 and Encap1500 groups compared to both control and soluble groups (Figure 4-4A, C, $P < 0.0001$ two-way ANOVA, $P < 0.01$ for control vs. Encap150 and Encap750, $P < 0.01$ for soluble vs. Encap750, $P < 0.001$ for control vs. Encap1500, $P < 0.001$ for soluble vs. Encap150 and Encap1500). Since there was no significant difference between Encap100 and control or soluble groups, there appears to be a dose response of encapsulated Tβ4 on the number of recruited cells. The
migration distance was significantly higher in all groups with encapsulated Tβ4 compared to control and soluble groups (Figure 4-4B, D, P < 0.0001). All groups with encapsulated Tβ4 showed tube formation while there was no tube formation in the control and soluble groups (Figure 4-4E). There were no significant differences in branch density and branch length amongst the Tβ4 groups (Figure 4-4E, F, P > 0.05 for both). It is likely that the doses selected in this study already resulted in the maximum migration distance, branch density and branch length for the time period studied. Thus to observe the dose response in these parameters, lower concentrations of Tβ4 may be required.

In explants from adult rats, there was no significant difference in the recruited cell density with respect to the Tβ4 treatment groups and the controls (Figure 4-5A, C, P = 0.6361). However, there was a trend for the increase in the migration distance with the increase in the amount of encapsulated Tβ4 (Figure 4-5B, D). The migration distance was significantly higher in Encap1500 compared to both control and soluble groups (Figure 4-5D, P = 0.0095 one-way ANOVA, P < 0.05 for both control and soluble vs. Encap1500). The branch densities of groups with encapsulated Tβ4 were all significantly higher compared to both the Tβ4-free control and soluble groups (Figure 4-5E, P = 0.0406). Interestingly, the branch length peaked at 100ng, amongst the encapsulated Tβ4 groups (Figure 4-5F, P < 0.05 for Encap100 vs. Encap1500). In this case, the higher doses of Tβ4 resulted in the increased number of short branches that sprout from longer branches (Figure 4-5B). Branching is a necessary event in angiogenesis [205], as this leads to the formation of more complex vascular structures.

Overall, the common trend for both adult mouse and adult rat cardiac explants was that the cell migration distance and the branch density increased when Tβ4 was control released from the hydrogels compared to its application in the culture medium or the Tβ4-free controls (Figure 4-4, Figure 4-5). The tubes formed from the cardiac explants were found to be CD31-positive (Figure 4-6), thus indicative of endothelial cell-based tubes.
Figure 4-4. Cell recruitment and tube formation from adult mouse cardiac explants were enhanced by hydrogels with encapsulated Tβ4.

(A) Representative fluorescence microscopy images of cells migrated from the mouse explants (arrows indicate part of the explants). (B) Representative fluorescence microscopy images of tubes sprouted from the mouse explants. (C) Recruited cell density. (D) Maximum distance of cell migration from the explants. (E) Branch density (where a branch is the distance between two nodes, or a node and the end of a tube). (F) Average length of the branches. N/A indicates no tube formation for the experimental group. Experimental groups include hydrogels without Tβ4 (Control), with soluble Tβ4 in culture medium during cultivation period (Soluble), or with 100ng (Encap100), 150ng (Encap150), 750ng (Encap750), and 1500ng encapsulated Tβ4 (Encap1500). Analysis performed after 7 days in culture. * indicates statistically significant difference compared to control; # indicates statistically significant difference compared to soluble group (P < 0.05 one-way ANOVA with post-hoc Tukey tests).
Figure 4-5. Cell recruitment and tube formation from adult rat cardiac explants were enhanced by hydrogels with encapsulated Tβ4.

(A) Representative microscopy images of cells migrated from the rat explants (arrows indicate part of the explants). (B) Representative microscopy images of tubes sprouted from the rat explants. (C) Recruited cell density. (D) Maximum distance of cell migration from the explants. (E) Branch density (where a branch is the distance between two nodes, or a node and the end of a tube). (F) Average length of the branches. The experimental groups include collagen-chitosan hydrogels without Tβ4 (Control), with soluble Tβ4 in culture medium during cultivation period (Soluble), with 100ng encapsulated Tβ4 (Encap100), 150ng encapsulated Tβ4 (Encap150), 750ng encapsulated Tβ4 (Encap750), and 1500ng encapsulated Tβ4 (Encap1500). Analysis performed after 7 days in culture * with bracket indicates statistically significant difference between the two experimental groups; * indicates statistically significant difference compared to control; # indicates statistically significant difference compared to soluble group (P < 0.05 one-way ANOVA with post-hoc Tukey tests).
Figure 4-6. Tβ4-recruited tubes were generated by endothelial cells.
Representative fluorescence microscopy images showing CD31 immunostaining. There were CD31-positive tubes (red indicates CD31-positive staining; blue indicates staining of the nuclei) on collagen-chitosan hydrogels with 1500ng encapsulated Tβ4 (Encap1500), while there was a lack of tube formation on hydrogels without Tβ4 (Control).

Immunostaining demonstrated that the recruited cell population consisted mostly of fibroblasts (Vim+), myofibroblasts (Vim+/SM-MHC+), endothelial cells (Vim+/CD31+) and smooth muscle cells (SM-MHC+/Vim-) (Figure 4-7, Figure 4-8). There were no significant differences in the % CD31, % vimentin and % smooth muscle myosin heavy chain (SM-MHC) positive cells amongst the groups (P = 0.3652, P = 0.5435, P = 0.9292 for CD31, vimentin and SM-MHC in rat; P = 0.5218, P = 0.2019, P = 0.4866 for CD31, vimentin and SM-MHC in mouse). Based on the cell markers, the percentages of different cell types in the recruited cell population were calculated and found to be comparable amongst different groups for both mouse and rat explants (Table 4-4, Table 4-5). The recruitment of endothelial cells and smooth muscle cells into the hydrogel suggests a great potential for the hydrogels with encapsulated Tβ4 to be used for
promoting angiogenesis. There were no recruited Troponin-T-positive cardiomyocytes in the cell population in all experimental groups (data not shown).

Figure 4-7. The recruited cell population from mouse cardiac explants contained endothelial cells, fibroblasts and smooth muscle cells as indicated by CD31, vimentin, and SM-MHC immunostaining. Representative fluorescence microscopy images showing (A) CD31, (B) Vimentin, and (C) SM-MHC staining. Analysis of immunostaining images showing (D) %CD31-positive cells, (E) %Vimentin-positive cells, (F) %SM-MHC-positive cells. No significant differences were found amongst the experimental groups.

Table 4-4. The presence of Tβ4 does not influence the composition of migrating cells in mouse cardiac explants.
Percentages of different cell types in the recruited cell population from mouse cardiac explants as estimated from immunostaining in Figure 4-7. Experimental groups include hydrogels without Tβ4 (Control), with soluble Tβ4 in culture medium during cultivation (Soluble), with 100ng encapsulated Tβ4 (Encap100), and with 1500ng encapsulated Tβ4 (Encap1500).

<table>
<thead>
<tr>
<th></th>
<th>Endothelial Cells</th>
<th>Smooth Muscle Cells</th>
<th>Myofibroblasts</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28 ± 6</td>
<td>22 ± 11</td>
<td>0 ± 14</td>
<td>41 ± 8</td>
</tr>
<tr>
<td>Soluble</td>
<td>24 ± 5</td>
<td>2 ± 2</td>
<td>25 ± 12</td>
<td>49 ± 13</td>
</tr>
<tr>
<td>Encap100</td>
<td>26 ± 1</td>
<td>10 ± 7</td>
<td>22 ± 10</td>
<td>42 ± 9</td>
</tr>
<tr>
<td>Encap1500</td>
<td>31 ± 2</td>
<td>9 ± 2</td>
<td>27 ± 12</td>
<td>33 ± 11</td>
</tr>
</tbody>
</table>
Figure 4-8. The recruited cell population from rat cardiac explants contained endothelial cells, fibroblasts and smooth muscle cells as indicated by CD31, vimentin, and SM-MHC immunostaining.

Representative fluorescence microscopy images showing (A) CD31, (B) Vimentin, and (C) SM-MHC staining. Analysis of immunostaining images showing (D) %CD31-positive cells, (E) %Vimentin-positive cells, (F) %SM-MHC-positive cells. No significant differences were found amongst the experimental groups.

Table 4-5. The presence of Tβ4 does not influence the composition of migrating cells in rat cardiac explants.

Percentages of different cell types in the recruited cell population from rat cardiac explants as estimated from Supplemental Fig. 3. The experimental groups include collagen-chitosan hydrogels without Tβ4 (Control), with soluble Tβ4 in culture medium during cultivation period (Soluble), with 100ng encapsulated Tβ4 (Encap100), and with 1500ng encapsulated Tβ4 (Encap1500).

<table>
<thead>
<tr>
<th></th>
<th>Endothelial Cells</th>
<th>Smooth Muscle Cells</th>
<th>Myofibroblasts</th>
<th>Fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25 ± 5</td>
<td>16 ± 3</td>
<td>25 ± 10</td>
<td>34 ± 12</td>
</tr>
<tr>
<td>Soluble</td>
<td>21 ± 3</td>
<td>12 ± 2</td>
<td>25 ± 8</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Encap100</td>
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<td>13 ± 3</td>
<td>31 ± 10</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>Encap1500</td>
<td>30 ± 4</td>
<td>16 ± 3</td>
<td>25 ± 8</td>
<td>29 ± 9</td>
</tr>
</tbody>
</table>
4.3.3 In vivo rat subcutaneous injection studies

Figure 4-9. In vivo subcutaneous injections of collagen-chitosan hydrogels in rats led to cell infiltration and angiogenesis.

Representative histological images of collagen-only hydrogel samples with 1500ng encapsulated Tβ4 (Collagen-Encap1500) and collagen-chitosan hydrogel samples with 0ng (Control), 100ng (Encap100), 1500ng (Encap1500) encapsulated Tβ4 after 7 days. Hematoxylin and eosin, trichrome, FVIII and SMA staining are shown. Note that the SMA staining strikingly indicates the presence of myofibroblasts in the control hydrogel compared to SMA+ blood vessels in cases with encapsulated Tβ4.

The collagen-only and collagen-chitosan hydrogel solutions were gelled under body temperature when injected subcutaneously in the upper back of adult rats, as indicated by the formation of a lump at the injection site under the skin after 5-8 minutes. On Day 7, the hydrogel samples were extracted to evaluate the angiogenic effect of encapsulated Tβ4. The degradation of the
collagen-chitosan hydrogels was negligible in vivo at 7 days post-injection, since 21±6nmol and 20±1nmol biotin was measured at Day 0 and Day 7 respectively after the injection of biotinylated collagen-chitosan hydrogel (P = 0.9283). Interestingly, there was significantly decreased cell infiltration (P < 0.0001), hemoglobin concentration (P < 0.0001), FVIII-positive vessel density (P < 0.0001), SMA-positive vessel density (P < 0.0001), and SMA-positive vessel diameter (P = 0.0058) in the collagen-only hydrogel with 1500ng encapsulated Tβ4 (Collagen-Encap1500 group), as compared to all the collagen-chitosan hydrogel groups (Figure 4-9, 4-10). There was similar amount of cell infiltration in all collagen-chitosan hydrogel groups (Figure 4-10A, P = 0.9142), as evaluated from hematoxylin and eosin staining and trichrome staining (Figure 4-9). However, the hemoglobin concentration within the hydrogel was significantly higher in the Tβ4 groups as compared to the control group (Figure 4-10B, P < 0.001 for Control vs. Encap100, P < 0.01 for Control vs. Encap1500). This suggests that there was more infiltration of blood vessels that are connected to the host circulation when Tβ4 was encapsulated in the hydrogel. FVIII-positive vessel density was significantly higher in Encap1500 group compared to control group (Figure 4-9, 4-10C, P < 0.01 for Control vs. Encap1500), thus showing the angiogenic effect of Tβ4. Furthermore, this suggests a dose response for blood vessel density since Encap1500 group showed significantly higher FVIII-positive vessel density than control group, while Encap100 group did not. The area fraction of FVIII-positive staining was also significantly higher in Encap1500 group compared to control group (data not shown, P = 0.0397). There was significantly increased SMA-positive vessel density in the collagen-chitosan hydrogels with encapsulated Tβ4 (Figure 4-9, 4-10E, P < 0.05 for both Encap100 and Encap1500 vs. Control), compared to the collagen-chitosan control group. The collagen-chitosan control exhibited high levels of SMA staining outside the blood vessel structures, consistent with the significant infiltration of myofibroblasts. The FVIII-positive vessel diameters were similar in all groups (Figure 4-9, 4-10D, P = 0.2720). The SMA-positive vessel diameters were similar in all collagen-chitosan groups (Figure 4-9, 4-10F, P = 0.0993).
Figure 4-10. Angiogenic effect of encapsulated thymosin β4 in collagen-chitosan hydrogels in vivo was dose-dependent.

(A) Infiltrated cell density. (B) Hemoglobin concentration within the hydrogels. (C) Density of FVIII-positive blood vessels. (D) FVIII-positive vessel diameter. (E) Density of SMA-positive blood vessels. (F) SMA-positive vessel diameter. * indicates statistically significant difference (P < 0.05 one-way ANOVA with post-hoc Tukey tests)

4.4 Discussion

Functional vasculature is essential for tissue formation and maintenance [138]. Recent strategies to stimulate the regeneration of functional vasculature in ischemic tissues often involve the injection of endothelial cells and precursors [206-208], or the injection of angiogenic growth
factors such as VEGF [209-210]. Cell injection is limited by the selection and expansion of an appropriate cell source, lack of cell engraftment and low cell survival [211]. The survival of vascular cells within implants depends on the rapid stimulation of vascular organization [138] which can be stimulated by delivery of angiogenic factors provided that they are not rapidly cleared from the implantation site [212].

Tβ4 was previously shown to enhance the survival of vascular cells and cardiomyocytes [118, 213]. More importantly, it induced endogenous endothelial cell migration to the ischemic site following cardiac injury, which in turn stimulated neovascularization [92, 116]. As such, the ability to recruit endogenous cells using Tβ4 may eliminate the need for cell injection. Tβ4 also down-regulated the expression of inflammatory molecules [214], which can potentially reduce adverse remodelling post-MI. Being a peptide, Tβ4 has additional advantages over conventional angiogenic growth factors because of its small size, high activity, and minimized immunogenicity [215]. Recent work by Smart et al. [92] suggested that the use of Tβ4 is superior to other angiogenic growth factors since it alone can initiate angiogenesis and sustain vascular stability through the recruitment and differentiation of endothelial cells and smooth muscle cells.

