Synthesis and Characterization of Tissue-Engineered Collagen Hydrogels for the Delivery of Therapeutic Cells

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Abstract

The expanding field of tissue engineering provides a new approach to regenerative medicine for common ailments such as cardiovascular disease and type-I diabetes. Biomaterials can be administered as a delivery vehicle to introduce therapeutic cells to sites of damaged or diseased tissue. A specific class of biomaterials, termed hydrogels, is suitable for this application as they can provide a biocompatible, biodegradable scaffold that mimics the physical properties of the native soft tissue.

Injectable hydrogels are increasingly being developed for biomedical applications due to their ability to be delivered in a minimally invasive manner. One potential use for such materials is in the delivery of therapeutics such as cells or growth factor-releasing particles. In this study, the first aim was to determine the interactive effects between collagen-based hydrogels and additives (cells and microspheres) for cardiac regeneration. The results demonstrated that the addition of either cells or microspheres to a collagen-based hydrogel decreased its gelation time and increased its viscosity. Increased cross-linker concentrations resulted in lower cell viability. However, this cell loss could be minimized by delivering cells with the cross-linker neutralizing agent, glycine.

As a potential application of these materials, the second aim of this study was to develop a hydrogel for use as an ectopic islet transplant site. Specifically, collagen-chitosan hydrogels were synthesized and characterized, with and without laminin, and tested for their ability to support angiogenic and islet cell survival and function. Matrices synthesized with lower chitosan content (20:1 collagen:chitosan) displayed greater cell compatibility for both angiogenic cells and for islets and weaker mechanical properties, while matrices with higher chitosan content (10:1 collagen:chitosan) had the opposite

effect. Laminin did not affect the physical properties of the matrices, but did improve angiogenic cell and islet survival and function. Overall the proposed collagen-based hydrogels can be tailored to meet the physical property requirements for cardiac and islet tissue engineering applications and demonstrated promising cell support capabilities.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
AM	Acetomethoxy
ANOVA	Analysis of variance
APC	Allophycocyanin
BCA	Bicinchoninic acid
BM	Basement membrane
BSA	Bovine serum albumin
CAC	Circulating angiogenic cell
CAD	Coronary artery disease
CD	Cluster of differentiation
CPD	Critical point drying
CVD	Cardiovascular disease
E	Elastic modulus
EBM	Endothelial basal medium
ECD	Electron coupled dye
ECM	Extracellular matrix
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
G'	Storage modulus
G''	Loss modulus
GAG	Glycosaminoglycan
HA	Hydroxyapatite
HBSS	Hank's buffered salt solution
Hz	Hertz
IBMX	1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione
IE	Islet equivalents
IgG	Immunoglobulin G
LM	Laminin
MES	2(-N-morpholino) ethanesulfonic acid
MI	Myocardial infarction
mL	milliliter
NHS	N-hydroxysuccinimide
nm	Nanometer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PECy7	Phycoerythrin-Cy7
PEG	Poly(ethylene glycol)
PGA	Poly(glygolic acid)
PLA	Poly(lactic acid)

PLGA	Poly(lactic-co-glycolic acid)
RGD	Argenine-glycine-asparatic acid
RIPA	Radio-Immunoprecipitation Assay
SAS	Statistical analysis system
SD	Standard deviation
SDF-1	Stromal-cell derived factor 1
SEM	Scanning electron microscopy
3D	Three dimensional
TMB	3,3′,5,5′-tetramethylbenzidine
U	Units
μg	Microgram
μL	Microliter
VEGFR2	Vascular endothelial growth factor receptor 2

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Statement of Contribution

Dr. Erik Suuronen developed the concept and the design of both studies. For the first study (Synthesis and Characterization of a Collagen-Based Hydrogel for Cardiac Regeneration) Dr. Chao Deng developed the matrix formulation used. Drew Kuraitis synthesized the alginate microspheres. Branka Vulesevic ran the flow cytometry and imaged the circulating angiogenic cells using the confocal microscope. Dr. Donna Padavan aided with analysis of the rheological properties and the chemical pathway.

For the second study (Synthesis and Characterization of a Collagen-Based Hydrogel for Delivery of Insulin-Producing Tissue) Dr. Chao Deng developed the matrix formulation used. Cara Ellis from the University of Alberta Diabetes Institute isolated neonatal porcine islets. Dr. Donna Padavan performed the mechanical testing, water contact angle testing, imaged the matrices using scanning electron microscopy and aided with the degradation studies. Branka Vulesevic ran the flow cytometry and Dr. Joanne McBane ran the insulin ELISA and BCA. The following facilities were used for experimental testing: University of Western Ontario for the use of their Instron Mechanical Tester, Carleton University for their scanning electron microcopy facilities and the University of Ottawa for their contact angle equipment. All other experiments were conducted at the University of Ottawa Heart Institute.

CHAPTER 1: INTRODUCTION

1.1 Overview

A major shortcoming of tissue engineering for regeneration of damaged or diseased tissue is the lack of vasculature [1]. Formation of vasculature is essential for the treatment of cardiovascular disease and diabetes [2]. Cardiac tissue is not capable of self-healing and requires intervention to stimulate regeneration of ischemic myocardium. Diabetes is an endocrine disorder leading to hindered insulin secretion in response to elevated glucose levels. A complication associated with diabetes is dysfunctional neovascularization [3]. Furthermore, current islet transplantation strategies are only moderately effective, in part due to poor revascularization of the cells [4].

Cardiovascular disease and type-I diabetes require strategies targeted at regenerating vasculature in order to achieve successful treatments. Such enhancements may include angiogenic cell delivery via biomaterial scaffolds harnessing natural extracellular matrix (ECM) components and mechanical properties of the native tissue to guide the therapeutic cells to stimulate vascularization [1].

1.2 Thesis Objectives

- To synthesize and optimize collagen-based hydrogels for the following applications:
    - the delivery of therapeutic angiogenic cells to restore function to ischemic myocardium for treatment of heart disease, herein referred to as cardiovascular injectable matrix
    - as a vehicle to transplant islet cells for the treatment of type-I diabetes, herein referred to as islet graft scaffold

- To characterize the hydrogels physical properties using various techniques:
    - Cardiovascular injectable matrix: rheology
    - Islet graft scaffold: rheology, mechanical testing, degradation, scanning electron microscopy, and water contact angle
- To assess the *in vitro* cell support capabilities of the hydrogels:
    - Cardiovascular injectable matrix: ability to support human circulating angiogenic cells (CACs) survival and phenotype
    - Islet graft scaffold: ability to support CAC survival and porcine islet cell survival and function (insulin secretion).

1.3 Organization of Thesis

The studies outlined in this thesis are focused on characterizing and optimizing collagen-based hydrogels for two different ailments, cardiovascular disease and type-I diabetes. This thesis is organized into five chapters. A detailed review of tissue engineering, biomaterials and applications for hydrogels are discussed. In Chapter 3, the methods, results, and discussion for the first study involving the synthesis and characterization of a cardiovascular injectable matrix are outlined. In Chapter 4, the methods, results, and discussion for the second study involving the synthesis and characterization of an islet graft scaffold are presented. Lastly, final conclusions, future work and recommendations are addressed.

CHAPTER 2: LITERATURE REVIEW

2.1 Tissue Engineering

The failure or impaired function of an organ or tissue places a large burden on the health care system worldwide [5]. Commonly, end-stage patients require organ transplantation from another person and often remain on a wait list while their condition worsens. The ability to improve treatment methods and intervene before the organ or tissue becomes further damaged would reduce the strain on the healthcare system and the need for organ transplantations.

Tissue engineering is a relatively new approach to regenerative medicine for the repair of damaged or diseased cells, tissues and organs. One tissue engineering approach relies on the use of a three-dimensional (3D) scaffold to direct tissue regeneration at the appropriate site and to provide a suitable environment for cells to survive and function [6]. The need for such interventions stems from the lack of suitable autologous or allogenic grafts. Graft inadequacies are due to the risk of disease transmission and immune rejection for allogenic sources and insufficient healthy donor tissue from autologous sources [7]. Tissue engineering brings forth the ability to implement treatment methods in a manner not reliant on autologous or allogenic grafts. Materials that mimic the healthy native tissue are used to aid the regeneration of the tissue and restore function to the damaged region. Future advances in the field of tissue engineering involve biodegradable polymers, drug and cell delivery, angiogenesis and biomimetic techniques [6].

2.2 Biomaterials

Biomaterials are utilized for numerous applications whereby synthetic and modified natural materials are coupled with biological systems [6]. Ultimately, biomaterials are used to control the microenvironment of the cells or tissue through optimization of nutrient and waste diffusion, delivery of bioactive molecules, mechanical properties, degradation rate, and cell-surface interactions [7]. The field of biomaterials is multidisciplinary drawing on expertise from chemistry, chemical engineering, biomechanics, mechanical engineering, material surface science, biology, bioengineering, ethics and government regulated medical standards [6, 8]. There are several classes of biomaterials including ceramics and metals (for hard tissue applications) and polymers, which are versatile in terms of altering the materials' mechanical properties. Polymers can be adapted to mimic physical properties of soft tissues (e.g. skin and organs) or harder tissues (e.g. tendons and cartilage) [5]. Additionally, there are two types of biomaterials: natural and synthetic, which are described in more detail in the sections that follow.

The choice of biomaterial for a particular application rests on many factors. The biomaterial must possess properties similar to that of the native tissue and be compatible with the structure and environment of the implantation site. Specifically, the material should mimic the mechanical properties of the target tissue and maintain similar porosity and microstructure. In addition, the material should provide a 3D environment with similar ECM components present in the tissue. The material should be biocompatible with little to no induction of host immune response [1, 6-9]. The degradability of materials is pertinent to tissue engineering as the matrices should support tissue re-growth

as the material degrades. Ideally, the tissue regeneration will match the material degradation rate and the material will not release toxic degradation products into the body [6, 9, 10]. Furthermore, vascularization of a transplanted biomaterial is crucial for the majority of applications to ensure successful support for the cells and tissue engraftment [11].

2.2.1 Natural Biomaterials

Natural biomaterials are comprised of materials purified from living animals such as collagen, chitin, chitosan, keratin, silk and elastin, and from plants, including starch, cellulose and pectin. Natural materials offer desirable characteristics including biodegradability and biocompatibility as their final degradation products materials are solely carbon dioxide and water [9, 12]. Furthermore, natural materials typically induce only mild (if any) inflammatory responses upon implantation [9]. Commonly used natural biomaterials incorporate native ECM components; collagen, fibrinogen, hyaluronic acid, glycosaminoglycan (GAGs) and hydroxyapatite (HA) into the 3D structure to ensure biocompatibility and mechanical properties similar to that of the target tissue [7, 13].

Disadvantages to natural biomaterials include inconsistencies between production methods as the purity of the final product depends largely on the isolation and processing technique. These discrepancies cause slight alterations to the mechanical properties and thus the response of the cells and tissue will be affected [9]. Furthermore, the mechanical properties of natural biomaterials are typically less amenable to applications where greater strength (i.e. yield, fatigue, tensile, compression, and impact) is required. To improve the mechanical integrity of the materials for such uses, cross-linking methods

are necessary. Careful consideration must be taken when cross-linking agents are introduced into the biomaterial network as they may directly affect the material and the cells it is designed to support.

2.2.2 Synthetic Biomaterials

Synthetic biomaterials are comprised of human-made materials. Synthetic materials have superior mechanical properties and thermal stability, which can be accurately fine-tuned to mimic native tissue. Altering the chemistry and synthesis techniques allows for precise management of the macroscopic architecture, including porosity, degradation rate and material stiffness. These properties are particularly important for cell support and functionality [6].

Common synthetic materials include polyethylene glycol (PEG), poly(lactic acid) (PLA), poly(glygolic acid) (PGA), poly(lactic-co-glycolic) acid (PLGA), and poly(caprolactone) (PCL). PEG is non-ionic biocompatible biomaterial capable of reducing immune response effects through resistance to protein adsorption and cell attachment [9]. PGA, PLA and PLGA are synthesized through a ring opening polymerization reaction with glycolide, lactide, or combination of the two, respectively. Varying ratios of each component in PLGA aids to tailor the physical properties of the synthetic polymer. The degradation products of these materials can be absorbed into the body, eliminating toxic effects [9]. PCL is synthesized via ring opening reaction of caprolactone and is a semi-crystalline polymer and provides biocompatibility on par with PLA and PGA [9].

Synthetic biomaterials are produced by controlled techniques eliminating variation by lot, which allows for synthesis of materials with consistent mechanical properties and microstructure [9]. While synthetic materials can be used to mimic the physical properties of the native tissue, they do not replicate the chemical and biological environment for cells to survive and function, thus limiting the therapeutic potential for cell delivery [6]. Another drawback of some synthetic biomaterial implants is the host response elicited by the immune system. When a biomaterial is introduced into the body the foreign body reaction occurs through a sequence of events involving protein adsorption, macrophage and neutrophil attachment to the surface, and eventually development of a giant cell formation, which produces a collagen capsule to wall the implant off from the body [6]. In addition, degradation products from synthetic materials may cause inflammatory responses [9].

2.3 Hydrogels

Hydrogels are attractive for soft tissue engineering applications as they are easily tailored to mimic native tissue properties. Hydrogels are insoluble polymer networks that are capable of swelling and retaining a considerable amount of water [6, 10]. Hydrogels are versatile in that they can be administered as an injectable matrix intended to solidify *in situ* [14-16] or they can be pre-formed and implanted as a solid scaffold [17]. Cross-linking hydrogels allows for tuning the physical properties of the material. The choice of cross-linker and its concentration will contribute to the mechanical properties of the hydrogel [6]. Several synthesis techniques are available for the preparation of hydrogels; the main classifications falling under the categories of chemically cross-linked or physically cross-linked hydrogels.

2.3.1 Chemically Cross-linked Hydrogels

Hydrogels can be chemically cross-linked using polymerization or chemical reactions. Polymerization reactions are more commonly used and can be initiated by irradiation via ultra- violet light, gamma-rays, x-rays or redox initiators [10]. Spatial and temporal control during hydrogel synthesis can be achieved by using photoinitiators in order to produce materials with a specific geometry. Redox initiators do not provide the same cross-linking precision but are generally less toxic [6].

Chemical reactions can be utilized to cross-link hydrogels with other functional groups, including hydroxy, carbonic, and amine groups [10]. The most common cross-linking agents include carbodiimide derivatives (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide; EDC/NHS), dialdehyde derivatives (glutaraldehyde and formaldehyde), carbonyldiimidazole, and disulfide derivatives [5, 9, 18, 19]. Glutaraldehyde is a well-known chemical cross-linker but has proven to be cytotoxic [20, 21]. EDC/NHS provides a zero-length cross-linking mechanism to chemically cross-link biomaterials without remaining in the polymer network. EDC activates carboxylic groups to form an intermediate ester before coupling the carboxylic group to an amine group. NHS acts to stabilize the intermediate to ensure the cross-linking reaction proceeds. The EDC and NHS molecules can be washed out of the hydrogel system after the reaction has reached completion [18]. EDC/NHS may provide a less toxic alternative for chemical cross-linking [21], particularly for pre-formed scaffolds materials.

2.3.2 Physically Cross-linked Hydrogels

Physically cross-linked hydrogels are of particular interest as they are synthesized without the use of toxic chemicals or initiators. This makes them particularly well-suited for cell biocompatibility and for delivery of sensitive materials such as cytokines [10]. Hydrogels can be physically cross-linked by ionic interactions, where anionic or cationic polymers are cross-linked to polymers of opposite charge. This technique is widely used for natural materials such as chitosan, alginate and hyaluronan [9, 10]. Biomaterials can also be synthesized using crystallization techniques where chains of the polymer network are crystallized and operate as cross-linking sites [10]. Another method of physical cross-linking is via hydrophobic interactions to form self-assembled block and graft co-polymers. These materials are characterized as having a water-soluble backbone attached to hydrophobic chains with hydrophilic moieties [9, 10].

2.3.3 Collagen-Based Hydrogels for Vascular Repair

Natural hydrogels containing materials such as collagen, chitosan and glycosaminoglycans (GAGs), such as chondroitin sulfate, are promising for tissue engineering applications as they provide a biocompatible environment, which can be tailored to mimic the physical properties of the native tissue [7, 13]. Collagen serves as a suitable base for hydrogel synthesis as it is the most abundant structural protein in the human body and provides a porous material on which many cell types adhere to [16, 22]. Specifically type-I collagen attributes tensile strength to skin and internal organs [12]. Chitosan is a biodegradable, antimicrobial cationic polysaccharide, readily found in shells of crustaceans. It is widely used for biomedical applications including tissue engineering, drug delivery and gene therapy [12, 23]. It was previously demonstrated the addition of

chitosan to collagen hydrogels improves the angiogenic potential of the matrix [17]. Chondroitin sulfate largely contributes to the mechanical integrity of the material, particularly under compressive loading [22, 24, 25].

CACs can further enhance angiogenesis in ischemic tissue when introduced in a collagen-based matrix [26, 27]. CACs have the potential to vascularize the islet transplant site to aid in islet survival and function upon transplantation. It has been previously reported that increasing chitosan content in collagen-chitosan based matrices improves the mechanical integrity of the hydrogel and improves CAC differentiation and angiogenesis [17] . Moreover, it was also shown in the first study of this thesis work that CAC viability can be enhanced when introduced into a collagen-matrix in media supplemented with glycine prior to thermogelation. Glycine shields CACs from the toxic effects during the chemical cross-linking reaction by coupling with the carbodiimide [14].

2.4 Cardiovascular Disease and Regenerative Therapies

2.4.1 Cardiovascular Disease (CVD)

Cardiovascular disease (CVD) is the leading cause of death in the developed world even with current improvements to surgical techniques and pharmaceutical treatments [9], which prolong life, but do not biologically address the heart's functional deficit. The build-up of plaque on the artery walls of the heart causing a narrowing is referred to as coronary artery disease (CAD). The reduction in blood flow can ultimately lead to myocardial infarction (MI). MI results in damage to non-healing cardiac tissue: specifically, heart muscle has a very limited ability to regenerate. As the body attempts to heal, granulation tissue is deposited by macrophages forming a scar [16]. The lack of a

sufficient number of organ donors severely limits the option of heart transplantation for end-stage patients. This necessitates the development of alternative treatment options, including tissue engineering to restore damaged or diseased myocardium [9].