Biomolecules that are administered through injections in soluble forms are susceptible to degradation [216]. This leads to a need for multiple injections and increased dosages to maintain the therapeutic concentration [216]. On the other hand, the encapsulation of peptides can allow for sustained release and localized effect. As a result, there is an incentive to provide a protective environment for the controlled release of Tβ4. Kraehenbuehl et al. [138] previously developed a synthetic matrix metalloproteinase (MMP)-responsive poly(ethylene glycol) (PEG)-based hydrogel to co-encapsulate vascular cells and Tβ4. The incorporation of Tβ4 increased MMP-2 and MMP-9 secretion of the encapsulated human umbilical vein endothelial cells (HUVECs), which in turn triggered the release of Tβ4 [138]. The central actin-binding domains in Tβ4 were responsible for the elevated levels of proteases. The released Tβ4 further improved HUVEC attachment and formation of vascular-like networks. While this hydrogel is advantageous for the delivery of vascular cells, it cannot be used to deliver Tβ4 alone without cells.
Here, we report the development of a bioactive collagen-chitosan composite hydrogel designed for the controlled release of encapsulated Tβ4 for ultimate application in cardiac repair strategies. The hydrogel was evaluated in its ability to recruit endogenous vascular cells from the epicardium of the heart in vitro and promote angiogenesis when subcutaneously injected in vivo.

Polyanion-polycation complexes formed from collagen and chitosan have been studied previously, particularly for drug release [217]. The gelation is thought to occur in two steps: the formation of a network of collagen fibrils, followed by the deposition of chitosan around the collagen fibrils and the subsequent electrostatic interaction and hydrogen bonding between chitosan and collagen molecules. The neutralization of the acidic stock collagen I solution by the addition of NaOH causes the shrinking of the polymeric network of collagen and formation of a polyelectrolytic coordination compound and gel [218]. While collagen supports cell survival, it has been shown in previous studies that chemically untreated collagen has fast biodegradation rate and low mechanical strength [217]. The addition of chitosan can improve mechanical strength and reduce the rate of degradation by collagenase [217]. Chitosan molecule has hydroxyl and amino groups and collagen has carbonyl and amino groups [217]. As such, there are two types of interactions between chitosan and collagen when forming complexes [219]. The first type of interaction is the electrostatic interaction when a polyanion/polycation complex is formed between two types of polyelectrolytes [219]. The second type of interaction occurs when there is an excess of chitosan, with hydrogen bonding between chitosan and collagen molecules [219]. Although negatively charged at physiological pH and likely associated with positively charged chitosan in the hydrogel, Tβ4 did not significantly affect the hydrogel structure (Figure 4-2B, C).

Moreover, hydrogels composed of collagen only were incapable of encapsulating Tβ4 for sustained release. All of the encapsulated Tβ4 in collagen hydrogels was released in the first 3 days of incubation in PBS. This is consistent with previous studies that also reported the inability of sustained release using collagen gels [194]. In contrast, the release of Tβ4 from collagen-chitosan composite hydrogels followed an almost linear profile from Day 3 to Day 28 (Figure 4-1D). The controlled release is likely due to the hindered diffusion caused by the presence of interconnected pores (Figure 4-2) and the association of negatively charged Tβ4 with positively charged chitosan. Due to the cationic nature of chitosan, it has been used to develop controlled release systems for negatively charged molecules [220]. Moreover, it was
determined previously that collagen-chitosan blends at different chitosan/collagen weight ratios of 0.2, 0.5, 0.8 and 1.0 were all positively charged with zeta potentials of 7.6-14.3mV at pH 6 and 2.7-4.1mV at pH 7 [221].

The release from the hydrogel was not due to degradation since there was no significant difference in mass of the collagen-chitosan hydrogel over 28 days (Figure 4-1A) in PBS. For the hydrogel samples in this study, the thickness was in the range from 0.5-1mm and the diameter was determined to be 8.0-11.3mm based on the volume of gel solution used. Also, the swelling ratios of the hydrogel in PBS and medium were relatively low (Figure 4-1B, 4-1C). As such, the hydrogel can be roughly approximated as a non-swelling thin slab [202]. Based on the diffusional exponent \(n\) values obtained for the Ritger-Peppas empirical equation (Table 4-2) and the relationship between \(n\) and aspect ratio where \(n = 0.5\) indicates Fickian diffusion for thin slabs [202], the release of peptides from the collagen-chitosan hydrogel was dependent on both drug diffusion and interactions between the peptides and the hydrogel. For the entire experiment duration (Day 0 to Day 28), poor correlation coefficients were observed for the kinetic parameters based on the zero-order model equation. However, relatively good correlation coefficients were obtained for the kinetic parameters based on Higuchi’s square-root equation, especially in the case of encapsulated polyalanine in hydrogel. The release profiles of peptides were best-fitted with the Ritger-Peppas empirical equation. This suggests that the release of peptides is not completely based on diffusion, which is generally the case in Higuchi’s square-root kinetics. Since the release of neutral polyalanine has an \(n\) value of 0.488 (close to 0.5), it is likely diffusion-controlled. For the release of Tß4, polyglutamic acid and polylysine, the \(n\) values slightly deviated from 0.5 likely due to the electrostatic interactions. These interactions included the repulsion or attraction of charged molecules within the hydrogel when negatively or positively charged peptides were encapsulated, which then affected the release kinetics [194]. Diffusion hindrance caused by small pore sizes in hydrogel and interactions between diffusing molecules and the hydrogel have been shown to account for the deviation from Fick’s law [222]. Since these peptides have similar molecular weights (750-5000Da) but different charges, and their release profiles were significantly different (\(P = 0.0002\)), the hindered diffusion was likely due to both the size and charge of the molecules. The structure (Figure 4-2) and mechanical properties (Table 4-3) of the collagen-chitosan hydrogel were not affected by the addition of different peptides.
The range of initial concentrations of Tβ4 was chosen based on previous studies, in which 100ng/mL Tβ4 was used for epicardial cell migration [116]. The high dose (1500ng loading) was an order of magnitude higher. Thus, 134-873ng/mL Tβ4 (Figure 4-1D, note that hydrogels were incubated in 500μL media) were released into the cell culture environment in 7 days during cell migration studies using different Tβ4 loading. These seemed to be appropriate concentrations of Tβ4 for inducing epicardial cell migration and angiogenesis, as evident in the increased recruited cell density and migration distance in mouse cardiac explants for the groups with encapsulated Tβ4 compared to the control and soluble groups (Figure 4-4C, D). The soluble group involved the addition of 1500ng Tβ4 into culture medium (i.e. 3000ng/mL). This Tβ4 amount was chosen to act as a control for the case where the Tβ4 was assumed to be fully released for the Encap1500 group. Tube formation was observed for the encapsulated groups, while there was none for the control and soluble groups (Figure 4-4E, F). Similar trends were found in rat explants for migration distance and branch density (Figure 4-5). Thus, these in vitro results showed that the Tβ4 released from the encapsulation system was more potent than Tβ4 in soluble form, although the concentration of soluble Tβ4 used in the soluble control group at a given time point was much higher. For example, while only 1500ng Tβ4 in total was used in the Encap1500 group, the corresponding soluble control involved utilization of 4500ng Tβ4 when all culture medium changes are accounted for. The reason for this improved potency might be that the encapsulated Tβ4 was localized to the cell culture surface and in turn capable of attracting large number of cells from the cardiac explant towards the hydrogel to grow and form tubes.

The dose response of encapsulated Tβ4 was observed, since Encap100 group did not show significantly higher recruited cell density compared to control and soluble groups (Figure 4-4C). This is expected since the release of Tβ4 for Encap100 group resulted in the instantaneous concentration of only 90ng/mL on Day 3 and 44ng/mL on Day 7, which are concentrations lower than those used for the same duration (3 days) in previous studies with soluble Tβ4 [116]. Consistent with the report by Smart et al. [92], in which Tβ4 was shown to be necessary for vascular stability, we observed the recruitment of both endothelial and smooth muscle cells (Figure 4-7, Figure 4-8, Table 4-4, Table 4-5). This is an advantage of Tβ4 release compared to the use of VEGF alone that promotes only endothelial cell migration [223].

In addition, Tβ4 improved angiogenesis in vivo when the collagen-chitosan hydrogels with different amounts of encapsulated Tβ4 were subcutaneously injected in adult rats (Figure 4-9, 4-
All collagen-chitosan groups showed similar cell infiltration (Figure 4-10A), but significantly higher hemoglobin concentration (Figure 4-10B) and SMA-positive blood vessel density (Figure 4-10E) were shown in the Encap100 and Encap1500 groups compared to control, thus indicating an angiogenic effect. It has been previously shown that Tβ4 stimulates angiogenesis by increasing the protein stability of hypoxia-inducible factor (HIF)-1α, which indirectly induces the expression of VEGF [127]. It was also found that the actin-binding motif of Tβ4 is essential for its angiogenic activity [125]. Moreover, the higher SMA-positive blood vessel density in the Encap100 and Encap1500 groups compared to control (Figure 4-10E) supports the ability of Tβ4 to induce both vascular growth and stability as shown previously [92]. In contrast, a large number of non-vascular SMA+ myofibroblasts was found in the control group (Figure 4-9) and they do not participate in blood vessel formation. Since there was negligible in vivo degradation of the collagen-chitosan hydrogel at 7 days post-injection, the angiogenic effect in vivo can be attributed to the controlled release of encapsulated Tβ4 due to size and charge of the peptide, rather than a release due to enzymatic degradation. However, there were no significant differences in FVIII-positive vessel diameter and SMA-positive vessel diameter amongst the collagen-chitosan groups (Figure 4-10D, F) suggesting a need to further optimize the time of experimental endpoint and the dosage of Tβ4 in vivo, or to add other factors as well. Importantly, there was significantly lower cell infiltration and angiogenesis in the collagen-only hydrogel with encapsulated Tβ4 compared to all collagen-chitosan hydrogel groups (Figure 4-9, 4-10). This was due to two factors. First, the sustained release of Tβ4 from collagen-chitosan hydrogels was necessary for the proper delivery of the peptide, since the collagen-only hydrogel was shown to release nearly all of the encapsulated Tβ4 within 3 days in vitro. The quickly released Tβ4 was likely washed or diffused out from the injection site, rendering the Tβ4 ineffective for eliciting angiogenesis. In the in vivo environment, collagen is likely subjected to enzymatic degradation, in turn leading to even faster release and washout of the encapsulated Tβ4. Second, it has been shown previously that the addition of chitosan to collagen improved the ability of the hydrogel to support angiogenic progenitor phenotypes in vivo [200].

The bioactive hydrogel described here is capable of releasing encapsulated Tβ4 and other peptides. The released Tβ4 acts as a chemoattractant for vascular cells from the epicardium of cardiac explants. Subsequent to the cell recruitment, the bioactive hydrogel acts as a
biochemical stimulus to support cell organization and tube formation [138]. This encapsulation system can prevent burst effects, degradation of biomolecules and rapid clearance by the body, which are common issues with soluble biomolecule delivery. Moreover, it allows localized and sustained presentation of Tβ4. As such, the collagen-chitosan hydrogel with encapsulated Tβ4 can potentially be used as a long-term sustained bioinstructive material in vivo. The envisioned clinical application of this system is injection in the infarct area post-MI to attract cells from the epicardium to repopulate and regenerate the area. In this setting the application of biomaterial alone may act to stabilize the ventricle wall and attenuate pathological remodeling according to previous analysis [46], while controlled release of Tβ4 may enhance angiogenesis. Current work is being performed to evaluate the ability of the controlled release of Tβ4 to directly improve cardiac function in vivo by using Tβ4 as both an angiogenic factor for promoting the growth of functional vasculature and a therapeutic agent for enhancing cardiac cell and tissue survival. The encapsulation of Tβ4 can also potentially be used to prevascularize hydrogels that are subsequently used for cardiac tissue engineering.

4.5 Conclusions

In this study, we developed a collagen-chitosan composite hydrogel for the controlled release of Tβ4. The encapsulated Tβ4 promoted cell migration and tube formation from adult mouse and rat cardiac explants in vitro. The hydrogels with Tβ4 also improved angiogenesis in a dose-dependent manner when subcutaneously injected in adult rats. Moreover, the developed hydrogel is capable of the controlled release of other peptides with molecular weights of 750-5000Da.

4.6 Acknowledgements

This study was supported by the Heart and Stroke Foundation Grant-in-Aid (T-6946), NSERC Discovery Grant (RGPIN 326982-10), NSERC Discovery Accelerator Supplement (RGPAS 396125-10), NSERC Strategic Grant (STPGP 381002-09) and NSERC Alexander Graham Bell Canada Graduate Scholarship (CGS-D to L.L.Y.C.). We thank Mr. Larry Meng and Mr. AJ Wang for help with subcutaneous injections, and Dr. Yu-Ling Cheng and Mr. Billy Cheng for help with rheological properties of hydrogels.
4.7 Commentary

In this chapter, I discussed the development of an injectable hydrogel for treatment of MI, which is the most common cardiac disease in adults around the world. In this disease, cardiomyocytes within the left ventricular wall die, leaving a thin scar that cannot withstand the stress and leading to heart failure. The use of scaffolds, as in Chapter 3, for this purpose would not be suitable since patch implantation would cause more damage to the MI patients. Rather, the injection of hydrogel offers a minimally invasive method to treat MI. The injection of hydrogel has been shown to be beneficial for cardiac repair due to mechanical effects [46]. In this study, Tβ4 was incorporated into the hydrogel to further promote vascularization. Tβ4 is the only peptide shown to simultaneously repair the myocardium and the vasculature, as necessary in the case of MI.

Tβ4 has been evaluated for cardiac repair in soluble form [118]. However, systemic administration requires multiple injections at high doses. Clinical trials currently administer Tβ4 by intravenous bolus daily for first 3 days, then weekly for 4 weeks (ClinicalTrials.gov Identifier: NCT01311518). Collagen gel with incorporated Tβ4 was also previously tested, but showed no additional benefits compared to soluble Tβ4 [118]. This may be because collagen gel does not support controlled release of the peptide, as shown in this chapter. In this study, encapsulation of Tβ4 into collagen-chitosan hydrogel allowed its controlled release. Tβ4 was mixed with collagen and chitosan in the hydrogel solution. Upon gelation, it was encapsulated within the collagen-chitosan complex formed by a combination of hydrogen bonding and polycation-polyanion interactions. Since Tβ4 is negatively charged (isoelectric point of 5.1 [224]), it is expected to have electrostatic interactions with cationic chitosan (at 70-95% deacetylation) [225].