2.4.2 Cardiovascular Tissue Engineering

The application of cells with regenerative capabilities to repair and/or replace tissue and restore function is being intensively investigated for the treatment of heart disease. However, current efforts to promote regeneration in ischemic and/or infarcted tissue by using stem or progenitor cells have been modestly effective, due in part to low cell retention and survival [28, 29]. For several reasons, including inflammation, scar tissue and a lack of blood supply, the ischemic myocardium is an undesirable environment for cell transplantation [28, 30, 31]. Therefore, cell therapies for cardiac disease will benefit from the development of innovative strategies to enhance the therapeutic potential of the delivered cells.

Injectable matrices are attractive vehicles for the delivery of cells to damaged or diseased tissue. These materials are aqueous prior to injection and can be engineered to solidify *in situ* once exposed to physiological temperature, pH or light [32]. Injectable matrices form to the shape and size of any cavity and can be designed to provide an environment for the adhesion, differentiation and function of therapeutic cells [33]. A collagen type-I based matrix is promising for cardiac tissue engineering due to its biocompatible and biodegradable characteristics, and also to complement the heart's extracellular matrix, which is composed primarily of collagens [26, 34].

Despite its promise, there are considerations that need to be made in developing such a matrix for application in the heart (or other tissues). For example, control of the final physical properties of the hydrogel is required which can be easily manipulated by altering cross-linking methods [35]. EDC/NHS has been reported to be a suitable candidate cross-linker because of the minimal toxicity of its breakdown products, and its ability to regulate the physical properties of collagen hydrogels [20, 36]. While EDC/NHS can be washed out the polymer system after the cross-linking reaction, it can be toxic to cells in its unreacted form [18, 37], and methods are needed to inhibit or minimize these effects if EDC/NHS cross-linked hydrogels are to be used as *in situ* gelation materials for cell delivery.

The effects of altering the cross-linking methods can be monitored by the materials' rheological behaviour prior to, during, and after thermogelation. This behaviour is indicative of the time a surgeon would have to handle the material prior to injection, as well as how long the material will take to solidify at physiological temperatures *in situ*. It is desirable for the material to solidify quickly after injection, in order to minimize the dissipation of the material from the injection site. This is particularly relevant in the heart, where the contractile beating forces may limit the retention of any delivered therapy. The viscosity of the hydrogel formed is of interest, as it has been shown that the degradation rate of collagen-based matrices is inversely related to its viscosity [38]. In addition, changes in collagen matrix stiffness can induce differences in vessel formation [2, 39], thus potentially playing a role in the efficacy of the therapy. While the rheological properties of *in situ* gelling hydrogels are routinely characterized, it is seldom reported how these properties (gelation in particular) are

affected by the addition of the therapeutic agent (e.g. cells, nano- or micro- particles) they are designed to deliver.

It has previously been reported that the use of injectable matrices for the delivery of therapeutic cells enhanced the retention of the transplanted cells and function of the target tissue [27, 40]. The possibility of further enhancing the therapeutic benefit of such matrices may come from the addition of nano- or micro-particles for the delivery and/or release of cells and cytokines/growth factors [41]. For cardiac tissue regeneration, delivery of growth factors, such as stromal cell-derived factor-1 (SDF-1) and vascular endothelial growth factor (VEGF), can augment the recruitment of endogenous progenitor cells thus improving the efficacy of the therapy [22]. Several reports describe the effective use of such encapsulation strategies for regenerative medicine applications (for review see [42-44]). Despite these promising results, improvements in cell survival and function are still desirable, and the effect that cells and delivery particles have on the properties of the injectable matrix are not well-characterized. The effect each additive has on the original materials' properties must be fully characterized, as well as the corresponding cell-material response in order to develop an ideal matrix. Cells respond to chemical, physical and biological cues and changes to these components of the hydrogel can significantly alter the cell-material interactions directly affecting the cell survival and function. Alternately, cells and other additives may modify the physical and chemical properties of the material to the extent where the material no longer meets the desired characteristics of cardiac tissue. Once the effects of additives on the matrix are fully characterized, the material that is capable of supporting the cells (or other deliverables),

whilst maintaining the desirable physical properties, can be selected for the specific application, in this case as a cardiovascular injectable matrix.

A version of this section has been published in 'Tunable Collagen Hydrogels are Modified by the Therapeutic Agents they are Designed to Deliver'. Journal of Biomaterials Science- Polymer Edition. 2012 (See Appendix A for copyright permission).

2.5 Type-I Diabetes and Regenerative Therapies

2.5.1 The Endocrine Function of the Pancreas

The pancreas is a complex organ containing heterogeneous clusters of cells referred to as islets of Langerhans as depicted in Figure 1 [45]. These clusters contain several cell types including beta cells, alpha cells, delta cells and polypeptide producing cells. Approximately 75% of islets are comprised of beta cells, 20% alpha cells, with delta cells contributing the majority of the remaining cells, and a very small percentage are polypeptide producing cells [46, 47].

The pancreas has two main roles, an exocrine and an endocrine function, [45], as outlined in Figure 1. The exocrine function is responsible for excreting metabolic enzymes, which is not affected by type-I diabetes.

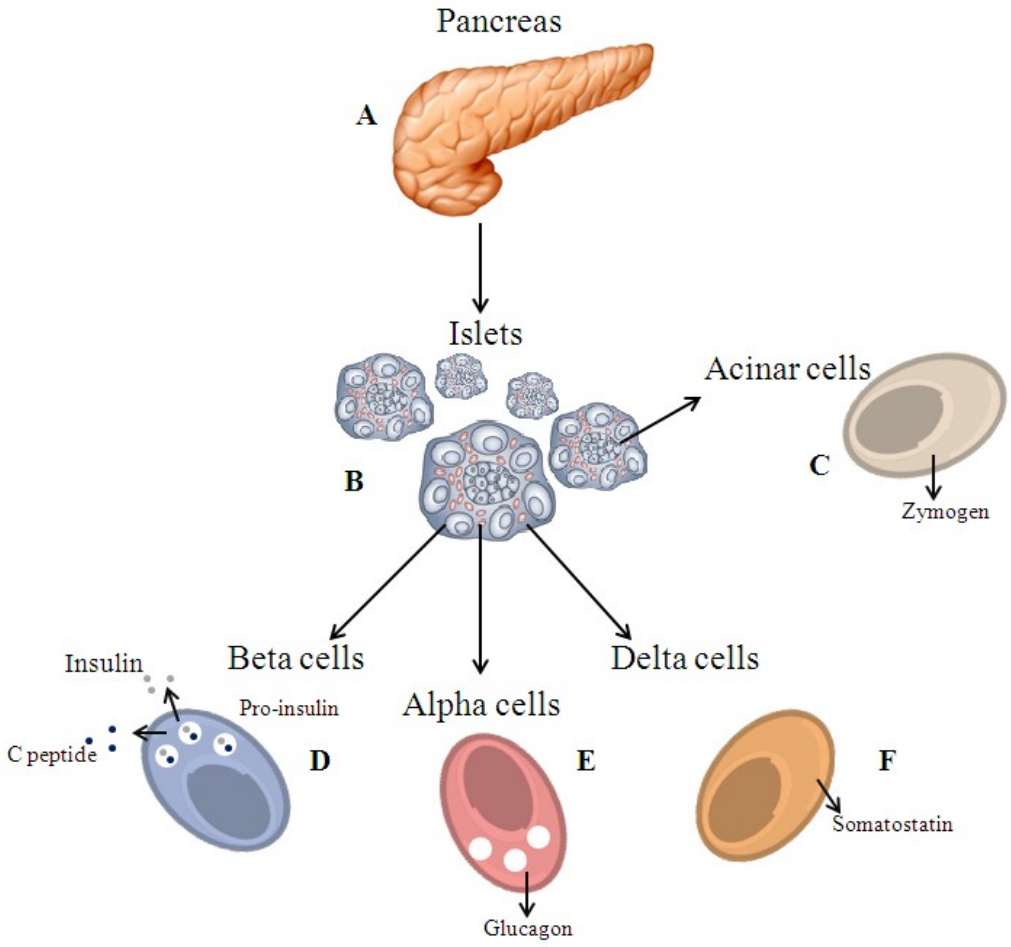

Figure 1: Exocrine and endocrine function of the pancreas [45]

(A) In the human body, the pancreas is located under the liver, behind the stomach and above the small intestines. The pancreas connects to the duodenum, (the first segment of the small intestine) via the pancreatic duct where exocrine pancreatic enzymes are released into the small intestine to facilitate digestion. (B) The pancreas is comprised of islets, a heterogeneous cell population, which contribute to the exocrine function (acinar cells) and the endocrine function (beta cells, alpha cells and delta cells). These cells are native to a highly vascularized environment within the pancreas. (C) Acinar cells are responsible for secreting digestion enzymes such as zymogen (a proenzyme). (D) Beta cells release the hormone insulin in conditions of high blood glucose levels. Insulin is produced by cleaving pro-insulin within the cell prior to being excreted by the cell. (E) Alpha cells release the hormone glucagon in conditions of low blood glucose levels. (F) Delta cells produce the enzyme somatostatin which aids in the regulation of insulin and glucagon production.

The endocrine function of the pancreas is associated with the secretion of hormones from islet cells, namely insulin and glucagon. Islet cells operate as neuroendocrine organs to maintain homeostatic control of metabolism, predominantly through stabilization of blood glucose levels [47]. These hormones play a key role in the digestion of protein, carbohydrates and lipids. Insulin, an anabolic hormone, enhances the uptake of glucose, fatty acids and amino acids into cells and tissues, while glucagon, a catabolic hormone, augments the release of these compounds from storage into the bloodstream [48].

Beta cells produce pro-insulin, a non-functional precursor to insulin. As shown in Figure 1, pro-insulin is cleaved within the cell to form insulin fragments, A and B chains, and C-peptide via the enzyme kallikrien. The remaining A and B chains link via disulfide bonds to form an insulin molecule in its functional form. The C-peptide and insulin are then secreted from the cell [45, 48].

2.5.2 Diabetes Mellitus

Blood glucose concentration is sustained within a biological healthy range through secretion of insulin from beta cells. Diabetics have hindered beta cell function and are unable to naturally regulate glucose levels in the blood. Diabetes is prevalent in North America and causes a greater risk of developing additional detrimental complications including heart disease, stroke, kidney disease, blindness, and amputation [49]. There are two types of diabetes: type-I (also referred to as insulin-dependent or juvenile diabetes) and type-II (also termed non-insulin dependent or adult-onset diabetes). In Canada alone, nine million people have diabetes, 10% of whom have type-I diabetes [50].

Type-I diabetes, is characterized by the autoimmune destruction of beta cells leading to the inability to produce insulin to control blood glucose levels [51, 52]. Islet cells are attacked by the host's immune system which leaves the islets compromised [51]. Type-I diabetics must monitor their blood glucose concentration and self inject insulin three to four times daily to maintain levels within a healthy range [46]. Type-II diabetes is characterized by the inability to produce insulin in sufficient amounts or the inability to properly utilize the insulin that the pancreas secretes. This type of diabetes is diagnosed more commonly in adults; especially those who make unhealthy lifestyle choices including smoking, inactivity, and poor diet. Current treatment methods include oral medication to stimulate beta cell function and occasionally insulin is administered [53]. In addition to medication, dietary control and exercise are crucial factors used to manage this disorder.

2.5.3 Current Islet Transplantation Methods

Since Study 2 specifically targets treatment for type-I diabetes, it will be the focus from this point forward. Type-I diabetes is characterized by destruction of islet cell function. Transplantation of functional islet cells provides a means to restore natural glucose regulation to the patient. Ideally, this treatment method would eliminate the need for daily insulin injections.

2.5.3.1 The Edmonton Protocol

Islet transplantation therapy aims to deliver donor islets to restore insulin secretion and regulate normal blood glucose levels in type-I diabetics. An early islet transplantation effort involved transplanting porcine pancreatic tissue; the tissue was able to survive for

several months, but patients were still reliant on insulin injections to monitor glucose levels [54]. Current transplantation methods procure the transplant islets from human cadaveric donors. The Edmonton Protocol was developed in the year 2000 and is seen as the gold standard of islet transplantation as it greatly improved upon previous transplantation methods. Throughout the ten years preceding the development of the Edmonton Protocol, islet transplantation methods were coupled with immunosupression, using antilymphocyte globulin along with cyclosporine, azathioprine and glucocoricoids. The earlier treatment technique achieved only 8% insulin independence at one year post transplant [55].

The Edmonton Protocol introduced a novel glucocoricoid-free immunosupression technique to circumvent glucocoricoid's toxicity to beta cells and its ability to enhance insulin resistance. Participants with type-I diabetes with severe hypoglycemia were selected and administered immunosuppression medication prior to surgery. Beta cells were transplanted via the portal vein at a concentration of 4000 islet equivalents (IE) per kilogram of patients' body weight. Antibiotics and vitamin supplements were administered following beta cell transplantation from cadaveric donors, coupled with further doses of immunosuppression medication [55]. Treatments were successful at achieving insulin independence after islet transplantation, which significantly reduced blood glucose level fluctuations. Overall, islet transplantation provides glycemic and metabolic control, diminishing the need for exogenous insulin over time [55].

Further research into the Edmonton Protocol focused on improving control of labile diabetes symptoms. Labile diabetes is characterized by rapid changes from one blood glucose level extreme to the other and often causes hypoglycemia, low blood

glucose levels. Ryan *et al*. transplanted islets into 15 labile diabetic patients. One year post transplant, 80% of participants remained insulin independent. Glucose levels were controlled and glycemic lability and hypoglycemic complications were resolved in 93% of transplant recipients. Six participants later required insulin therapy, three of whom continued to show stable blood glucose levels [56, 57].

2.5.4 Islet Tissue Engineering

Only 15% of islet transplant recipients remain insulin independent at five years post-transplant [4]. The decline in insulin independence rates seen in clinical islet transplantation is currently not fully characterized, but is likely complex. Detrimental factors include recurrent beta cell autoimmunity, allograft rejection, metabolic exhaustion, chronic islet toxicity of immunosuppressive drugs, poor revascularization of the islets, and moreover the limitations of the intra-portal site [58, 59]. Transplantation of islets into the portal vein has been associated with life-threatening intraperitoneal bleeding [60], portal vein thrombosis and hepatic steatosis [61, 62]. The liver may also contribute to the gradual attrition of chronic islet graft function [61]. Therefore, the search for an alternative, safer site for islet transplantation is an important issue to address. Some sites of interest include skeletal muscle, the peritoneal cavity and the omentum [63]. As described in the sections to follow, several bioengineered cell-based, implantable material strategies are under development for the long-term treatment of diabetes.

2.5.4.1 Mircoencapsulation

Microencapsulation is a strategy to provide a barrier between implanted islets and the host. Transplanted cells are separated from the surrounding host environment and protected from the immune response by a spherical semi-selective membrane [64]. Calcium alginate microspheres have been used to encapsulate islet cells. The encapsulated microenvironment was coated with a semi-permeable membrane, poly(L-lysine) or barium chloride, and implanted in the body [65, 66]. The semi-permeable membrane allows for nutrient and waste diffusion, while limiting the entrance of antibodies or other host immune cells [66], which is a major problem with islet transplantations.

As the human donor supply of islets will not be sustainable for the required diabetes treatments, xenogeneic sources are of interest. Microencapsulation has been utilized for xenogeneic islet transplantation. Porcine islets were used for treatments for type-I diabetes induced in rats using streptozotozin to destroy beta cell function. Islet cells were isolated from porcine sources and added to a calcium alginate solution to form microcapsules by air-jet methods, then complexed with barium chloride [66, 67]. Barium-complexed alginate beads demonstrate a negligible immune response after implanting empty beads in a rodent model [65] .

While islet microencapsulation offers a successful strategy to reduce immune attacks to transplanted islets, it is not entirely successful in that the membranes are not perfectly semi-selective and can allow a small quantity of immunomolecules to enter the capsule. Furthermore, antigens from the encapsulated cells' surface can be released upon cell death and leak out of capsule causing a local immune response at the transplant site

[64]. Another shortcoming of islet encapsulation is that it does not aid to re-establish the vasculature and cell-ECM interactions lost during islet isolation, which is essential for their survival and function.

2.5.4.2 Hydrogel Scaffolds

Hydrogels are attractive for the delivery of islet cells for the treatment of type-I diabetes, as they have the potential to provide a desirable environment, similar to native tissue, for cells to survive and be retained *in situ.* Introducing islets into a hydrogel containing native stromal ECM and peripheral basement membrane (BM) components will aid to restore cell-ECM interactions lost during cell isolation via enzymatic digestion [68-71]. The primary mechanism of islet cell death post-isolation is via anoikis due to the harsh detaching from the ECM. Islets express integrins that are capable of binding to argenine-glycine-asparatic acid (RGD) sequences on ECM components such as collagen type-I and laminin [51, 68, 72, 73]. Laminin is one the most abundant BM proteins in islet cells' *in situ* environment [74] and has been shown to enhance insulin secretion and glucose responsiveness of islet cells [75-77]. Laminin also functions to stimulate angiogenesis and endothelial cell differentiation and aid in development of new blood vessels *in vivo* [22].

Physical properties of the hydrogel cell delivery vehicle are crucial factors contributing to cellular response to the material, affecting the survival, function, differentiation and adhesion of CACs and islets [18, 78]. Cells respond not only to their biological and chemical environment, but also to their physical surroundings [11]. Several studies demonstrate a relationship between collagen matrix stiffness and cell response inducing vessel formation [2, 11, 79]. Embedding islets into a 3D environment

that promotes vascularization, and supports islet survival and function constitutes a promising technique to develop a successful islet graft, which is the second aim of this thesis.