Although it was shown here that the controlled release of Tβ4 can support the migration of cells and tube outgrowth from epicardial explants, and promote angiogenesis when subcutaneously injected, it is necessary to further show the utility of the developed controlled delivery system for in vitro cardiac tissue engineering and in vivo cardiac repair. Since a portion of the cell population that migrated out from the epicardial explants due to Tβ4 release was endothelial cells, and these cells formed tubes, there was an incentive to combine findings of this chapter with the use of topographical cues to grow organized vasculature for use in cardiac tissue.
engineering. This is described in the next chapter. Hydrogels can be easily molded, thus allowing the incorporation of patterns into the system. In addition, the developed hydrogel in this chapter can be directly injected for cardiac repair. Its feasibility to be used for the treatment of MI is described in Chapter 6.
Chapter 5

5 Perfusable Branching Microvascular Bed Generated in vitro through Control of Substrate Topography and Presentation of Angiogenic Factors

5.1 Introduction

Tissue engineering may offer alternative treatment options for tissue and organ replacement but challenges related to vascularization in vitro and in vivo remain. Functional vasculature is required to grow tissues beyond 0.02cm thick, due to the diffusional limitations in oxygen supply [74]. Strategies developed to engineer vascularized tissues generally involved: 1) co-culture of tissue-specific cells with endothelial cells or precursors [83, 206-208, 226], 2) incorporation of pre-existing endothelial networks or blood vessels [71], or 3) engineering of proangiogenic scaffolds with peptides, growth factors or geometric cues [33-36, 78, 90-92, 94, 116, 166, 227-231].

The incorporation of cells, endothelial networks or blood vessels can accelerate the vascularization of the implanted biomaterial or engineered tissue and its functional anastomosis with the host vasculature in vivo [71, 232-233]. Asakawa et al. [83] created an in vitro pre-vascular network by co-culturing endothelial cells between cell sheets that were stacked to form three-dimensional tissues. The vascular structures connected to the host vasculature and promoted in vivo vascularization of the tissues upon implantation [83]. Morritt et al. [71] cultivated cardiac cells in a subcutaneously implanted chamber containing an arteriovenous loop. A thick construct with extensive vascularization was formed by 4 weeks post-implantation [71].

In another approach, Madden et al. [33] fabricated scaffolds with interconnected pores of 30-40μm in diameter to promote angiogenesis post-myocardial infarction (MI). In addition, proangiogenic biomolecules were incorporated into tissue engineering biomaterials in soluble, encapsulated or immobilized form. Peptides and growth factors such as thymosin β4 (Tβ4) [92, 116], vascular endothelial growth factor (VEGF) [227-228] and hepatocyte growth factor (HGF) [227, 229-230] lead to increased vessel formation. To prevascularize an engineered cardiac tissue prior to implantation, an alginate scaffold with sustained release of several growth factors was transplanted onto the omentum for 7 days [36]. VEGF and angiopoietin-1 were also
immobilized onto collagen scaffolds to improve the proliferation of endothelial cells \textit{in vitro} and angiogenesis \textit{in vivo} [34-35, 78]. While these methods suggest that improving vascularization improves engineered tissue function, current methods for vascularization are limited due to the lack of control over cell position and the ultimate organization of the vasculature, leading to the formation of vascular networks with random spatial distribution [86]. Despite these existing approaches, strategies to engineer complex metabolically active organs such as the heart and liver, which consist of multiple cell types spatially distributed around an organized vascular network [86], remain limited.

Recent studies focused on improving the organization of growing vasculature using microcontact printing and microgrooved substrates [234-236]. It was found that the growth, differentiation and apoptosis of endothelial cells can be controlled by the spreading of the cells [236]. Bovine capillary endothelial cells cultured on 10µm wide lines of fibronectin spread to \( \sim 1000\mu m^2 \) and formed capillary tube-like structures with a central lumen, while those cultured on 30µm wide lines spread to \( \sim 2200\mu m^2 \) and proliferated rather than forming tubes [236]. Co et al. [234] controlled the spatial arrangement of endothelial cells and fibroblasts by using soft-lithography-based micropatterning of alternating cell-adhesive and cell-resistant lanes. A cell-resistant polyelectrolyte was micropatterned on chitosan films to confine the growth of human vascular endothelial cells on cell-adhesive areas, and these cells formed capillary tube-like structures. The substrate was then immersed into chitosan to coat the cell-resistant electrolyte to render the areas adhesive to fibroblasts [234], thus allowing the micropatterning of two cell types.

Here, we devised a method to engineer a prototype vascular network consisting of two branching vessels that connect to form a microvascular bed suitable for rapid vascularization of engineered tissues \textit{in vitro}. The novelty of our approach lies in the integrated use of topographical cues and sustained release of angiogenic factors to guide the outgrowths from an artery and a vein resulting in a connected microvascular bed (\textbf{Figure 5-1A}). Rather than forcing the isolated endothelial cells into defined positions of a polymeric or a hydrogel matrix, we used biomaterials to create a niche that directed sprouting and anastomosis of the vasculature from the two branching vessels \textit{in vitro}. We demonstrate the utility of this vascular network for improving the function of engineered vascularized myocardium. Functional properties of myocardium are determined by cardiomyocytes, a cell type sensitive to oxygen limitations due to their high metabolic activity. Direct perfusion of engineered cardiac tissues has been shown to improve
cell viability [74, 237]. However, it also causes non-physiologic shear stress on cardiomyocytes, thus affecting their phenotype [23]. The presence of a microvascular bed in an engineered cardiac tissue *in vitro* may provide a biomimetic milieu for functional cell assembly, since cardiomyocytes would orient themselves around the vasculature and be shielded from the shear stress of blood perfusion in the application of the engineered cardiac tissue in vitro and in vivo.

Our strategy involved the release of Tβ4 from the substrate coating, so as to mimic the in vivo environment in which biomolecules are present to promote formation of vascular network. Tβ4 is an angiogenic and cardioprotective peptide that enhances cardiomyocyte survival by induction of coronary vascularization and upregulation of Akt activity [116, 118]. This peptide was previously shown to both initiate angiogenesis and sustain vascular stability due to its ability to recruit and differentiate endothelial and smooth muscle cells [92]. Tβ4 was shown to induce endogenous endothelial cell migration and stimulate neovascularization at the ischemic site after cardiac injury [92, 116]. Compared to angiogenic growth factors, Tβ4 has advantages such as small size, high activity, minimal immunogenicity and high solubility in water [215, 227]. Soluble peptides and growth factors are easily degraded and washed out, thus requiring multiple doses at increased levels to maintain bioactivity [216]. We previously developed a collagen-chitosan hydrogel that was capable of releasing Tβ4 at nearly zero order kinetics over 28 days [238]. Collagen promotes cell attachment, survival and proliferation [197]. Chitosan is a positively charged polysaccharide that is also commonly used for tissue engineering and drug delivery [199]. The electrostatic interactions between negatively charged Tβ4 and positively charged chitosan contribute to sustained release of this angiogenic molecule [238]. Here we used Tβ4 to generate vascularized cardiac tissues due to its angiogenic and cardioprotective potential, as well as its ability to be encapsulated in collagen-chitosan hydrogel for highly localized and sustained bioactivity.

### 5.2 Methods

#### 5.2.1 Preparation of hydrogel

The hydrogel solutions were prepared by mixing 10X PBS, Tβ4 (Prospec, cat # HOR-275, stock solution of 1mg/mL in distilled water), chitosan in distilled water (75-90% deacetylation, Protasan, NovaMatrix, cat # G113), collagen I (BD Biosciences, cat #354236, 4.08mg/mL rat tail collagen I in acetic acid), and 1N NaOH. The volume of 10X PBS in the hydrogel solution was
one-tenth of the final volume, and the volume of NaOH was 0.025 times the volume of collagen I. The volumes of Tβ4, chitosan and collagen I were calculated based on the final desired concentrations of 20μg/mL (Encap100 group) or 150μg/mL (Encap1500 group), 1.25mg/mL and 2.5mg/mL respectively. Distilled water was added in place of Tβ4 for the Control group.

5.2.2 Fabrication of micropatterned PDMS substrates

Polydimethylsiloxane (PDMS) substrates were fabricated as previously described [239], using standard soft lithography. The resulting substrates consisted of lanes of 25μm, 50μm or 100μm in width and 65μm in height. The substrates were cut into 5mm x 5mm and fixed to the bottom of individual wells using PDMS mixture. Hydrogel solution, 10μL, was pipetted evenly onto each PDMS substrate to coat the surface. The substrates were incubated at 37°C for 1 hour to allow the coating to gel.

5.2.3 Isolation and cultivation of explants

Cardiac and vascular explants were isolated from mice (cardiac tissues, thoracic arteries and inferior vena cava from 5-week old YFP transgenic mice, 129-Tg(CAGEYFP) 7AC5Nagy/J; Jackson Laboratory) and rats (femoral arteries/veins from Sprague-Dawley rats) according to a protocol approved by the University of Toronto Committee on Animal Care. Human umbilical arteries and veins were kind gifts from Dr. John Davies at Tissue Regeneration Therapeutics. The explants were placed on the coated PDMS substrates. For studies with vascular explants, one piece of artery and one piece of vein were placed on the two sides of the substrate at ~0.5-1mm apart. For studies with cardiac explants, the top part of the heart containing the dense vasculature was used. The mouse explants were cultivated in culture medium consisting of Dulbecco’s Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 15% fetal bovine serum (FBS). To accelerate capillary outgrowths, some explants were cultivated in culture medium supplemented with 20ng/mL hepatocyte growth factor (HGF) or 100ng/mL vascular endothelial growth factor (VEGF). The rat explants were cultivated in culture medium consisting of DMEM with 1% penicillin/streptomycin, 1% HEPES and 10% FBS. The human explants were cultivated in Clonetics EGM-2 Endothelial Cell Growth Medium-2 with Bullet Kit (Lonza, CC-3162), suitable for the culture of human endothelial cells.
5.2.4 Image analysis

At different time points (Day 7, 9, 11, 14, 21), the samples were imaged under optical or fluorescence microscope (Olympus IX2-UCB) and the images were analyzed using ImageJ. Cell density was measured by counting the cells and normalizing the cell number with the image area. Branch density was evaluated by counting the number of branches (between two nodes or between a node and an end) within the capillary outgrowths, and normalizing the branch number with the image area. Branch length was evaluated by drawing a line on top of the branch, which is either between two nodes or between a node and an end, and measuring the length of the line by ImageJ. Branch width was determined by drawing a line across the width of the branch, and measuring the length of the line by ImageJ. The width of each branch was determined and averaged for 3 measurements at randomly selected locations along the branch.

5.2.5 Immunohistochemical analysis

The samples were fixed in 10% formalin and stained with specific antibodies against CD31 (Abcam, ab28364, 1:50), VE-cadherin (Abcam, ab33168, 1:50), von Willebrand factor (Abcam, ab6994, 1:100), SMA (Abcam, ab5694, 1:100), NG2 Chondroitin Sulfate Proteoglycan (Millipore, MAB5384, 1:200), EphB4 (Hycult Biotech, HM1099, 1:50), Ephrin-B2 (GenScript, Ab-330, 1:100), Troponin T (Fisher, MS-295-P, 1:100) and connexin-43 (Abcam, AB1728, 1:100). Nuclei were stained with Hoechst dye or DAPI (1:100). The staining was carried out as described previously [240], and signals were visualized by incubating the samples with rhodamine or Texas red-labelled secondary antibodies (1:100; rhodamine goat anti-mouse, Jackson ImmunoResearch, 115-025-166; rhodamine goat anti-rat, Jackson ImmunoResearch, 112-026-003; Texas red goat anti-rabbit, Abcam, ab6719).

5.2.6 Dextran perfusion

Rhodamine-labelled dextran (Invitrogen, D-7139) in distilled water, 200μg/mL, was injected into the artery explant using a 30G1/2 needle. Fluorescence microscopy was used to capture the perfusion of dextran through the capillary outgrowths.

5.2.7 Transmission electron microscopy

After 21-day cultivation, samples were fixed in glutaraldehyde for 1 hour and washed in PBS. Osmium tetroxide was added to the samples for 1 hour. The samples were then serially
dehydrated in 70%, 90% and 100% ethanol. After dehydration, the samples were imbedded in resin, sectioned, mounted on glass slides and stained.

5.2.8 VEGF ELISA

Culture medium was collected at 12 and 14 days of explant cultivation on hydrogel. VEGF ELISA was performed on the culture medium samples using Murine VEGF ELISA Development Kit (Peprotech, 900-K99), to determine the VEGF secretion between Day 12 and 14. The time points were chosen based on the plateau in cell outgrowth and the initiation of the majority of tube formation.

5.2.9 Seeding cardiomyocytes onto capillary structures

After the culture of mouse arterial and venous explants for 14 days with HGF supplementation to form connected capillary outgrowths, 100000 neonatal rat cardiomyocytes were seeded on the capillary structure for each sample. The cardiomyocytes were cultivated for 7 days in 1mL culture medium consisting of DMEM with 1% penicillin/streptomycin, 1% HEPES and 15% FBS. At the end of the 7-day cultivation, the engineered cardiac tissues were removed from the PDMS substrates and their functional properties were measured as previously described [241].

Briefly, functional properties of the engineered cardiac tissues were determined by evaluating two parameters: excitation threshold (ET) and maximum capture rate (MCR), as described previously [242]. The constructs, consisting of the hydrogel, vascular bed and cardiac tissue, were gently removed from the PDMS substrates using tweezers and placed between two carbon electrodes placed 1cm apart. The electrodes were connected to the electrical stimulator through platinum wires. ET is the minimum voltage required to induce synchronous contractions of at least 75% of the cells in field of view, when using monophasic square pulses at 1Hz and 2ms. MCR is the maximum pacing frequency at 200% of ET, and was measured by increasing the stimulation frequency until the contractions stopped or became asynchronous.

5.2.10 Cardiomyocyte isolation

Cardiomyocytes were isolated from 2-day-old neonatal Sprague-Dawley rat hearts by serial collagenase digestion and preplating, as described previously [7] and approved by the University of Toronto Committee on Animal Care. Briefly, the neonatal rats were first euthanized. The
hearts were removed and quartered. The cells were isolated by an overnight treatment with trypsin in HBSS at 4°C followed by a series of collagenase digests. The cells in the supernatant from the digests were collected, centrifuged, resuspended in culture medium, and pre-plated into T75 flasks to separate non-adherent cardiomyocytes from adherent non-myocytes. Then, the non-adherent cardiomyocytes were collected and seeded in 10μL culture medium containing DMEM with 1% penicillin/streptomycin, 1% HEPES and 15% FBS.

5.2.11 Statistical analysis

Statistical analysis was performed with Graphpad Prism 5.0 software. One-way ANOVA with post-hoc Tukey tests and two-way ANOVA with Bonferroni post-tests were used to compare the differences among groups, with statistical significance considered if P < 0.05. The data are presented as mean ± standard error.

5.3 Results

In this study, we applied the Tβ4-encapsulated hydrogel onto micropatterned poly(dimethylsiloxane) (PDMS) substrates with 25μm, 50μm or 100μm wide grooves (Figure 5-1A, Figure 5-2). Subsequently, mouse, rat or human arterial and venous explants were placed at the two ends of the substrate to promote outgrowth of capillaries and form an organized vascular structure that is anchored by two parent explants. We achieved connected and oriented capillaries between the explants by Day 14 with a supplementation of HGF or VEGF in the culture medium during culture, and by Day 21 without any growth factors. In addition, cardiomyocytes cultivated on the engineered vascular structures formed beating tissues with improved functional properties and cell morphology compared to those cultivated on the hydrogel-coated substrates alone.