CHAPTER 3: STUDY I Synthesis and Characterization of a Collagen-Based Hydrogel for Cardiac Regeneration

3.1 Study I: Materials and Methods

3.1.1 Preparation of Cardiovascular Injectable Hydrogel

As previously determined [80], a cross-linking mixture containing 0.1M 2(-N-morpholino) ethanesulfonic acid (MES) buffer (pH~6.0; EMD Chemicals, Gibbstown, NJ) and a 1:1 molar ratio of EDC/NHS (13mM; Sigma) was prepared and added to 1% porcine type-I collagen (w/v; Nippon Ham, Tskuba, Japan) in a glass centrifuge tube on ice. The cross-linked collagen solution was diluted with PBS (Sigma) and 20% chondroitin sulfate-C in Ca^{2+}/Mg^{2+} free PBS (Wako, Osaka, Japan) was added. Lastly, the pH was adjusted to 7.2±0.2 using NaOH, and matrices were thermogelled for 30 minutes at 37°C. This matrix mix containing 13mM cross-linker concentration was considered the standard to which others were compared. To examine the effect of cross-linker concentration, the matrix mixture was prepared by this same protocol, but with the addition of 6.5mM, 13mM or 26mM of the 1:1 molar ratio EDC/NHS cross-linking solution. Similarly, for investigating cell concentration effects, 2.5×10^5, 5.0×10^5 or 1.0×10^6 CACs were added per 500µL of standard matrix solution (13mM EDC/NHS).

3.1.2 Preparation of Cardiovascular Injectable Hydrogel Containing Alginate Microspheres

Blank alginate microspheres, with an average diameter of 38.5 µm were created as previously described [81], by forcing 1.25% sodium alginate (Sigma) through a J1 Encapsulation Device (Nisco, Brampton, Canada) at 1mL/minute, with 9.8L/min nitrogen gas. Through a physical cross-linking reaction, microspheres were formed in 2% $CaCl_2$ for 20 min, then washed in PBS and stored at -80°C until further use. Collagen matrix was prepared following the same protocol as described above (section 3.1.1), except

400μL of PBS containing 0.4g of microspheres was added to 1% porcine type-I collagen following the addition of the cross-linking solution (13mM).

3.1.3 Preparation of Cardiovascular Injectable Hydrogel for Circulating Angiogenic Cell Viability Assessment

CACs (in PBS) were incorporated into the collagen hydrogel prior to thermogelation. Matrices with cell densities of 2.5×10^5, 5.0×10^5, and 1.0×10^6 cells per 500μL of matrix were prepared with a 13mM cross-linker concentration. Matrices with cross-linker concentrations of 6.5mM, 13mM and 26mM were prepared with 5.0×10^5 cells per 500μL of matrix. In a 24-well plate, 500μL of each matrix was added and thermogelled at 37°C for 30 min. After gelation, 1 mL of endothelial basal medium (EBM) was added on top of each matrix and the plate was incubated for 24 hours at 37°C. For testing the effects of glycine on cell viability, matrices with a cross-linker concentration of 13mM were prepared as above with 5.0×10^5 CACs suspended in a solution of 0.15M glycine (Sigma) or PBS per 500μL matrix.

3.1.4 Isolation and Culture of Circulating Angiogenic Cells

Procedures for the isolation of human CACs were approved by the Human Research Ethics Board of the University of Ottawa Heart Institute. Total peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy human volunteers by Histopaque 1077 (Sigma, Oakville, Canada) density-gradient centrifugation, as previously described [82]. Cells were cultured on fibronectin-coated plates (2×10^{-5} g/plate) in EBM (Clonetics, Guelph, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics). After four days of culture, the adherent population was lifted from plates with phosphate buffered saline (PBS). Cell count and viability were

determined using a Vi-Cell counter (Beckman Coulter, Mississauga, Canada) by the Trypan blue exclusion method prior to embedding in matrices, and this was considered as the baseline viability.

3.1.5 Circulating Angiogenic Cell Live/Dead Viability Assay

A live/dead assay was used to simultaneously assess for viable and non-viable CACs after 24 hours using a live/dead cell staining kit (Invitrogen, Burlington, Canada). Calcein-AM (green) and ethidium homodimer-1 (red) were used to simultaneously stain for viable and non-viable cells, respectively. Sections were examined using a 20× objective on an Olympus IX80 laser scanning confocal microscope operated by FV1000 software v1.4a. Two independent counters quantified the images and the values were averaged. Green cells were considered alive, while red were considered dead and yellow as apoptotic.

3.1.6 Circulating Angiogenic Cell Flow Cytometry Phenotype Analysis for Cardiovascular Injectable Hydrogel

The phenotype of surviving cells 24 hours after seeding (in glycine) and gelation within the matrix was evaluated. Matrices were digested with 1% collagenase type-I (Invitrogen) for 1 hour at 37°C, and filtered to collect the cells. Cells were rinsed and counted using a Vi-Cell analyzer (Beckman Coulter). Cells (3×10^5) in 200µl of EBM were then stained for 30 min at 4ºC with antibodies against the following antigens: CD31-FITC (Beckman Coulter), CD34-PECy7 (BD Biosciences, Mississauga, Canada), CD133-APC (Miltenyi Biotec, Auburn, USA), VEGFR2-PE (R&D Systems, Minneapolis, USA) and L-selectin-ECD (Beckman). Samples were also stained with appropriate IgG isotype-matched controls. Immediately prior to analysis, the viability stain 7-actinomycin D (7-AAD)

(Invitrogen) was added to samples to a final concentration of 8.3μg/ml. Cells were analyzed and quantified with a BD FACSAria cell sorter (BD Biosciences).

3.1.7 Rheological Characterization

The rheological properties of the collagen-based matrix were measured using a Brookfield R/S Plus Rheometer as previously described [17]. Each sample (1.5mL) was subjected to a constant shear rate of 5 s^{-1}, at a frequency of 1Hz, for 15 minutes using the C50-2 spindle. A gap of 4 μm was set according to the spindle specifications, and the temperature was maintained at 37°C. The Rheo3000 v1.2 software was used to monitor the rheological properties in terms of viscosity (Pa·s) and time to gelation (s). The time at which maximum viscosity was reached was considered to be the material's time to gelation.

3.1.8 Statistical Analysis

Values are reported as mean ± standard deviation. Statistical analyses were performed in SAS® version 9.2 (SAS Institute Inc., Cary, NC). Comparisons of continuous data between groups were analyzed by a one-way ANOVA, and for comparisons of data between treatments for individual blood donors, paired *t* tests were used. All rheology trials were performed with n=6 and cell viability experiments with n=3. A probability value of $p<0.05$ was considered significant.

3.2 Study I: Results and Discussion

Cardiovascular regeneration therapies aimed at using injections of cells into tissues are hindered by a low rate of engraftment and limited survival and function of the transplanted cells [28, 30, 31]. Furthermore, the relocation of the transplanted cells from

the target site to non-specific tissues is an unwanted side effect [30, 83]. One strategy to enhance the retention and therapeutic potential of the transplanted cells is to develop biomaterials for use as cell delivery vehicles that can also provide more suitable transplant environments. Several *in situ* gelation biomaterials have been developed for cardiovascular tissue engineering [84]. However, while the physical properties of such materials are routinely characterized, less is known about how these properties may be changed once the therapeutic agent (e.g. cells, microspheres) has been added to the mixture.

3.2.1 Rheological Characterization: Cross-linker Effects

The concentration of the EDC/NHS cross-linker was varied in the collagen matrices to modulate the gelling behavior of the material. Rheological analysis showed an inverse relationship between cross-linker concentration and gelation time (Figure 2A, C). Doubling the EDC/NHS concentration (to 26mM) resulted in a reduction of time to gelation (0.7±0.1 fold; p=0.004), whereas halving the cross-linker concentration (to 6.5mM) resulted in a 1.8±0.1 fold increase in gelation time (p<0.0001) compared to the standard collagen matrix (13mM EDC/NHS). Also, there was a significant difference in the gelation time between the 6.5mM and 26mM cross-linker matrices (2.4±0.5 fold change; p<0.0001). Rheological data also demonstrated that cross-linker concentration and maximum viscosity were directly related (Figure 2B). The maximum viscosity reached for the 13mM and 26mM EDC/NHS matrices was increased (by 1.2±0.2 and 1.4±0.2 fold, respectively) compared to the 6.5mM EDC/NHS matrix (p=0.04 and p<0.001, respectively). This established a baseline to which the subsequent experiments using matrices with different additives could be compared.

The physical characteristics, and in particular the rheological properties of a material, are of importance in the development of injectable hydrogels. The time to gelation determines the amount of time the clinician will have to handle the materials, and the viscosity is related to the stability upon implantation. The rheological properties of the collagen-based matrix in response to different EDC/NHS cross-linker concentrations are consistent with previous studies [38]. As the concentration of cross-linker was increased, greater viscosity of the collagen hydrogel and a reduction in the time to gelation were observed. This can be attributed to a decrease in the swelling ratio, fewer free amino groups on collagen, increased resistance to enzymatic degradation and increased immobilization of chondroitin sulfate, as previously reported [38]. In addition to viscosity-time profiles, time sweep profiles (modulus vs. time) of the hydrogels were conducted to observe responses to various cross-linking concentrations and to the addition of deliverables (Figures 3C, 2C and 4D).

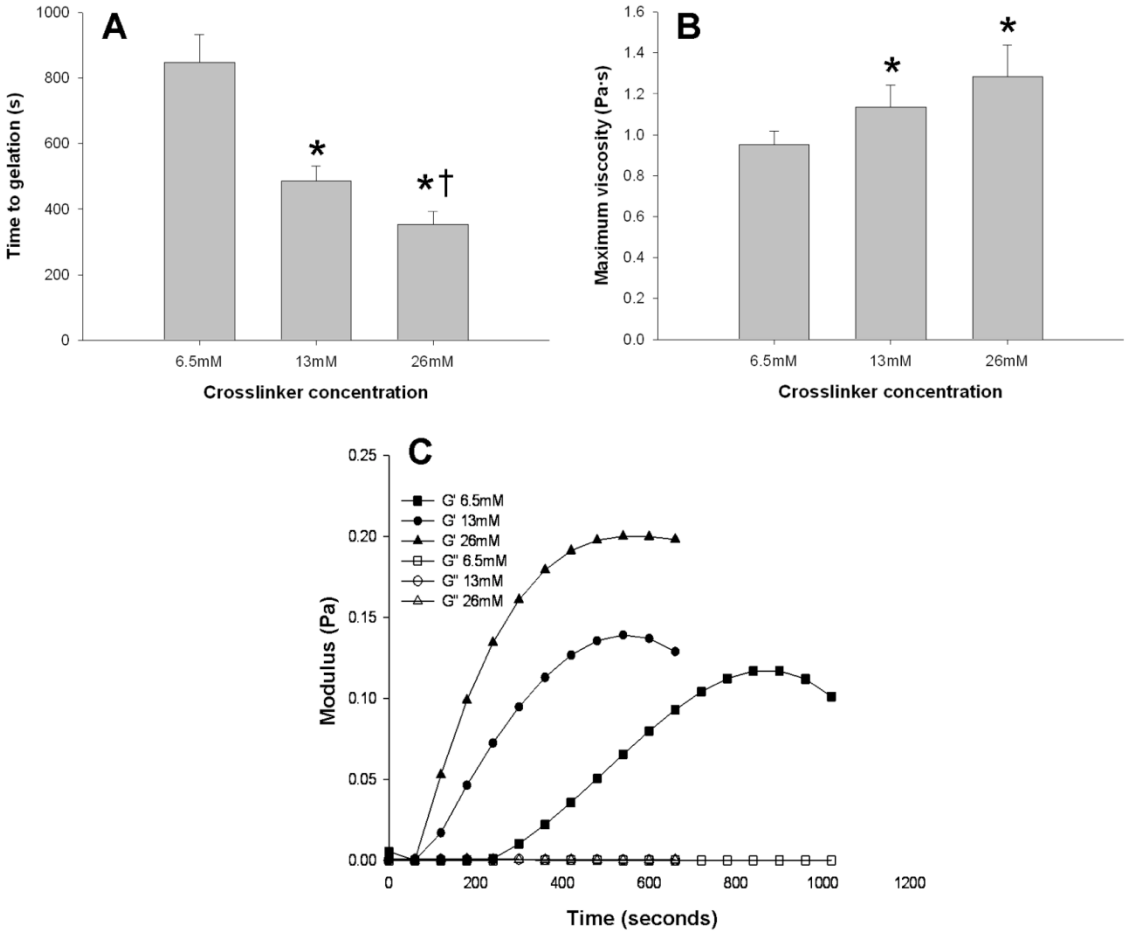

Figure 2: Effect of cross-linker concentration on gelation time and viscosity

Cross-linker concentrations of 6.5mM, 13mM and 26mM were analyzed (n=6). (A) Gelation time of the collagen matrix decreased with increasing cross-linker concentration. (B) Viscosity of the matrix increased with increasing cross-linker concentration. (C) Representative modulus vs. time curves illustrating G' (storage) and G'' (loss) values of the different hydrogels. *p≤*0.04 and* †p=*0.004 versus 6.5mM and 13mM concentrations, respectively.*

G" (loss modulus) values are initially slightly greater than G' (storage) values because most of the energy is lost as viscous heat, and samples are still in a semi-liquid state where viscous properties dominate. At time >100 seconds, a rapid increase of G' followed by a plateau occurs for all deliverables. The reaction period during which the storage modulus rapidly increases corresponds to the transition of collagen solution to hydrogel transformation. As the solution gels, G' begins to increase at a rate much higher than G" since the elastic properties of the gelling collagen hydrogel begin to dominate. The viscosity and modulus values obtained fall within a range reported previously to provide suitable injectability and allow adequate time for delivery of the material before a change in viscosity occurs [17].

3.2.2 Rheological Characterization: Circulating Angiogenic Cell Effects

The different numbers of cells tested (2.5×10^5, 5.0×10^5 and 1.0×10^6 cells) were chosen as a therapeutically relevant range for the treatment of rodent ischemic tissue [27, 85]. Overall, the rheological behavior of the collagen matrix was affected by the incorporation of CACs into the material (Figure 3). An increase in cell density corresponded to a decrease in gelation time (Figure 3A, C, D). The addition of 1.0×10^6 cells per 500μL of matrix resulted in a 22.6% and 18.2% reduction of gelation time compared to the matrix with no cells (p=0.001) and 2.5×10^5 cells (p=0.01), respectively. In addition, higher cell density appears to correlate with an increase in maximum matrix viscosity (Figure 3B, C, D). The maximum viscosity reached for all matrices with cells was greater than for the matrix without cells (p=0.03, p=0.001 and p<0.001, for 2.5×10^5, 5.0×10^5 and 1.0×10^6 cell concentrations, respectively).

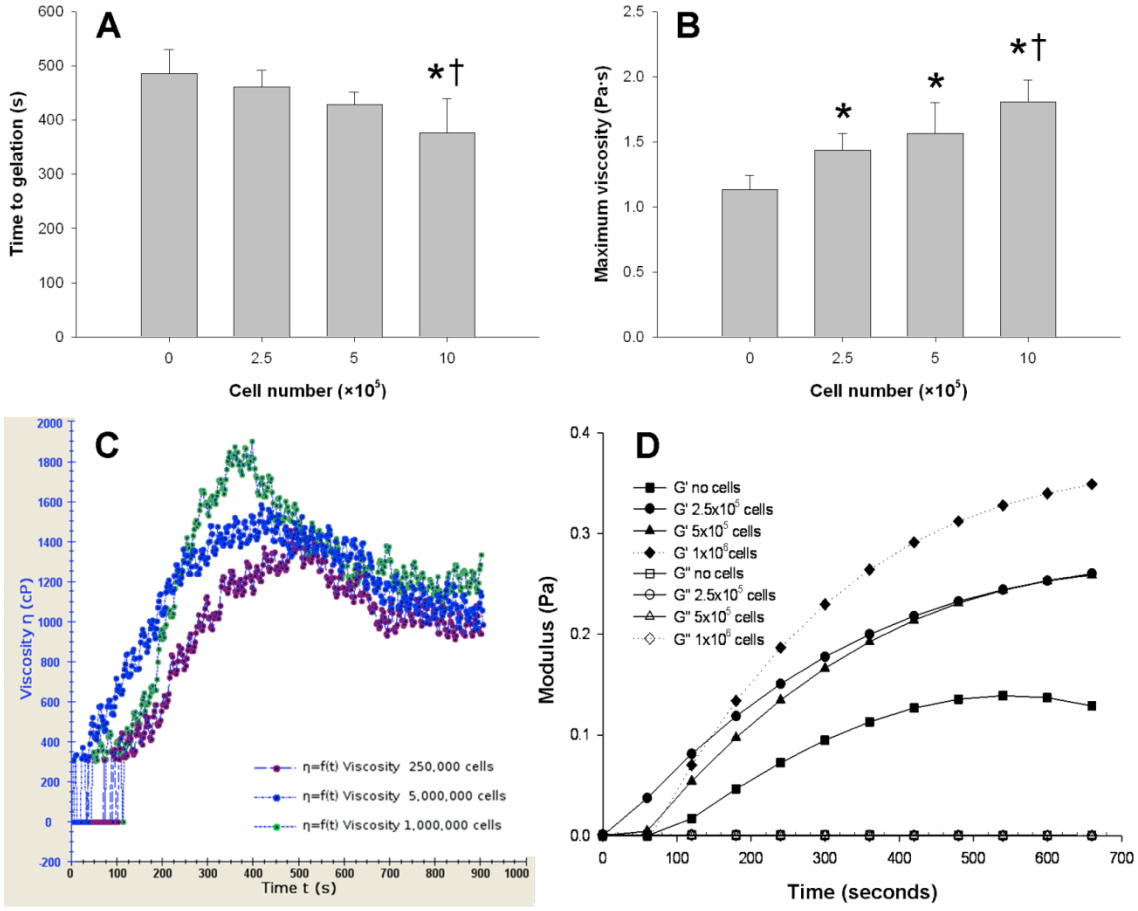

Figure 3: Effect of cell density on gelation time and viscosity

Matrices with cell densities of 2.5×10^5, 5.0×10^5, and 1.0×10^6 cells per 500µL of matrix (13mM EDC/NHS) were compared with the 13mM EDC/NHS matrix without cells (n=6). (A) The time to gelation decreased with increasing cell density. (B) Maximum viscosity of the matrix increased with higher cell density. (C) Representative viscosity-time curves for matrices with different cell concentrations. (D) Representative modulus vs. time curves illustrating G' (storage) and G" (loss) values of the different hydrogels. *$p\leq0.02$ and $^{\dagger}p\leq0.01$ *versus 0 and 2.5×10^5 cell concentrations, respectively.*

Rheological analyses are routinely performed to evaluate the development of injectable hydrogels as delivery vehicles, but how the addition of the deliverable (be it cells, microspheres, growth factors/cytokines or other) affects the material's properties is usually overlooked. It was previously shown that the incorporation of cells above a critical density could enhance the mechanical integrity of RGD-alginate hydrogels [86]. In addition, using fluorescence emission energy transfer technology, it was demonstrated that cells seeded within RGD-alginate hydrogels could modify the rheological properties of cell/polymer mixtures through specific interactions between the cells and the polymer [87]. In the present study, an increase in CAC concentration within the collagen matrix solution resulted in a decrease in the time to gelation and an increase in hydrogel viscosity. This suggests that the CACs may participate in the collagen cross-linking process. The uniform cell dispersion observed within the formed hydrogels (see Figure 7) suggests that interactions between integrin receptors on the surface of CACs and the adhesion ligands of collagen dominated over cell-cell interactions, which would have resulted in cell aggregation [87]. Combined with the previous RGD-alginate work [86, 87], the results with the collagen hydrogel demonstrate that the effect of cell-matrix interactions is not unique to specific materials, but rather is likely to play a role in regulating the physical properties of all hydrogels designed to interact with cells.