To determine if the inclusion of Tβ4 improved cell recruitment and accelerated formation of our vascular network, we exposed mouse arterial and venous explants to different Tβ4 amounts encapsulated in collagen-chitosan hydrogel (0ng for Control, 100ng for Encap100, 1500ng for Encap1500). An increased cell outgrowth density from the arterial and venous explants was observed with a higher dose of Tβ4 (1500ng vs. 0ng and 100ng; Figure 5-1B-D). Higher outgrowth density was observed in venous explants on control Tβ4-free gels, compared to
arterial explants. However, there were no significant differences between the outgrowth densities in the arterial vs. venous explants (Figure 5-1C vs. 5-1D) when Tβ4 was applied.

While Tβ4 can stimulate endothelial cell migration and differentiation on its own [116-117], it was previously reported that Tβ4 can also stimulate the synthesis and autocrine secretion of angiogenic growth factor VEGF [193]. Here, we observed enhanced VEGF secretion from explants cultivated on Tβ4 hydrogels compared to bare hydrogels (Figure 5-1E-F). Our basal culture medium contains serum, which may be the source of VEGF and was reported in the previous studies to stimulate quiescent endothelial cells to proliferate [243]. We measured the basal VEGF concentration in the culture medium to be 0.06±0.006ng/mL by ELISA. However, this level was increased by autocrine secretion in the presence of cultivated explants (Figure 5-1E-F). In general, basal levels of VEGF secretion were higher with the venous explants compared to the arterial explants cultivated without Tβ4 (Figure 5-1F vs. Figure 5-1E), which correlated with the increased cell density on the control hydrogel in the presence of venous explants compared to the arterial explants (white bars in Figure 5-1C and D). The VEGF amount measured in the culture medium for artery explants at Day 14 was increased with both application of 100ng as well as 1500ng of Tβ4 (P = 0.0034 one-way ANOVA, P < 0.05 for Control vs. both Encap100 and Encap1500). For vein explants, VEGF amount was increased with the 1500ng Tβ4 gel compared to the Control and 100ng Tβ4 in the hydrogel (P = 0.0036 for Control vs. Encap1500; P = 0.0055 for Encap100 vs. Encap1500). As such, there is a correlation between VEGF secretion and cell outgrowth. Encap1500 group (referred to as Tβ4 Gel) was used for further studies due to the observed dose response of both VEGF secretion and cell outgrowth to Tβ4.
Figure 5-1. Substrate topography and controlled release of angiogenic peptide Tβ4 guide capillary outgrowths from arteries and veins.

(A) Experimental setup. (i) PDMS substrates were first fabricated using standard soft lithography methods and (ii) coated with collagen-chitosan hydrogel with or without Tβ4. (iii) Arteries and veins were isolated and placed on the
two ends of the substrate, and (iv) cultivated for 2 weeks with HGF or VEGF supplementation, or 3 weeks without any growth factors. (v) Then, cardiomyocytes were seeded onto the engineered vascular bed and cultured for additional 7 days to grow a beating vascularized cardiac tissue. (B) Representative images of cell outgrowths from mouse artery and vein explants on non-patterned substrates at Day 14 of cultivation (* indicating location of explants, arrows indicating location of outgrowths). (C) Density of migrated cells from artery explants at various time points. (D) Density of migrated cells from vein explants at various time points. (E, F) Autocrine VEGF secretion was dependent on the Tβ4 dose and correlated to cell outgrowth density. (E) Total amount of VEGF measured with artery explants between Day 12 and 14. (F) Total amount of VEGF measured with vein explants between Day 12 and 14. (G-J) Tβ4 increased branch length of capillary outgrowths from mouse artery and vein explants at Day 14, with most pronounced effect on substrates with 50μm wide grooves. (G) Representative images of outgrowths. (H) Branch density on grooves with different widths (a branch is defined by the distance between two nodes or a node and the end of a tube). (I) Average branch length on grooves with different widths. (J) Average branch width on grooves with different widths. Micropatterned PDMS substrates had grooves of 25μm, 50μm and 100μm in width and 65μm in height. Experimental groups include hydrogel without Tβ4 (Control) and hydrogel with 1500ng encapsulated Tβ4 (Tβ4 Gel). * indicates statistically significant difference (P < 0.05 two-way ANOVA with Bonferroni post-tests for cell density, branch density, branch length and branch width; one-way ANOVA with post-hoc Tukey tests for VEGF expression).
Figure 5-2. Details of experimental setup.

(A-C) Cross sections of PDMS substrates with a coating of collagen-chitosan hydrogel. (A) 25μm grooves, (B) 50μm grooves, and (C) 100μm grooves. The height of the grooves was measured to be 65μm. The height of the hydrogel coating on top of the ridges was around 8μm. The grooves were filled with hydrogel for 25μm and 50μm grooves, and the 100μm grooves were half-filled. (D-E) Cross section images showing the mouse artery (D) and vein (E) on the flat PDMS substrate (* indicates vascular explant, dotted line indicates boundary between explant and PDMS substrate). The height and width of the mouse artery were around 250μm and 880μm respectively. The height and width of the mouse vein were around 330μm and 630μm respectively.

We next aimed to determine if we can guide and control the sprouting from the mouse arterial and venous explants through the integrated use of topographical cues (25, 50 and 100μm wide grooves) and controlled release of Tβ4. Previously, exogenous, soluble Tβ4 increased sprouting
from coronary artery rings in an angiogenesis assay [117], but the directionality of the outgrowths was not controlled. Here we hypothesized that we could accelerate the guided sprouting on microgrooves by the addition of Tβ4. We used a gel with a 1500ng dose of Tβ4 to coat the microgrooves in our hybrid system containing topography and chemical signals, since a 1500ng dose of encapsulated Tβ4 increased VEGF secretion and cell outgrowths from arteries and veins as compared to no Tβ4 or a 100ng dose (Figure 5-1). We evaluated three parameters to describe the sprouting process including: 1) branch density, 2) branch length and 3) branch width. Increased branch density correlates with higher level of sprouting often found during angiogenesis. Increased branch length is necessary for rapid connection between the parent explants. Ideally, branch width should be similar to that of the capillaries in the native vasculature.

The branch density and branch length within the outgrowths from mouse arterial and venous explants (Figure 5-1H, I) were both increased with the addition of encapsulated Tβ4 in the hydrogel coating (P = 0.0002 two-way ANOVA for branch density, P < 0.01 Bonferroni post-test for Control vs. Tβ4 Gel groups on 100μm grooves; P < 0.0001 two-way ANOVA for branch length, P < 0.05 for Control vs. Tβ4 Gel on 25μm grooves, P < 0.001 for Control vs. Tβ4 Gel on 50μm and 100μm grooves). Branch density for the Tβ4 Gel groups was not affected by the groove width (Figure 5-1H, P = 0.7078). However, the branches were significantly longer on 50μm grooves compared to other groove widths (Figure 5-1I, P < 0.001 for 50μm grooves vs. both 25μm and 100μm grooves). The lower branch lengths on substrates with smaller groove size were likely related to the hindrance of tube growth by spatial limitations. At larger groove widths, the capillary outgrowths tended to branch more due to the wide channels available for expansion of the vascular structure, thus lowering the branch length (Figure 5-1G, I). There was no significant difference in tube widths between Control and Tβ4 Gel groups (Figure 5-1J, P = 0.9699), showing that the width was not dependent on Tβ4 dose. Tubes grown on substrates with 25μm, 50μm and 100μm grooves showed comparable widths of ~10-12μm (P = 0.3782 one-way ANOVA), typical of capillaries in the native mouse myocardium (~10-20μm) [244]. Thus, we utilized 50μm wide grooves in further studies. There were no significant differences in branch density, length and width between arterial and venous explants, and the results were shown as lumped rather than separately for arterial or venous.
Figure 5-3. Engineering of a connected microvascular bed using topographical cues and hydrogels with encapsulated Tβ4.

(A) Fluorescence microscopy images showing the time course of capillary outgrowths extending between a YFP+ mouse artery (right side) to vein (left side) during in vitro cultivation on substrates with 50μm grooves and hydrogel coating containing 1500ng encapsulated Tβ4. The connection was achieved at Day 21. Arrows indicate locations of artery and vein explants. (B-G) Soluble angiogenic growth factors enhance the outgrowths. (B) Branch density at different time points when cultivating the explants in culture medium with no growth factors, or supplemented with VEGF or HGF. (C) Average branch length at different time points with no growth factors, or supplemented with VEGF or HGF. * indicates statistically significant difference between groups (P < 0.05 two-way ANOVA with Bonferroni post-tests). (D-G) Fluorescence microscopy images of YFP+ mouse artery and vein outgrowths with soluble growth factor supplementation. The connection was achieved at Day 14 using (D-E) VEGF supplementation and (F-G) HGF supplementation.
Our results clearly indicate that the enabling factor in tube formation was the presence of topographical cues, while the presence of an angiogenic factor Tβ4 simply accelerated the formation of the tubes and enhanced their length (Figure 5-1). Introduction of topographical cues was essential for formation of branches with open lumens, as collagen-chitosan gel alone led to cell outgrowth, but no formation of the luminal structures (Figure 5-1B). Topographical cues themselves, could act in enabling the tube formation by one of the following factors: contact guidance, change in the local mechanical properties of the environment or by locally increasing the concentration of autocrine growth factors.

When an artery and a vein were placed at the opposite ends of a PDMS stamp with Tβ4 hydrogel, the outgrowths from each vessel followed the topographical cues approaching one another with time (Figure 5-3A, Day 7 and Day 14). By Day 21, the capillary outgrowths from the artery explants connected to those from the vein explants, thus forming an arteriovenous loop with capillaries that were aligned in the direction of the microgrooves (Figure 5-3A). The average branch length (Figure 5-3C) was similar to the capillary length of ~600μm in the native rat myocardium [245]. The achieved branch density was much lower than the capillary density of more than 4000 capillaries per mm² in the native mouse heart [246]. However, multiple engineered vascular structures could be stacked and cultivated for an additional period to increase capillary density. Additionally, hypoxia could be utilized to increase sprouting from the formed vascular bed.

We next aimed to determine if we can further accelerate the sprouting rate to achieve connection between the artery and vein in a shorter time frame. For this purpose, we chose to introduce the angiogenic growth factors, VEGF and HGF, to the culture medium at 100ng/mL [247] or 20ng/mL [248] respectively. The doses of VEGF and HGF were chosen based on concentrations used previously for angiogenesis assays [247-248]. Culture medium was changed at 100% every other day. Both VEGF and HGF have been shown to accelerate vascularization in vitro and in vivo. VEGF is a potent mitogen for arterial, venous and lymphatic vascular endothelial cells [176]. It promoted the infiltration of endothelial cells into collagen gels to form capillary-like structures [249], and induced sprouting of rat aortic rings [250]. In addition, we found that the increased sprouting in our system was correlated with autocrine VEGF secretion (Figure 5-1B-F). HGF is secreted by fibroblasts and vascular smooth muscle cells [248]. HGF stimulates the
migration and proliferation of vascular endothelial cells, and accelerates their organization into capillary-like tubes \textit{in vitro} [248]. It also induces the formation of blood vessels \textit{in vivo} [248]. Previous studies demonstrated that the HGF-induced angiogenesis \textit{in vivo} involved the induction of VEGF expression in endothelial cells [230, 251].

\textbf{Figure 5-4. Oriented capillary outgrowths from rat artery and vein explants.}  
(A) Representative images of outgrowths at Day 14 for control and hydrogel with 1500ng of encapsulated Tβ4.  (B) Branch density (a branch is defined by the distance between two nodes or a node and the end of a tube) at Day 14. (C) Average branch length at Day 14. Micropatterned PDMS substrates had grooves of 25μm, 50μm and 100μm in width. Experimental groups include hydrogel without Tβ4 (Control), hydrogel with 100ng encapsulated Tβ4 (Encap100), and hydrogel with 1500ng encapsulated Tβ4 (Encap1500).  

The addition of HGF or VEGF to the culture medium during cultivation increased the length of the branches significantly at Day 14 (\textbf{Figure 5-3C}, P < 0.0001 two-way ANOVA, P < 0.05 for
Tβ4 Gel vs. Tβ4 Gel+VEGF, P < 0.001 for Tβ4 Gel vs. Tβ4 Gel+HGF). As a result, the vessel outgrowth rates were accelerated with the addition of HGF or VEGF, and connections between an artery and a vein could be attained after 14 days of culture (Figure 5-3B-G). There was no difference between VEGF and HGF in accelerating anastomosis, branch density or branch length in this system (Figure 5-3B, C).

Figure 5-5. Oriented capillary outgrowths from mouse cardiac explants.
(A) Representative images of outgrowths at Day 14. (B) Branch density at Day 14. (C) Average branch length at Day 14. Micropatterned PDMS substrates had grooves of 25μm, 50μm and 100μm in width. (D-F) Branch density at different time points on PDMS substrates with grooves of (D) 25μm, (E) 50μm and (F) 100μm in width. (G-I) Branch length at different time points on PDMS substrates with grooves of (G) 25μm, (H) 50μm and (I) 100μm in width. Experimental groups include substrates with a coating of hydrogel without Tβ4 (Control), hydrogel with 100ng encapsulated Tβ4 (Encap100), and hydrogel with 1500ng encapsulated Tβ4 (Encap1500).
Figure 5-6. Capillary outgrowths from mouse arteries and veins contained open lumens formed by endothelial cells and remained intact upon removal from the substrate. (A-F) Confocal microscopy showing lumens of YFP+ capillary outgrowths. (A) Full view z-stacked x-y image. (B) Cross sectional lumens in the y-z plane at the location indicated by the dotted line labelled as yz in the z-stacked image. (C-D) Cross sectional lumens in the x-z plane at the locations indicated by the dotted lines labelled as (C) xz-1 and (D) xz-2 in the z-stacked image. (E-F) Longitudinal lumens at the locations as indicated by orange boxes labelled as (E) xy-a and (F) xy-b in the z-stacked image. (G) Confocal microscopy images of von Willebrand factor staining (red): (i) Full view z-stacked x-y image; (ii) Cross sectional lumens in the y-z plane at the location as indicated by the dotted line labelled as yz in the z-stacked image; (iii) Cross sectional lumens in the x-z plane at the
location as indicated by the dotted line labelled as xz in the z-stacked image; (iv) Longitudinal lumen at the location as indicated by the orange box labelled as xy in the z-stacked image. (H) Lumens formed by endothelial cells as shown by transmission electron microscopy. (I-J) Representative immunostaining for (I) CD31 and (J) VE-cadherin (red indicates positive staining, blue indicates counterstaining of the nuclei with DAPI, arrows highlighting where the protein is localized, dotted lines outlining a capillary outgrowth). (K-L) Capillary network maintained its structural integrity after removal from the PDMS substrate. Fluorescence microscopy images showing capillary outgrowths (K) before and (L) after removal.