3.2.3 Rheological Characterization: Microsphere Effects

The addition of 1.25% alginate microspheres to the 13mM EDC/NHS collagen matrix resulted in a 10% reduction in gelation time (p=0.03) and a 23% increase in maximum viscosity (p=0.02; Figure 4). The sequence of reactions for the gelation of collagen hydrogels (PATH A) with the addition of chondroitin sulfate (PATH B) and

microspheres (PATHS C) are shown in Figure 5. Collagen hydrogel was synthesized via a 1:1 molar ratio of EDC/NHS in MES to a 1% type-I porcine collagen on ice forming an intermediate ester. Additional collagen in the system binds to the intermediate ester forming the major product, consisting of a collagen hydrogel containing amide bonds (PATH A in Figure 5). Simultaneously, EDC/NHS activates the carboxylic groups on chondroitin sulfate, forming an intermediate ester, thereby allowing any remaining collagen in the system to react with it to form a collagen-chondroitin hybrid hydrogel (PATH B in Figure 5). Similarly, the addition of alginate microspheres results in a possible third product. We believe the remaining carboxylic groups on the microspheres that are unbound following the $CaCl_2$ cross-linking reaction are activated by EDC/NHS forming an alginate microsphere-NHS intermediate. Any additional collagen remaining in the system will bind to this intermediate, forming a collagen-alginate microsphere hybrid hydrogel (PATH C in Figure 5).

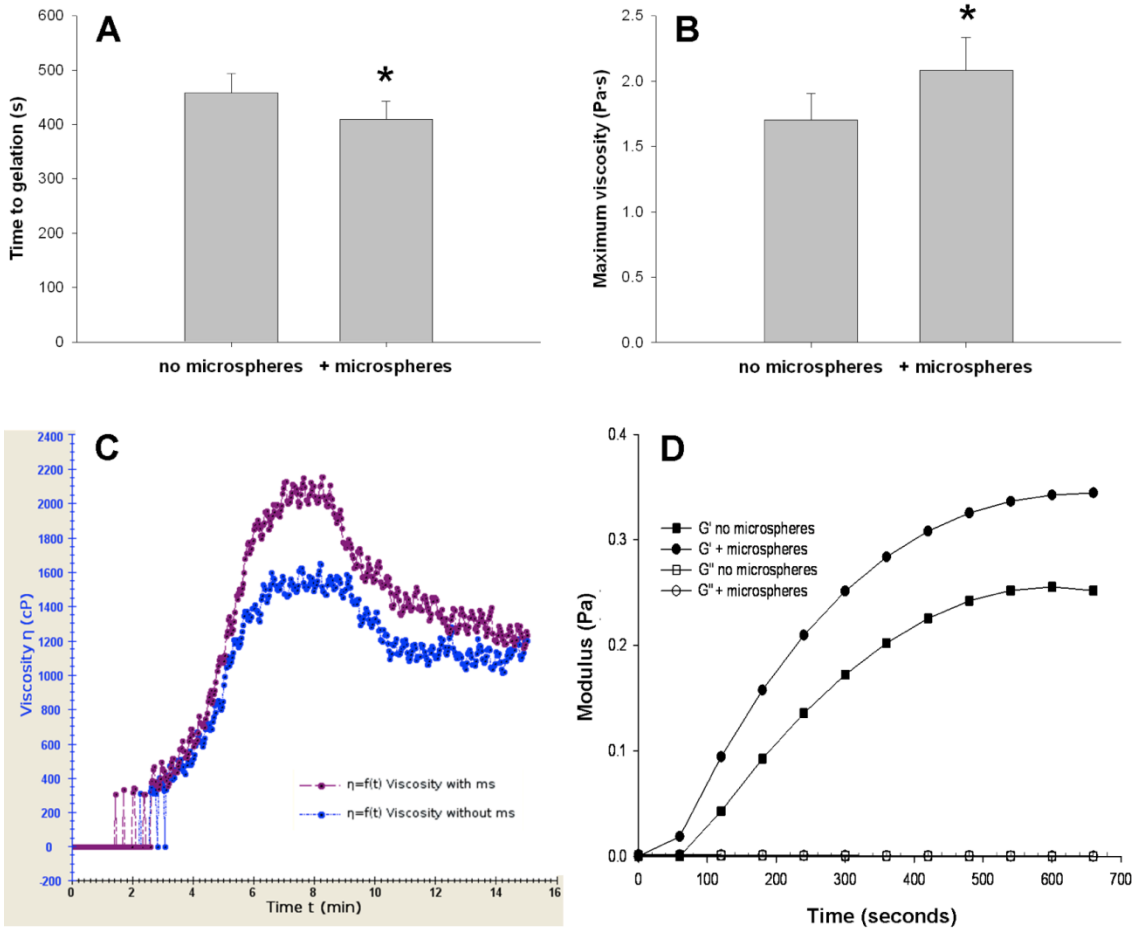

Figure 4: Effect of the addition of microspheres on gelation time and viscosity

*(A) The addition of alginate microspheres to the collagen matrix reduced the time to gelation (n=6). (B) The addition of alginate microspheres to the collagen matrix increased the maximum viscosity reached (n=6). (C) Representative viscosity-time curves for matrices with and without the addition of alginate microspheres (ms). (D) Representative modulus vs. time curves illustrating G' (storage) and G" (loss) values of the different hydrogels. *p≤0.03*