Our strategy was not species dependent. Similar results were shown using rat femoral artery and vein explants (Figure 5-4). Furthermore, we were also able to achieve oriented capillary outgrowths from mouse cardiac tissue explants (Figure 5-5), suggesting that it is not necessary to isolate specific arteries and veins. However, there are advantages of using vascular explants over cardiac, or other specific tissue explants. First, arteries and veins are more readily available for isolation. Second, the anchorage of the resulting capillary structure by parent vascular explants could allow implantation and perfusion of the structure. The maximal branch density obtained using a single mouse or rat artery or vein was similar at ~10/mm² (Figure 5-1, 5-4). Cardiac explants in contrast gave us ~30/mm² (Figure 5-5) likely due to the presence of large number of blood vessels in cardiac explants that could all con-currently sprout.

Confocal microscopy demonstrated that the mouse capillary outgrowths generated by the arteries and veins isolated from YFP+ mice had characteristics of developed vascular structures with open lumens (Figure 5-6A-F). Images from transmission electron microscopy (TEM) also demonstrated the presence of lumens formed by the cells in the peripheral position (Figure 5-6H). The cells that formed the capillary outgrowths were positive for the common endothelial cell markers, including CD31, VE-cadherin and von Willebrand factor (Figure 5-6G, I-J). This is typical of capillaries, which are made of a single layer of endothelial cells. Importantly, formation of lumens by von Willebrand factor-positive endothelial cells was clearly evident (Figure 5-6G) by confocal microscopy z-stacks. Smooth muscle actin- and NG2-positive cells that were associated with these capillary outgrowths appeared in the peripheral position, typical of pericytes (Figure 5-7). Smooth muscle actin positive cells were also found in between the vessels and the basement of the grooves (Figure 5-7B).
Figure 5-7. SMA- and NG2- positive pericyte-like cells appeared in the peripheral position on the capillary outgrowths.

Representative images showing (A) SMA and (B) NG2 staining of the capillary outgrowths from YFP+ mouse explants at Day 21 (red indicates positive staining, green indicates YFP expression due to the use of YFP+ mouse explants, blue indicates counterstaining of the cell nuclei with DAPI). Arrows indicate the presence of SMA+ or NG2+ cells near the outgrowths.

Since the microvascular bed originates from the artery and vein explants, we investigated if the cells in the newly generated microvascular bed exhibited the phenotype of either arterial or venous endothelial cells. Ephrin-B2 and EphB4 have been shown to selectively mark arterial and venous endothelial cells [252]. As expected, outgrowths on the artery and vein sides of the engineered capillary structure were positively stained for Ephrin-B2 and EphB4 respectively at Day 21 (Figure 5-8). Interestingly, there were also Ephrin-B2 positive cells within the capillaries on the vein side, and EphB4 positive cells on the artery side, suggesting an integration
of the arterial and venous outgrowths. It was demonstrated that Ephrin-B2 and EphB4 can physically interact in growing blood vessels [253] consistent with the lack of localization of these markers to either venous or arterial side observed here. Ephrin-B2 is known to be upregulated during pathological and physiological angiogenesis [252, 254]. Ephrin-B2 also regulates the internalization and signalling activity of VEGFR2 [255], the receptor of VEGF165 that was upregulated using the Tβ4 gels here.

Figure 5-8. Identification of venous and arterial endothelial cells.
Representative images showing EphB4 (venous marker) and Ephrin-B2 (arterial marker) staining of the capillary outgrowths on the artery side and on the vein side at Day 21 (red indicates positive staining, green indicates YFP expression due to the use of YFP+ mouse explants, blue indicates counterstaining of the cell nuclei with DAPI). Arrows indicate EphB4 or Ephrin-B2 positive cells.

The capillary structure was easily removed without damage to the morphology by peeling the hydrogel coating off the PDMS substrate (Figure 5-6K-L). More importantly, the engineered vascular structures were perfusible with fluorescently-labelled dextran.
Figure 5-9. Oriented and connected capillary outgrowths were formed from human umbilical artery and vein explants.

Day 21 microvascular bed on micropatterned PDMS substrates with a coating of collagen-chitosan hydrogel containing 1500ng encapsulated Tß4. (A) Brightfield images of outgrowths on PDMS substrates with 25µm, 50µm or 100µm groove width. (B) Viable staining of outgrowths on PDMS substrates with 25µm, 50µm or 100µm groove width (green represents CFDA staining of live cells). (C) Fluorescence microscopy image showing positive CD31 staining of endothelial cells that make up the capillary outgrowths on PDMS substrate with 50µm groove width (red indicates positive CD31 staining, blue indicates counterstaining of nuclei with DAPI). (D) Brightfield image showing connection of capillary outgrowths between the artery (right side) and vein (left side) explants on PDMS substrate with 50µm groove width. Connected outgrowths indicated between two dotted lines. Arrows indicate the parent explants. (E) Confocal microscopy image of CFDA stained sample showing lumens of the capillary outgrowths: (i) z-stacked x-y image; (ii) cross sectional lumens in the y-z plane at the location as indicated by the dotted line in the z-stacked image.
Neonatal rat cardiomyocytes were seeded around Day 14 microvascular bed and cultivated for additional 7 days. HGF was added to the culture medium during culture of capillary outgrowths. (A) A movie frame of the beating cardiac tissue from the group cultivated on Tβ4 gel with capillaries, showing the position of the cardiomyocytes relative to the capillary outgrowths that run parallel between the parent explants (* indicating location of vascular explant, YFP+ capillary outgrowths shown in green and indicated by arrows, cardiomyocytes shown as brightfield with an added dark background to enhance contrast). Functionality of engineered cardiac tissues was evaluated by measuring (B) excitation threshold and (C) maximum capture rate. (D) Troponin T immunostaining illustrates striations in cardiomyocytes grown on microvascular bed. The Tβ4 hydrogel alone exhibits poorly developed cells (Troponin T staining shown in red, Hoechst dye staining of the cell nuclei shown in blue). (E) High magnification confocal microscopy images showing Troponin T immunostaining (red). (F) Connexin-43 staining images showing more cell-cell junctions in the cardiac tissues grown on the microvascular bed (arrows indicating positive punctate Connexin-43 staining, Connexin-43 staining shown in red, Hoechst dye staining of the cell nuclei shown in blue).

Experimental groups include seeding of cardiomyocytes on PDMS substrates with a coating of Tβ4-free hydrogel (Control Gel), with a coating of Tβ4-encapsulated hydrogel (Tβ4 Gel), and with Day 14 capillary outgrowths on a coating of Tβ4-encapsulated hydrogel (Tβ4 Gel with capillaries).
Using the same strategy, we engineered an oriented capillary bed using human umbilical arteries and veins (Figure 5-9A, B). The capillary outgrowths were composed of CD31-positive endothelial cells (Figure 5-9C). The outgrowths connected between the parent arterial and venous explants by Day 21 (Figure 5-9D). Confocal microscopy showed that the capillaries contained lumens (Figure 5-9E).

To determine whether cardiac tissues with improved function could be engineered using the established capillary structure, cardiomyocytes were cultivated for 7 days on the PDMS substrate containing the mouse vascular explants and the associated capillary outgrowths created after 14 days of culture with HGF supplementation (Figure 5-10). Culture medium without HGF was used to cultivate the cardiomyocytes. Beating cardiac tissues were formed on the engineered vascular structures after 7-day cultivation. This type of co-culture precisely defines the position of endothelial cells and cardiomyocytes, with cardiomyocytes in parenchymal spaces around the capillaries as in the native heart (Figure 5-10A). Improved cardiac function was observed in our novel engineered tissues. The cardiac tissues grown on vascular structures had significantly lower excitation threshold compared to those grown on Control and Tβ4 Gel coated PDMS substrates without vascular structures (Figure 5-10B, P = 0.0036 one-way ANOVA, P < 0.01 post-hoc Tukey test for Control vs. Tβ4 Gel with Capillaries, P < 0.05 for Tβ4 Gel vs. Tβ4 Gel with Capillaries). The maximum capture rates for the cardiac tissues were similar with or without vascular structures (Figure 5-10C, P = 0.2226 one-way ANOVA), and comparable to the values from our previous studies [241, 256]. Troponin T staining showed that cardiomyocytes grown on vascular structures contained better organized sarcomeres compared to those grown on hydrogel coating only (Figure 5-10D, E). There was increased Connexin-43 staining in the group with cardiomyocytes cultivated on vascular structures (Figure 5-10F), thus indicating better cell-cell junctions.

5.4 Discussion

The phenotype of cardiomyocytes in this system could further be enhanced by application of electrical [242, 257] or mechanical [258] stimulation. Here we focused on proving that the engineered vascular network is suitable for cultivation of parenchymal cells, and we focused on the effects of the network alone. Endothelial cell networks previously led to synchronized contraction of cardiomyocytes and increased connexin-43 expression due to improved survival.
and spreading of the cardiomyocytes [259]. In future studies, the vascular structures could be connected to a perfusion system to engineer more complex and metabolically active cardiac tissues. This strategy can provide a native-like supply of oxygen and nutrients while preventing the cells from experiencing hydrodynamic shear. Importantly, anchoring of the parent artery and vein explants provides a means for surgically connecting the host vasculature to the engineered vascular structure, thus potentially allowing the engineered tissue to be fully integrated with the native tissue.

Organized vasculature is important for the integration of the blood vessels with other tissue-specific cell types to create complex tissues [86], especially cardiac tissues that require small intercapillary distances for metabolically active cardiomyocytes [260]. In the native myocardium, millions of capillaries run in parallel to reduce the total capillary resistance [261]. Moreover, organized vasculature allows easy isolation, manipulation and implantation of the vascular structures [86].

A number of notable approaches were used previously to create branching microchannels via microfabrication in polymers such as polystyrene [262], poly(ester amide) elastomers [263], or silk fibroin [264]. Microvessels that were in some cases endothelialized were also created in hydrogels such as collagen gels [265-266], alginate [267], as well as Matrigel or fibrinogen using gelatin as a sacrificial layer [268]. Embedding of endothelial cell laden alginate microfibers into smooth muscle cell laden agar based matrix was used for defined vascular cell co-culture before [269]. These approaches rely on providing defined areas for attachment of endothelial cells. In polymeric devices, the parenchymal space is often occupied by polymer rendering cell seeding in the area around microvasculature difficult. Although hydrogels are more amenable to co-culture, most are often fragile and difficult to handle, especially when fabricated in forms of microfibers or long hollow cylinders. In contrast, we did not force the cells to attach in microfabrication defined attachment regions. Instead, the approach we used here was inspired by nature, where topographical cues, hydrogel and angiogenic factors were used to create a supportive niche for controlled and directed sprouting of arterial and venous explants.

Vascularization during development or tissue regeneration in vivo involves endothelial cell proliferation and angiogenic sprouting in which a sub-set of endothelial cells, called tip cells, acquire motile behaviour under the influence of VEGF family of growth factors [270-271].
During embryonic vasculogenesis and sprouting angiogenesis, anastomosis is accomplished via connection of extended cellular processes followed by lumen propagation through intercellular and intracellular vacuole fusion [272-273]. Here, we used angiogenic factor (Tβ4) to enhance the outgrowth cell density on the collagen-chitosan hydrogel and increase autocrine VEGF secretion, while topographical cues were critical for formation and propagation of extended cellular processes. In this approach, endothelial cells are not forced to attach and proliferate on a pre-fabricated structure, instead they migrate and proliferate, under direction of externally added and autocrine cytokines, and assemble into luminal structures directed by the topographical cues. Thus, the approach described here enables engineering of a prototype vascular network consisting of two branching vessels suitable for cultivation of different parenchymal cell types or potentially for direct anastomosis to the host vasculature.

5.5 Conclusions

In summary, we have shown that Tβ4 in collagen-chitosan hydrogels in conjunction with the application of topographical cues guided endothelial outgrowths from an artery and a vein, and aided the organization of capillaries into functional microvasculature. The resulting capillary structure was used as a vascular bed for growing vascularized engineered cardiac tissues with improved function. The engineered microvascular bed was perfusible and removable from the PDMS substrate. Its functionality and ease of manipulation facilitate its potential use in future tissue engineering and in vivo applications.

5.6 Acknowledgements

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5.7 Commentary

In this chapter, I applied the hydrogel developed in the previous chapter onto a micropatterned substrate to create a biomimetic niche with combined topographical cues and localized presence of angiogenic factor, suitable for the engineering of a microvascular bed using nature’s approach. This study was motivated by the moldability of hydrogels, which allowed the combined use of topographical cues and the Tβ4 hydrogel, and the presence of tube-forming endothelial cells recruited from epicardial explants in the previous chapter.

The extension of formed tubes shown here was not due to a gradient of Tβ4, since no gradient was used. However, Tβ4-containing hydrogel was only used on the side of the artery or vein explant that faced the microgrooves. Thus, the localized presence of Tβ4 acted as a chemoattractant for endothelial cells to migrate in one direction into the Tβ4 hydrogel, and then their formation into tubes was controlled by topographical cues.

This study is the first to demonstrate that angiogenic biomaterials can be combined with topographical cues to mimic the in vivo conditions that promote the formation of organized vasculature. This further motivates the development of hydrogels that incorporate both topographical cues and controlled delivery of angiogenic factors. For example, a disordered microvascular structure between the intact artery and vein is often present in the damaged heart. A hydrogel with aligned carbon nanotubes and encapsulated Tβ4 could be injected between the artery and vein to induce the reorganization of the vasculature.
Chapter 6

6 Controlled Release of Thymosin β4 from Injected Collagen-Chitosan Hydrogels Promotes Angiogenesis and Prevents Tissue Loss after Myocardial Infarction

6.1 Introduction

After myocardial infarction (MI), cardiomyocytes die followed by a cascade of events that remodel the heart, including fibrosis of the infarct area. This is a compensatory mechanism that enables the ventricle to withstand the systolic pressure, but it leads to the eventual thinning of the scar tissue [181]. Wall thinning increases myocardial wall stress, in turn increasing the expansion of the border zone and progression of the LV remodelling [274]. This ultimately causes decrease in cardiac function and congestive heart failure.

A main requirement in cardiac repair is revascularization of the infarcted myocardium. Vascularization is essential in providing nutrients and oxygen to the tissue in order to support cell viability and maintenance of a thick ventricular wall [181]. To induce vascularization, cells or angiogenic biomolecules can be injected in vivo. Bone marrow cells [275-276] and endothelial progenitor cells [277] were previously found to participate in the repair of the damaged cardiac muscle through induction of angiogenesis. Cell-free biomaterials such as fibrin [47, 57], collagen [57], alginate [278] and decellularized ventricular extracellular matrix [279] have been injected for cardiac repair. In addition, angiogenic biomolecules including vascular endothelial growth factor [67] and hepatocyte growth factor [280-281] have been delivered, either in soluble forms or incorporated in biomaterials, to promote angiogenesis in the infarcted myocardium. It was previously found that the injection of bio-inert non-degradable poly(ethylene glycol) (PEG) gel provided passive structural reinforcement post-MI, which was insufficient to prevent ventricular remodelling [274]. This may be related to the inability of the

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5 Copyright © 2012 Future Medicine Ltd. Contents of this chapter have been published in Regenerative Medicine: Chiu LLY et al. Controlled release of thymosin β4 from injected collagen-chitosan hydrogels promotes angiogenesis and prevents tissue loss after myocardial infarction. Regenerative Medicine. 2012 July; 7(4):523-533. Reuse with permission from Future Medicine Ltd. A link to the published paper can be found at: www.futuremedicine.com/doi/abs/10.2217/rme.12.35
gel to increase vascularization to restore the blood supply to the infarct region [274], as compared to other injected materials with or without cells that have shown improvement in cardiac function due to increased vascularization [57-58].

In this study, we injected a collagen-chitosan hydrogel with encapsulated thymosin β4 (Tβ4) after LAD ligation in rats. The hydrogel itself can serve as a mechanical support to the host tissue by conforming to the geometry of the ventricular space and thus deforming with the contracting environment [57]. Moreover, the injection of biomaterials offers a less invasive strategy for myocardial reconstruction, as compared to the implantation of scaffold patches or engineered cardiac tissues [57]. While collagen supports cell retention and survival, it was shown that the compression modulus of collagen-chitosan hydrogels was greater than collagen-only hydrogels [63]. This is important for the stabilization of the ventricular wall in order to reduce dilatation [63]. Tβ4 is an important angiogenic and cardioprotective peptide used for inhibiting myocardial cell death, inducing vessel growth, and activating endogenous cardiac progenitors [282]. It is the first known molecule that is capable of initiating myocardial and vascular regeneration 	extit{in vivo} simultaneously [282]. The benefits of using a cell-free hydrogel with a controlled release of a bioactive molecule for cardiac repair include the elimination of a need to expand cells and support cell survival post-injection. Tβ4 encapsulation also allows the localization of bioactivity and prevents washout of the peptide from injection site, thus prolonging bioactivity.

In previous studies, Tβ4 was applied as multiple intraperitoneal injections in PBS or an intracardiac injection in collagen gel [118-119]. We have recently achieved controlled release of Tβ4 from collagen-chitosan composite hydrogels at a nearly zero order kinetics, which was not possible from collagen-only hydrogels [238]. This developed collagen-chitosan hydrogel with encapsulated Tβ4 was shown to promote the migration of endothelial and smooth muscle cells from heart epicardial tissue explants, and induce angiogenesis upon subcutaneous injection in rats [238].
6.2 Materials and methods

6.2.1 Preparation of hydrogel solutions

The following stock solutions were prepared: collagen I (BD Biosciences, cat #354236, 3.89mg/mL rat tail collagen I in acetic acid), chitosan (75-90% deacetylation, MW = 150-200kDa, Protasan, NovaMatrix, G113, 5.92mg/mL in distilled water), and Tβ4 (Prospec, cat #HOR-275, 1mg/mL in deionized water). The stock solutions were mixed in a single step on ice, with the addition of PBS (10X, one tenth of the final volume) and sodium hydroxide (1N, 0.025 times the volume of collagen), such that the final concentrations of collagen I, chitosan and Tβ4 were 2.5mg/mL, 1.25mg/mL and 0μg/mL (Control group) or 30μg/mL (Thymosin group) respectively. The solutions were gently vortexed and kept on ice.

6.2.2 Degradation of hydrogel

For in vitro degradation study, the hydrogels were incubated at 37°C in 500μL PBS or culture medium consisting of 83% Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L glucose, 4mM L-glutamine, 15% fetal bovine serum (FBS), 100units/mL penicillin, 100μg/mL streptomycin and 1% HEPES buffer. At Day 7 and 21, hydrogels were removed and lyophilized as described previously [238]. For Day 0 samples, hydrogels were immersed in PBS or culture medium for 30 minutes prior to lyophilization. The dry weights at different time points were compared for hydrogel degradation.

For in vivo degradation study, the mixture of collagen and chitosan was labelled using the Lightning-Link biotin labelling kit (Innova Biosciences, cat #704-0015). Thus, biotin was conjugated to both collagen and chitosan. Briefly, the mixture of collagen and chitosan was added to the lyophilized biotin conjugation material and the modifier reagent, and incubated for 3 hours at room temperature. Then, a quencher was added and the solution was incubated for 30 minutes to deactivate unreacted chemicals. Biotin quantification was performed on positive control hydrogels labeled with biotin, and it was determined that biotin was conjugated at 1.4±0.2mol biotin per mol collagen/chitosan molecules. The mixture was then used to prepare hydrogel solutions (50μL) that were injected subcutaneously into the upper back of adult Sprague Dawley rats, as described in [238]. The hydrogel was extracted at Day 0, 7 and 21. The biotin content of the hydrogel samples was quantified using the biotin quantification kit.
(Pierce Biotin Quantification Kit, Thermo Scientific, cat #28005), as described previously [238]. No biotin content was detected by Day 21.

6.2.3 Experimental animals

Animal procedures were approved by the University of Toronto Animal Care Committee. Lewis rats (200-225g, Charles River Laboratories) were used for the study (n=3-5/group).

6.2.4 Left anterior descending artery ligation and hydrogel injection

Myocardial infarction was induced in isoflurane-anaesthetized rats by a permanent left anterior descending artery (LAD) ligation. The LAD ligation model has been shown in various studies performed by Dr. Momen to generate a reproducible infarct size [283-285]. Briefly, a left thoracotomy was performed in the fifth intercostal space. After opening the pericardium, the proximal LAD coronary artery was encircled and ligated using a 6-0 polypropylene suture. Hydrogel solutions were injected immediately after the LAD ligation into the infarcted region of the heart from three spots in the border zone surrounding the infarct. The total volume injected was 50μL. The hydrogel solutions were preheated prior to injection at 30°C for 10min to increase the temperature of the solutions, which were kept on ice, and to initiate the gelation process. The gelation time of the Control and Thymosin hydrogels was previously determined to be 266±24s [238]. Thus, the pre-gelling process was necessary to render the material capable of gelation immediately upon injection into the heart, in turn ensuring that the hydrogel was retained in the myocardium. The chest was closed with 4-0 Dexon suture. Experimental groups included: MI Only (LAD ligation without hydrogel injection), Control (LAD ligation with the injection of collagen-chitosan hydrogel without Tβ4), and Thymosin (LAD ligation with the injection of Tβ4-encapsulated collagen-chitosan hydrogel). Sham controls (n=2) were performed by opening the chest without performing LAD ligation.

6.2.5 Heart morphology and histology

At 21 days after hydrogel injection, rats were euthanized by an isoflurane overdose and a subsequent cervical dislocation. Hearts were fixed in 10% formalin solution for 24 hours, rinsed in PBS, and photographed. A rat heart slicer (Zivic, HSRS001-1) was used to cut two 1mm thick heart sections below the suture, with the first cut made exactly at the location of suture. The sections were then paraffin embedded and sectioned for staining with Masson’s trichrome, anti-
Factor VIII (FVIII) antibody, or anti-α-smooth muscle actin (α-SMA) antibody (n=2 sections per heart for each stain). Scar thickness, tissue loss, vessel density and vessel diameters were quantified using ImageJ software. Scar thickness was evaluated from Masson’s trichrome staining by measuring the thickness of the left ventricular wall at evenly spaced locations (n=5) at the infarct area. Tissue loss was measured from sections stained with Masson’s trichrome using ImageJ by first measuring the total red-coloured area from each section, which represents the tissue area, and then comparing the total tissue area of the heart section to the average total tissue area of healthy heart sections (n=2 animals for Sham group). To measure the red-coloured area, ImageJ was used to split each image into greyscale images of the red, green and blue channels, and then perform a threshold on the green channel, which showed the best contrast between red-coloured tissue (dark intensity) and blue-coloured collagen scar (light intensity). The area fraction of the thresholded area, representing the red-coloured tissue, was measured in ImageJ and divided by the average area fraction of thresholded area in the Sham group to determine the percentage tissue loss. Vessel density was measured by counting the number of FVIII-positive or SMA-positive blood vessels within the infarct area of the sections stained with anti-FVIII or α-SMA antibody, and normalizing by the area of view. Vessel diameters were measured at the shorter axes of the blood vessels from sections stained with anti-FVIII or α-SMA antibody. All evaluations were performed in the middle of the infarct.

6.2.6 Immunostaining for cardiomyocytes

Slides containing the paraffin sections of the hearts were incubated at 58°C for 30 minutes. Then, the slides were heat treated for antigen retrieval by an incubation in reveal decloaker solution (Biocare Medical, RV1000M) in a decloaking chamber (Biocare Medical) at 95°C for 20 minutes. Blocking was performed using 10% fetal bovine serum (FBS) in PBS at room temperature in a humidified chamber for 40 minutes. Primary antibody (mouse Troponin T, Fisher, MS-295-P) was applied (dilution factor of 1:50) in PBS with 1.5% FBS and 0.5% Tween-20 at 4°C overnight. Secondary antibody (rhodamine goat anti-mouse, Jackson ImmunoResearch, 115-025-166) and DAPI were applied at dilution factors of 1:100 in PBS with 1.5% FBS and 0.5% Tween-20 at room temperature for 30 minutes. The slides were finally mounted with Fluoromount and imaged using fluorescence microscope.
6.2.7 Statistical analysis

All data were represented as mean ± standard error. Analyses were performed using SigmaPlot 12. Differences between experimental groups were analyzed by using one-way ANOVA in conjunction with Student-Newman-Keuls tests. P < 0.05 was considered significant.

6.3 Results

The collagen-chitosan hydrogel showed no degradation after 7 days in vitro and in vivo (Table 6-1), making it applicable in replacing the 7-day regimen of daily injections of Tβ4 as used for cardiac repair previously [119]. The mass of the hydrogel samples were similar at Day 0 and Day 7 after immersing them into PBS or culture medium (P = 0.5147 for PBS, P = 0.1501 for culture medium, t-tests). The mass did not change by Day 21 in PBS, while an increase in mass was found when the hydrogels were immersed in culture medium for 21 days, likely due to the absorption of proteins by chitosan. The biotin content of the biotinylated hydrogel samples were also similar at Day 0 and Day 7 after subcutaneously injecting the hydrogel solutions into rats (P = 0.9244 t-test). No biotin was detected in vivo at Day 21, suggesting a complete degradation of the hydrogels by 21 days in vivo.

Table 6-1. Degradation of collagen-chitosan hydrogel.

In vitro degradation study was performed by immersing the hydrogel into PBS or culture medium containing DMEM with 15% FBS, and then lyophilizing the samples to measure the dry weight. In vivo degradation study was performed by subcutaneously injecting biotinylated collagen-chitosan hydrogel into rats. Biotin content of the hydrogel after extraction at Day 0 and Day 7 was quantified.

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<th>Day 0</th>
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<td><strong>hydrogel after PBS</strong></td>
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<td><strong>incubation (mg)</strong></td>
<td>10.8 ± 2.5</td>
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<td><strong>In vitro dry weight of</strong></td>
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<td><strong>culture medium</strong></td>
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<tr>
<td><strong>incubation (mg)</strong></td>
<td>12.4 ± 1.7</td>
<td>14.7 ± 0.4</td>
<td>18.9 ± 0.6</td>
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<td><strong>In vivo biotin content</strong></td>
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<td><strong>(nmol)</strong></td>
<td>20.8 ± 6.5</td>
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The hydrogel solutions were injected into the infarct region of rat hearts after LAD ligation. At Day 21, wall thickness as evaluated from Masson’s trichrome staining (Figure 6-1A) was significantly increased with the injection of Tβ4-encapsulated hydrogel compared to both MI Only and Control groups (Figure 6-1B, P = 0.0008 one-way ANOVA, Thymosin vs. MI Only P < 0.001, Thymosin vs. Control P < 0.05). The injection of Tβ4-free hydrogel also increased wall
thickness compared to no injection (P < 0.01). A similar trend of increasing wall thickness can be seen from gross morphology (Figure 6-2). LAD ligation without hydrogel injection (MI Only group) led to severe thinning of the left ventricular wall (Figure 6-1A, Figure 6-2B), leaving a thin wall of ~0.3mm compared to 1.9±0.11mm (Trichrome) for Sham control. This is consistent with the left ventricular wall thickness of ~0.4mm as previously measured for the MI Only control group at 28 days post-MI [286]. Significant amount of viable heart muscle tissue was lost at 21 days post-MI without treatment (Figure 6-1C). The loss of tissue was attenuated with the injection of collagen-chitosan hydrogel with or without encapsulated Tβ4 (P = 0.001 one way ANOVA, MI Only vs. Control P < 0.01, MI Only vs. Thymosin P < 0.001). The Tβ4 treatment further decreased tissue loss compared to the injection of collagen-chitosan hydrogel only (Control vs. Thymosin P < 0.05).
Figure 6-1. Masson’s trichrome staining of heart sections shows significantly thicker LV wall and decreased percentage tissue loss with the incorporation of thymosin β4 into the hydrogel.

(A) Representative images of Masson’s trichrome staining. Scale = 1mm. (B) Thickness as evaluated from trichrome staining. Red line indicates the thickness for sham group. (C) Percentage tissue loss as evaluated from trichrome staining, by comparing the total tissue area of the sample to an average total tissue area of sham controls. * denotes statistically significant difference between groups (P < 0.05 one way ANOVA in conjunction with Student-Newman-Keuls tests)
Both FVIII-positive (Figure 6-3B) and SMA-positive (Figure 6-4B) vessel density were significantly higher in the Control and Thymosin groups compared to MI Only group (one-way ANOVA P = 0.0144 for FVIII, P < 0.0001 for SMA). Importantly, the average FVIII- and SMA-positive vessel diameters were significantly increased in the Thymosin group compared to MI Only group (Figure 6-3C, 6-4C, P = 0.0169 for FVIII, P = 0.0077 for SMA). A large percentage of the blood vessels found in the MI Only group were small capillaries with diameters less than 10μm (Figure 6-5). Furthermore, no large vessels (>50μm) were present in the MI Only group (Figure 6-5A, B). There were significantly more large FVIII-positive vessels (>50μm) in the Control and Thymosin groups compared to MI Only group (Figure 6-5A, P < 0.0001 one-way ANOVA, P < 0.001 for both MI Only vs. Control and Thymosin). The Thymosin group further showed significantly greater number of FVIII-positive vessels of >50μm in diameter as compared to Control group (P < 0.001 for Control vs. Thymosin group). This is an important indication of Tβ4 inducing vascularization, since vessel density and diameter could be skewed by the presence of small blood vessels. Thus, although both Control and Thymosin
groups showed increased blood vessel density compared to MI Only group (Figure 6-3, 6-4), the Thymosin group also had an improved vascular maturity consisting of larger diameter vessels (Figure 6-3, 6-4, 6-5).

Figure 6-3. FVIII staining shows significantly greater vessel density and diameter with the incorporation of thymosin β4 in collagen-chitosan hydrogel.

(A) Representative images of FVIII staining. Scale = 100μm. (B) Density of FVIII+ vessels within the infarct area. (C) Average diameter of FVIII+ vessels. * denotes statistically significant difference between groups (P < 0.05 one-way ANOVA in conjunction with Student-Newman-Keuls tests)
Figure 6-4. SMA staining shows significantly increased average vessel diameter with the incorporation of thymosin β4 in collagen-chitosan hydrogel. 

(A) Representative images of SMA staining. Scale = 100μm. (B) Density of SMA+ vessels within the infarct area. (C) Average diameter of SMA+ vessels. * denotes statistically significant difference between groups (P < 0.05 one-way ANOVA in conjunction with Student-Newman-Keuls tests)
Figure 6-5. Higher frequency of larger vessels found in the thymosin β4 group.

(A) Binning of FVIII+ vessel diameters.  (B) Binning of SMA+ vessel diameters.

Besides increased vascularization, cardiomyocyte presence within the infarct area was also better sustained with the injection of Tβ4-encapsulated hydrogel post-MI (Figure 6-6). Both Control and Thymosin groups showed increased presence of cells in the infarct area at Day 21, as compared to the cardiomyocyte-deprived infarct area in the MI Only group (Figure 6-6A). However, the Thymosin group further increased the percentage of Troponin-T positive cardiomyocytes (Figure 6-6B, P = 0.0453 one-way ANOVA, Thymosin vs. MI Only P < 0.05) in the infarct area.
Figure 6-6. Injection of thymosin β4-containing hydrogel enhances cardiomyocyte presence in the infarct zone.

(A) Representative images of Troponin-T staining (red indicates positive staining; blue indicates DAPI staining of nuclei). Scale = 100μm. (B) Percentage of Troponin-T positive cells within the infarct area. * denotes statistically significant difference between groups (P < 0.05 one-way ANOVA in conjunction with Student-Newman-Keuls tests)

There is a positive correlation between the SMA-positive blood vessel density and the wall thickness (Figure 6-7, r = 0.9319, P < 0.0001). This suggests that the Tβ4-induced stabilization and maturation of blood vessels reduces thinning of the left ventricular wall post-MI. Overall, both vascularization and the enhanced presence of cardiomyocytes played a role in retaining the
thickness of the infarct region in the left ventricular wall upon injection of collagen-chitosan hydrogel with encapsulated Tβ4.

Figure 6-7. Correlation between angiogenesis and wall thickness.

6.4 Discussion

Myocardial infarction leads to LV remodelling and dilation, death of cardiomyocytes, and ultimately heart failure [47]. LV remodelling post-MI contributes to the progression of heart failure [287], and is a main indication of mortality from heart failure [288]. Previous studies have shown that the injection of biomaterials into the infarct regions can prevent LV remodelling [278, 289-290]. The injection of biomaterials can reduce wall thinning by providing mechanical support to the infarct region and preventing scar expansion [289]. Besides giving mechanical support, the injection of biomaterials with or without bioactive molecules has been shown to promote cell infiltration to the infarct area, enhance vascularization, and support cell survival, in turn increasing wall thickness [48, 67].

Tβ4 has been shown to improve early cardiomyocyte survival after ischemic injury in the heart by the upregulation of ILK and Akt activity [291]. It also contributed to vascular repair in the heart by inducing the migration of coronary endothelial and smooth muscle cells from the heart epicardium [116, 282]. A recent study by Smart et al. [119] showed that epicardial cells can be primed with Tβ4 such that the cells give rise to new functional cardiomyocytes upon induction of myocardial infarction. In the study, an injection regimen of Tβ4 prior to myocardial infarction was required, involving intraperitoneal injection of Tβ4 daily for 7 days. In another study, Tβ4
was delivered intraperitoneally every 3 days for 2 weeks to reduce scar size post-MI [118]. Intraperitoneal administration of Tβ4 required multiple injections, resulting in the use of 600μg Tβ4 in total compared to 1.5μg used in our study. Tβ4 was also administered locally in a collagen gel within the cardiac infarct, but no significant difference in cardiac dilation was found between systemic and local delivery methods [118]. While intracardiac administration of Tβ4 in a collagen gel required only one injection, the effects of injecting control hydrogel and hydrogel with Tβ4 were not compared. The injection of biomaterial alone can lead to increased angiogenesis and reduced wall thinning, as shown in our study here. Thus, there is an incentive to fabricate a hydrogel delivery system for the controlled release of Tβ4 to eliminate washout of the peptide from the injection site, such that multiple injections [118-119] would not be necessary and the bioactivity of Tβ4 can be localized and sustained, resulting in the improved efficacy of the peptide.

Here, we evaluated a previously developed collagen-chitosan hydrogel with Tβ4 [238] to improve vascularization and promote cardiac cell survival post-MI. The developed hydrogel can deliver Tβ4 in a sustained manner over 28 days [238], at approximately zero order kinetics thus eliminating the need for multiple injections. The hydrogel does not degrade or swell significantly in PBS [238]. It has a porous structure, with gelation time of 200-300s, plateau storage modulus of ~30Pa and plateau loss modulus of ~3Pa. The collagen-chitosan hydrogel was capable of in vitro Fickian release of encapsulated polyalanine, which is a neutral peptide. The sustained release of positively and negatively charged peptides, including Tβ4, polyglutamic acid and polylysine, slightly deviated from Fick’s law due to interactions between the diffusing peptides and the hydrogel. In contrast, encapsulated Tβ4 in collagen-only hydrogel was quickly released into the PBS environment within the first 3 days. We have previously shown that the hydrogel with encapsulated Tβ4 can increase the migration of endothelial cells and smooth muscle cells from the epicardium of the heart in vitro, as compared to soluble Tβ4 or hydrogel without Tβ4 [238]. We have also shown enhanced angiogenesis when the Tβ4-encapsulated collagen-chitosan hydrogel was injected subcutaneously into rats, as compared to injection of collagen-only gel with encapsulated Tβ4 or injection of Tβ4-free collagen-chitosan hydrogel [238]. This demonstrated that the controlled release of Tβ4, rather than relying on the biomaterial alone or injection of the peptide in soluble form, is necessary to induce angiogenesis in vivo. The controlled release of Tβ4 was not assessed previously in the myocardial infarction...
model. In this study, we aimed to further investigate whether or not the Tβ4-encapsulated hydrogel can aid cardiac repair through inducing angiogenesis in the infarct region after MI. This was a study to show the feasibility of using the developed hydrogel for improving angiogenesis and cardiac cell maintenance post-MI. Long term data for cardiac function will follow in a separate study.

Soluble injection of Tβ4 in PBS was not performed as a control in this study since it has already been shown that multiple injections are required for this treatment mode to be effective. Rather, we focused on evaluating the controlled release of Tβ4 from our developed collagen-chitosan hydrogel in achieving cardiac repair. We also omitted the collagen-only gel with encapsulated Tβ4 here because no sustained release was achieved previously with collagen-only hydrogels, thus leading to no significant enhancement in angiogenesis when injected subcutaneously [238]. The encapsulated dose in this study was selected based on our previous study in which the angiogenic effect of encapsulated Tβ4 in collagen-chitosan hydrogel was dose-dependent when subcutaneously injected in rats, with the most infiltration of FVIII-positive blood vessels in the group with 1500ng encapsulated Tβ4 [238]. The selected concentration of 30μg/mL (1500ng Tβ4 in 50μL) was also similar to 40μg/mL Tβ4 previously encapsulated in collagen [118] or PEG hydrogels [139] for MI studies.

Since the degradation of the collagen-chitosan hydrogel was minimal over 7 days (Table 6-1), it is suitable for the controlled release of Tβ4 to replace daily injections of Tβ4 for 7 days as used in a previous study for cardiac repair [119]. The hydrogel degrades completely by 21 days, indicating its biodegradability in vivo (Table 6-1), thus motivating the 21 day duration of our in vivo experiments. In our previous study, we determined that the hydrogel remained bioactive by studying the outgrowths of cells from rat heart explants cultivated on top of the hydrogel with controlled release of Tβ4 for 7 days. Here, the injection of the Tβ4-encapsulated hydrogel increased the blood vessel density (Figure 6-3, 6-4), the average diameter of both FVIII- and SMA-positive vessels (Figure 6-3, 6-4), and the percentage of cardiomyocytes (Figure 6-6) within the infarct area, when compared to the untreated case. This is consistent with the angiogenic and cardioprotective property of the Tβ4 peptide. The combination of enhanced angiogenesis and sustained presence of cardiomyocytes led to an increase in wall thickness (Figure 6-1, 6-2, 6-7) which is important for reducing myocardial wall stress, preventing further wall thinning and dilatation, and improving cardiac function [274]. The importance of retaining
LV wall thickness can be clearly seen in the MI Only case, since the thin LV wall of 0.3±0.1mm correlated with a significant loss of overall cardiac tissue (Figure 6-1, 58±3% loss compared to Sham hearts). This is due to the high stress that causes cardiac dilatation. Tissue loss due to myocardial infarction was significantly higher without injection of Tβ4-free or Tβ4-encapsulated hydrogel, thus showing that collagen-chitosan hydrogel alone has biochemical and mechanical properties that attenuate pathological remodelling process. Importantly, the injection of Tβ4-encapsulated hydrogel further prevented tissue loss compared to Tβ4-free hydrogel (Figure 6-1C, 30±8% for Control vs. 13±4% for Thymosin). Thus, thicker LV wall and in turn reduced tissue loss, as supported by Tβ4-induced vascularization and cell survival, was achieved.

However, this in vivo study was only conducted for 3 weeks. A longer term effect of the controlled release of Tβ4 should be investigated to observe the density of stabilized blood vessels and the long-term improvement in cardiac function. It is expected that since the number of larger blood vessels was increased in the Thymosin group (Figure 6-5), the number of blood vessels at later end points would also be higher in this group compared to MI Only and Control groups. In contrast, small capillaries (<10μm in diameter) that were not supported by smooth muscle cells (Figure 6-3, 6-4, 6-5), as found in the MI Only group, might disintegrate with time. The enhanced vascularization through the injection of Tβ4-encapsulated collagen-chitosan hydrogel should continue to support cell infiltration and survival necessary for further improving LV wall thickness and in turn overall cardiac function.

### 6.5 Conclusions

We injected a previously developed collagen-chitosan hydrogel with encapsulated Tβ4 into the infarct area after LAD ligation in rats. The controlled release of Tβ4 increased the vessel density, improved the maturation of blood vessels and supported the survival of cardiomyocytes within the infarct area, which in combination led to the attenuation of LV wall thinning. Retaining thick LV wall post-MI is important for preventing heart failure due to the increase in wall stress.

### 6.6 Acknowledgments

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6.7 Commentary

In this chapter, I described the injection of the developed hydrogel from Chapter 4 into the rat hearts after LAD ligation was performed to mimic MI. In this feasibility study, it was shown that the controlled release of Tβ4 supported vascular maturation and prevented tissue loss as compared to no treatment and treatment with Tβ4-free hydrogel. This suggests that the bioactivity of the incorporated Tβ4 was necessary for cardiac repair, rather than relying on the mechanical properties of the hydrogel alone.

While many angiogenic hydrogels were studied previously, this is the first cell-free hydrogel with both angiogenic and cardioprotective abilities due to the bioactivity of Tβ4 peptide. Here, the dual capability of the hydrogel was found to be feasible for cardiac repair. In future studies, it would be of interest to separate the angiogenic and cardioprotective effects. This could be done by using synthetic segments of Tβ4 that are individually responsible for angiogenesis or cardioprotection. The use of the full Tβ4 peptide would be compared to these segments to observe any synergistic effect of angiogenesis and cardioprotection on cardiac repair (i.e. reduction in tissue loss).

Although Tβ4 modulates the inflammatory response, which is important for vascularization and cardioprotection, this was not evaluated in the thesis. Rather, I focused on evaluating the angiogenic and cardioprotective properties of Tβ4 as the two main experimental endpoints to show feasibility in using the Tβ4 hydrogel specifically for revascularization and maintenance of cardiomyocyte presence after MI. However, it would be of interest to also evaluate the immune response of injecting this Tβ4 hydrogel in future studies. This would indicate whether or not the hydrogel elicits a low but desirable level of inflammation necessary for angiogenesis. Also, the evaluation of inflammatory response would allow a closer look at the mechanism of Tβ4-induced angiogenesis and determine whether or not this was due to inflammation. Moreover, it would reveal the effect of modulated inflammatory response on cardiomyocyte survival, and whether inflammation works with or against angiogenesis to support cardioprotection. This thesis was
not concerned with the mechanism of angiogenesis and cardioprotection induced by the controlled release of Tβ4.
Chapter 7

7 Conclusions

The problem at the start of my thesis was the lack of vascularization approaches for cardiac tissue engineering and cardiac repair. At the time, most strategies for cardiac repair involved the transplantation of cells that either contribute to neovascularization (i.e. endothelial progenitor cells [292]), or participate in both myogenesis and angiogenesis through transdifferentiation (i.e. bone marrow cells [292-293]). A strong emphasis was put into finding a suitable cell source and developing expansion techniques for these cells to be used for growing engineered cardiac tissues in vitro or cardiac repair in vivo. In addition, extensive research was performed to create engineered cardiac patches with contractile properties similar to the native myocardium, through the application of electrical and mechanical stimulation during the cultivation of these patches. The transplantation of stem cells or progenitors showed marginal success in cardiac repair when tested in clinical studies, likely due to the washout of cells, while greater success was found with the grafting of engineered cardiac patches [36]. Both cell transplantation and grafting of engineered cardiac patches require cell isolation and expansion to attain large numbers of cells, making these potential treatment options for cardiac diseases costly and time consuming. At the same time, vascularization became increasingly important because of the high cell density needed for cardiac tissue engineering, and the high metabolic activity of cardiac cells.

Several important vascularization approaches for cardiac tissue engineering emerged, including the incorporation of angiogenic cells in the in vitro cultivation of engineered cardiac tissues [4, 72], the growth of cardiac tissues around an arteriovenous blood vessel loop [71], the prevascularization of engineered cardiac patches by transplanting them onto the blood vessel enriched omentum [36], and the implantation of thin cell sheets layer-by-layer in multiple surgeries to allow blood vessel infiltration over time [69]. However, it became immediately apparent that the use of cell-free biomaterials that support vascularization would be desired for cardiac repair. As such, I borrowed ideas from previous studies involving the development of angiogenic biomaterials, which were loaded or coupled with angiogenic factors (i.e. VEGF, basic fibroblast growth factor or platelet-derived growth factor) or contained microgrooves to improve perfusion and/or cell alignment, and applied them to vascularization in the cardiac tissue. In turn, the findings in my thesis, as described here, contribute to a further understanding of
vascularization using cell-free bioinstructive biomaterials, which can be applied to tissues other than the cardiac tissue.

In this thesis, I have developed two important controlled delivery systems of angiogenic factors for cardiac repair, and confirmed their effectiveness in inducing vascularization in vitro and in vivo. The systems are:

1) VEGF-conjugated collagen scaffolds for potential treatment of septal defects in children
2) Tβ4-encapsulated collagen-chitosan hydrogel for in vitro engineering of organized vascular bed and for potential treatment of myocardial infarction in adults

In the first aim, a collagen scaffold with covalently immobilized VEGF was created to be used for implantation in hearts with congenital heart defects. Scaffolds are suitable for treatment of congenital heart defects that often require surgical replacement of a full thickness defect. The VEGF-conjugated scaffolds increased the proliferation of endothelial and bone marrow cells that were cultured on the scaffolds in vitro. In vivo, the implantation of the VEGF-conjugated scaffolds to replace a full thickness defect of the right ventricular free wall showed enhanced cell recruitment, cell proliferation and vascularization compared to implantation of blank scaffolds. This in turn improved the formation of viable myogenic tissue and supported the integrity of the right ventricular free wall. Both in vitro and in vivo effects of conjugated VEGF were dose dependent with increased angiogenesis at a higher dose of VEGF.

In the second aim, a collagen-chitosan hydrogel with encapsulated Tβ4 was developed to be injected into cardiac infarcts after MI. Injectable hydrogels are suitable for minimally invasive administration into the infarct region post-MI for cardiac repair based on both mechanical and biochemical effects. The Tβ4-containing hydrogel was capable of controlled release of Tβ4 over 28 days with nearly zero-order kinetics from Day 3 to Day 28. The controlled delivery of Tβ4 induced the outgrowth of endothelial cells from mouse, rat and human vascular explants, which in turn formed capillary-like structures whose growth could be guided by topographical cues. By placing parent vascular explants on opposite sides of a Tβ4 gel-coated micropatterned substrate, a connected and perfusable microvascular bed was engineered based on the combined effects of topographical cues and localized release of an angiogenic peptide. In vivo, the injection of Tβ4-containing hydrogels into the rat cardiac infarct led to increased angiogenesis, protection of cardiac muscle tissue and attenuated left ventricular wall thinning necessary for cardiac repair.
Septal defects and myocardial infarction are the two most common cardiac diseases in children and adults respectively. In both systems described here, controlled delivery was used to enhance the bioactivity of the biomolecules compared to injection of soluble biomolecules, by improving their localized and sustained presence. In addition, this thesis focused on *in situ* cardiac repair using cell-free bioinstructive materials, which eliminates the need to find an appropriate cell source and expand large numbers of cells as in cases of cell transplantation or implantation of engineered cardiac tissues. While there were many previous studies involving the development of angiogenic biomaterials, the significance of my work is the application of these biomaterials in the heart. This involved the selection of biomaterials and biomolecules that are suitable for cardiac repair.

In the treatment of septal defects, the use of scaffolds was necessary to replace the full thickness defects in the heart. VEGF is the most potent factor in angiogenesis. While numerous studies have incorporated VEGF in biomaterials for controlled delivery, this is the first time that VEGF has been covalently immobilized to a scaffold for cardiac repair. Covalent immobilization of VEGF not only sustains its bioactivity, but also attracts infiltration of native vessels into the patch area due to localized bioactivity, thus accelerating the recellularization of the patch and its integration with the surrounding native tissue. The developed system here uses small amounts of VEGF to achieve angiogenesis, which is advantageous as excessive amounts of VEGF can lead to angioma formation.

Importantly, the bioactivity of VEGF was maintained after 28 days of scaffold aging *in vitro*, as demonstrated by the ability of aged VEGF scaffolds to promote cell proliferation. While it is expected that the degradation of the scaffolds occurs much more rapidly *in vivo*, in turn releasing soluble VEGF, this study demonstrates for the first time that growth factors have sustained bioactivity after a month if remained immobilized. This means that immobilized growth factors, not their release, can sufficiently and significantly promote vascularization. In addition, this motivates further controlling the biodegradability of biomaterials to prolong the presence of immobilized growth factors.

In the treatment of MI in adults, a hydrogel system was chosen to provide a non-invasive administration to the patient. Moreover, Tβ4 was selected as the biomolecule to be delivered because it is both angiogenic and cardioprotective, suitable for simultaneous vascular and cardiac
repair of the damaged heart post-MI. This is the first time that Tß4 has been reported to be released in a sustained manner using a controlled delivery system. As demonstrated here, the hydrogel could also be applied easily as a coating on micropatterned surfaces for the purpose of engineering vascularized cardiac tissues *in vitro*, which could then be transplanted to the heart post-MI. The hydrogel here can also be applied for the delivery of other molecules.

In addition, the application of the hydrogel onto microgrooved substrates and the study of capillary outgrowth and vascular organization in this system gave insights into the process of vascular repair. The finding that a combination of topographical cues and localized release of angiogenic peptide from hydrogel can lead to formation of an organized microvascular bed motivates the development of a fiber-containing hydrogel that can be injected *in vivo*. In the damaged heart, the arteries and veins are still present but with disordered microvascular structure. A hydrogel with aligned fibers can be injected in between to promote the growth of organized microvasculature through the combination of topographical cues from the incorporated fibers and angiogenic molecules released from the hydrogel.

The biomimetic approach to engineer a microvascular bed further allows the engineering of a nature-inspired vascularized cardiac tissue to be used as an *in vitro* model. The vascularized cardiac tissue can be subjected to ischemic conditions to mimic myocardial infarction. The envisioned outcome of this finding in the thesis is the use of an *in vitro* model of the vascularized cardiac tissue to create a mathematical model relating the parameters of angiogenesis (i.e. blood vessel density, diameter) to the level of cardiac regeneration (i.e. survival of cardiomyocytes, electrical signal propagation). This can lead to a standardized method to quantify and evaluate vascularization in cardiac repair.

Cardiac diseases are affecting large number of people around the world, in both developing and developed countries. Continual efforts to advance the development of simple bioinstructive biomaterials, particularly for vascularization, would be essential to provide cheap and effective treatment. The VEGF scaffolds and Tß4 hydrogels described in this thesis both showed feasibility in maintaining heart wall thickness critical for cardiac repair, by simply incorporating a single biomolecule. Importantly, these two biomaterials vary in function, and display utility for treatment of two common cardiac diseases.
Chapter 8

8 Recommendations for Future Work

In this thesis, I have developed two delivery systems for cardiovascular tissue engineering, including 1) a scaffold with covalently immobilized VEGF and 2) an injectable hydrogel with controlled release of Tβ4, which were both evaluated \textit{in vitro} and \textit{in vivo}. Future work includes 1) further characterization and optimization of the controlled delivery systems, 2) fundamental studies on the mechanism of capillary outgrowth and organization, as well as manipulation and use of the engineered microvascular bed that was grown on the Tβ4 gel, and 3) engineering of a vascularized human cardiac tissue.

8.1 Characterization and optimization of controlled delivery systems

While two controlled delivery systems were developed and evaluated in this thesis, further characterization will be required to confirm their safety and efficacy. Similar to many other angiogenic molecules, both VEGF and Tβ4 are angiogenic molecules that are linked to tumor growth. VEGF is expressed in human breast [294], colorectal [295] and prostate carcinomas [296], and is an important target for cancer therapies. Tβ4 was found overexpressed in human pancreatic cancer cells [297], colon carcinomas [298], and breast cancer cells [299]. However, the importance of angiogenic molecules to cell and tissue growth merits a further development of these controlled delivery systems, particularly to: 1) optimize the dose of the biomolecules at a given time point, 2) find other potential molecules for release from the developed delivery systems, 3) co-deliver multiple synergistic biomolecules, and 4) investigate the safety and effectiveness of the treatment with the controlled delivery systems in long-term \textit{in vivo} studies. Controlled and localized release of angiogenic molecules will be important for preventing potential side effects in comparison to systemic application.

8.2 Characterization, manipulation and utility of engineered microvascular bed

While the engineered microvascular bed in Chapter 5 was perfusable according to preliminary tests, it can be further characterized, manipulated and utilized in future studies.
In this thesis, the ability to perfuse the microvascular bed was merely tested by using a needle to manually inject fluorescently labelled dextran through the engineered vasculature. Perfusability was determined by the lack of leakage of the dextran from the capillaries of the microvascular bed during the injection. To more rigorously evaluate the perfusability and hemocompatibility of the microvascular bed, mouse whole blood or monocytes should be perfused using a micro-syringe pump at a suitable flow rate to mimic the native vasculature. The selection of the flow rate depends on the shear stress. The typical physiologic shear stress is 10-70 dynes/cm\(^2\) within arteries and 1-6 dynes/cm\(^2\) within veins [300]. Hemocompatibility needs to be further demonstrated by measuring monocyte attachment and cytokine release. As a first step to achieve continuous perfusion for the microvascular bed, a perfusion device (Figure 8-1) was designed to enclose the micropatterned substrate and to provide flow through the connection of the inlets and outlets to the parent vascular explants.

**Figure 8-1. Preliminary design of a perfusion device for long-term continuous perfusion of engineered microvascular bed.**

(A) The micropatterned substrate is placed inside a PDMS casing consisting of inlet and outlet connections via needles attached to tubings. (B) Vascular explants can be inserted through the needles, which are located directly on top of the micropatterned substrate such that the explants are in contact with the substrate.

Further, the mechanism by which combined topographical cues and release of Tβ4 led to the capillary outgrowth from vascular explants and the subsequent formation of an organized, perfusable vascular bed has not been thoroughly investigated. It was clear from the results in Chapter 5 that topographical cues enabled the formation of tubules with open lumens and the presence of Tβ4 accelerated the formation of the tubes. While it is clear that Tβ4 aided tube formation due to its angiogenic activity, there are three possible mechanisms by which
topographical cues could act to enable tube formation: 1) provide contact guidance, 2) change the local mechanical properties of the environment, and 3) locally increase the concentration of autocrine growth factors. It is highly likely that topographical cues provide contact guidance to the cell outgrowths from parent vascular explants, causing the endothelial cells to better align and elongate in the direction of the grooves and form tubes. Live microscopy will be necessary to track the growth of the capillaries to observe whether their growth is controlled by the speed at which the extending tip makes contact with the groove wall. The fabrication of curved features on the PDMS substrates may also give insights on how the grooves are guiding the growth of the capillaries. Topographical cues may have changed the local mechanical properties of the Tβ4 gel coating since the coating was only 8μm in thickness above the ridges but ~30-65μm in thickness within the grooves. Smooth PDMS substrates with different coating thicknesses will be required to study the effect of hydrogel thickness on cell outgrowth and tube formation. Atomic force microscopy measurements can be performed to determine substrate stiffness and the effects of grooves on modulating mechanical properties. In this thesis, it was found that there was an increased VEGF concentration in the culture medium when cultivating vascular explants on Tβ4-containing gels compared to Tβ4-free gels. To determine if there was a local increase in the concentration of autocrine growth factors such as VEGF due to the presence of grooves, diffusion of these molecules should be modelled using simulation software such as COMSOL Multiphysics.

The physiologic level of shear stress is necessary for the stabilization and healthy remodeling of blood vessels [300]. In future studies, the endothelial cell outgrowths from vascular explants can be cultivated under long-term flow to improve barrier function of the resulting vasculature. By connecting the parent vascular explants to flow during cultivation, it may be possible to influence the endothelial cell phenotype. Vasculature cultivated under no, low and high flow can be compared to determine the desired flow rate for the specific application (i.e. engineering of vasculature with quiescent or activated phenotype). Quiescent phenotype, as characterized by low platelet aggregation, low cell proliferation and apoptosis, and increased cell alignment, can be achieved under laminar blood flow with high shear stress [300]. On the other hand, activated phenotype can be achieved under turbulent blood flow with low shear stress. The activated vasculature has high platelet aggregation and leukocyte adhesion, as well as increased cell proliferation and apoptosis.
In this study, perfusion was not applied during the cultivation of cardiomyocytes. Although the growth of cardiomyocytes on the engineered microvascular bed improved the morphology and functionality of the resulting cardiac tissue, the improvements were due only to the ability of endothelial cells to promote cardiomyocyte survival and organization [301]. In co-culture of endothelial cells and cardiomyocytes, cardiomyocytes positioned themselves on the outside of tubes formed by endothelial cells [259]. Endothelial cells also reduced cardiomyocyte apoptosis and promoted the synthesis of Connexin-43, a gap junction protein of cardiomyocytes [259]. The direct perfusion of cardiomyocytes cultivated in scaffolds has been used previously to improve cardiac survival and function [12, 237]. However, direct perfusion can expose cardiomyocytes to hydrodynamic shear that lowers their functionality. Scaffolds with parallel array of channels were developed to protect cardiomyocytes from this non-physiologic condition [23, 302]. In nature, every cardiomyocyte is positioned between two capillaries and the endothelial cells are exposed to capillary blood flow. The perfusion of the vascularized engineered cardiac tissue in Chapter 5 with a flow of culture medium through the microvascular bed can better mimic the conditions of the native myocardium.

The engineered microvascular bed can also be used as a model system to study vascular diseases. It can be exposed to different shear stresses and other physiological conditions to create vascular disease models for both an understanding of the diseases and further use as an in vitro drug screening model.

8.3 Vascularized human engineered cardiac tissue

The ultimate goal in cardiac tissue engineering is to engineer a functional human tissue replacement to be implanted for cardiac repair. A necessary step to achieve this tissue replacement is to use human cardiomyocytes as the cell source. Human induced pluripotent stem cell (iPSC)-derived cardiomyocytes are a viable cell source for this purpose. A standardized and scalable process has been recently developed by Cellular Dynamics to manufacture human iPSC-derived cardiomyocytes, making large numbers of human cardiomyocytes more easily accessible for various applications.

In this thesis, human microvascular bed was engineered by inducing capillary outgrowths from human umbilical arteries and veins. In future studies, human iPSC-derived cardiomyocytes can be seeded at a high cell density of $10^8$ cells/cm$^3$ onto the human microvascular bed and cultured
for additional 7 days as was done for mouse vascularized cardiac tissues in this thesis. As a result, a vascularized human cardiac tissue can be engineered, resembling the native myocardium which consists of cardiomyocytes arranged between capillaries. Electrical field stimulation can be used to attain cardiac tissues with improved contractility.

In addition, the human engineered cardiac tissue can be used as an *in vitro* MI model. To mimic ischemic injury, the engineered tissue can be incubated in a hypoxia chamber. There are many applications for this *in vitro* model. For example, it can be used to screen potential angiogenic biomolecules, and study the interactive mechanisms of key angiogenic molecules. It can also be used to determine the level of angiogenesis required to regain proper cardiac function after MI. As well, potential biomaterials for cardiac repair can be tested using this *in vitro* model prior to preclinical studies.
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