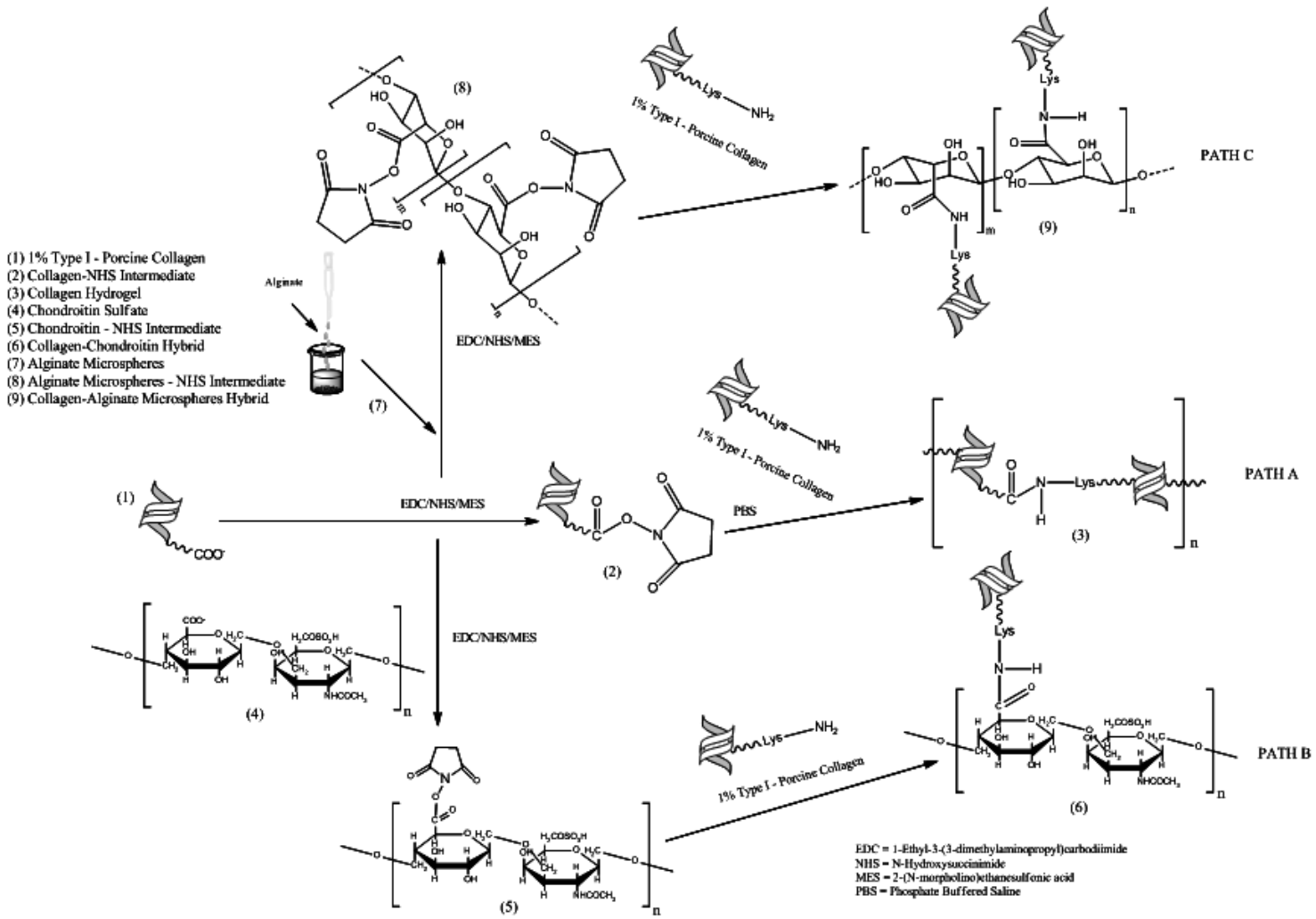

Figure 5: Reaction scheme of the pathways involved during gelation of the collagen matrix with chondroitin sulfate and microspheres PATH A (Collagen Hydrogel Pathway) = 1 → 2 → 3; PATH B (Collagen Chondroitin Pathway) = 1 → 4 → 5 → 6; and PATH C (Collagen – Alginate Microspheres Pathway) = 1 → 7 → 8 → 9. PATH A dominates, while simultaneous pathways (PATHS B and C) occur less frequently.

The design of nano- or micro-particles for the delivery of cells and cytokines/growth factors is a rapidly expanding area of investigation [41, 43]. Similar to the cell-matrix studies, the nano- and micro-particles research has primarily focused on the material (release kinetics in particular), and less so on the effect that the particles may have on the physical properties of their environment, including delivery matrices. In the present work, the addition of alginate microspheres to a collagen matrix resulted in decreased time to gelation and increased viscosity of the hydrogel. Cross-linking between the alginate and collagen is resulting from the formation of amide bonds, and from intermolecular hydrogen bonding of -OH groups [88]. Therefore, alginate-collagen reactions serve to accelerate formation of the hydrogel and enhance its viscosity; and this is consistent with the findings of Liu *et al*., which concluded that alginate microspheres can increase the tensile strength and modulus of collagen hydrogels [89].

3.2.4 Cross-linker Effects on Circulating Angiogenic Cell Viability

The viability of CACs after 24 hour culture in matrices was reduced when compared to baseline viability prior to seeding in matrix. CAC viability decreased by 43%, 65% and 78% in the 6.5mM, 13mM and 26mM EDC/NHS matrices, respectively (Figure 6A; $p \leq 0.04$). Increasing the concentration of EDC/NHS cross-linker had a negative effect on cell viability. A greater number of the seeded CACs remained viable in the 6.5mM EDC/NHS matrix (0.6±0.2 fold versus baseline) compared to the 13mM (0.4±0.2 fold) and 26mM (0.2±0.06 fold) EDC/NHS matrices (p=0.08 and 0.03, respectively).

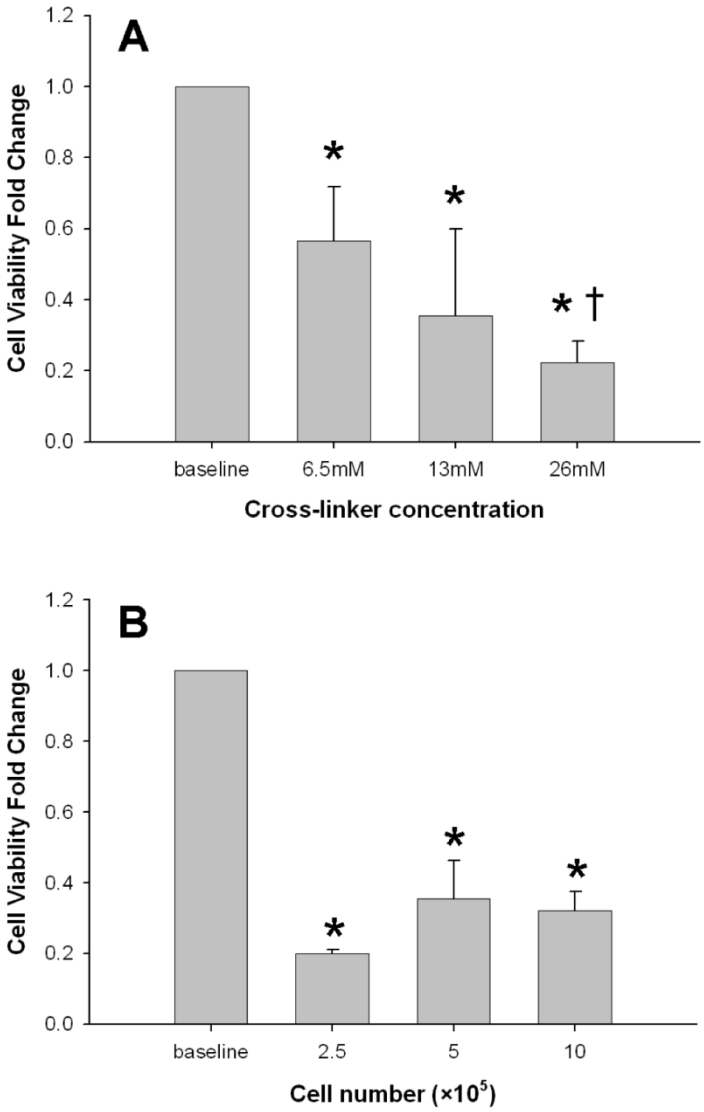

Figure 6: Effect of cross-linker concentration and cell seeding density on cell viability

*(A) Fold change in CAC viability (compared to baseline prior to culture) after 24 hour culture in matrices with different cross-linker concentrations (n=3). *p≤0.04 and †p=0.03 versus baseline and the 6.5mM EDC/NHS matrix, respectively. (B) Fold change in CAC viability (compared to baseline prior to culture) after 24 hour culture in matrices with different seeded cell densities all with 13mM EDC/NHS (n=3). *p≤0.002 versus baseline.*

While the literature regarding the effects that cells have on materials is limited, there are abundant studies reported that describe the effect that a material's properties have on a cell's phenotype and function (for review, see [90, 91]). For example, in fibrin matrices, the fibrin cross-link density has been shown to strongly influence endothelial cell process extension, while also influencing the material's response to cellular proteolytic activity [92]. Specifically regarding collagen-based materials, Crister *et al*. recently reported that the concentration of collagen in a matrix influenced its vascularization by transplanted progenitor cells when implanted subcutaneously *in vivo* [2]. Previous *in vitro* work had similarly demonstrated the effect of collagen concentration on endothelial cell function and vessel formation [39]. The present study demonstrates that cross-linker concentration in a matrix also affects the cells incorporated within it; CAC viability was reduced as the cross-linker concentration was increased. Although the EDC/NHS cross-linker has been reported as non-toxic [20, 36], this conclusion is based on studies of cells interacting with materials after the cross-linking event has been terminated. In injectable systems, such as the hydrogel tested herein, the cells are incorporated within the matrix mixture prior to gelation and are thus exposed directly to the cross-linker. Similar to the results of this study, direct exposure to EDC cross-linker negatively affected the viability of fibroblasts, in a concentration-dependent manner [37].

Although reduced cell viability within the collagen matrices was observed *in vitro,* previous *in vivo* studies have demonstrated successful revascularization and increased perfusion in ischemic hindlimbs of rats treated with cells delivered within injectable collagen-based hydrogels [27, 85]. Despite the possible effect of cross-linker toxicity on CACs, the use of the delivery matrix was still superior in maintaining more

numerous viable and functional cells in the target tissue, compared to the transplantation of CACs alone. This suggests that chemically cross-linked delivery scaffolds may further improve cell therapy if the effects of cross-linker toxicity can be minimized.

3.2.5 Cell Density Effects on Circulating Angiogenic Cell Viability

Cell density had no effect on preventing the reduction in the viability of CACs cultured in matrix for 24 hours. Compared to baseline (prior to seeding), CAC viability decreased by 80%, 65% and 68% in the matrices with 2.5×10^5, 5.0×10^5 and 1.0×10^6 cells, respectively (Figure 6B; $p\leq0.04$).

3.2.6 Effect of Glycine on Circulating Angiogenic Cell Viability

CACs were added to the 13mM EDC/NHS collagen matrix mix in a solution of 0.15M glycine or in PBS, the matrices were thermogelled, and cell viability after 24 hours was compared between glycine and PBS protocols. The addition of cells to the matrix in a glycine solution resulted in improved CAC viability (74.4±10.1%) compared to PBS (44.9±13.7%), representing a 1.7 fold increase (Figure 7; p=0.03). Compared to baseline (91.8±2.6%), the use of glycine prevented CAC death in the matrix (74.4±10.1%; p=0.2), whereas the viability of CACs added to matrix in PBS (44.9±13.7%) was significantly reduced (p=0.003). Figure 8 shows the reaction pathway involved during gelation of the collagen matrix when cells are suspended in glycine and added to 1% type-I porcine collagen (PATH D) in addition to PATHS A and B (described in detail in 3.2.3). The addition of glycine and cells with the collagen-NHS intermediate, leads to a matrix consisting of collagen-glycine and cells.

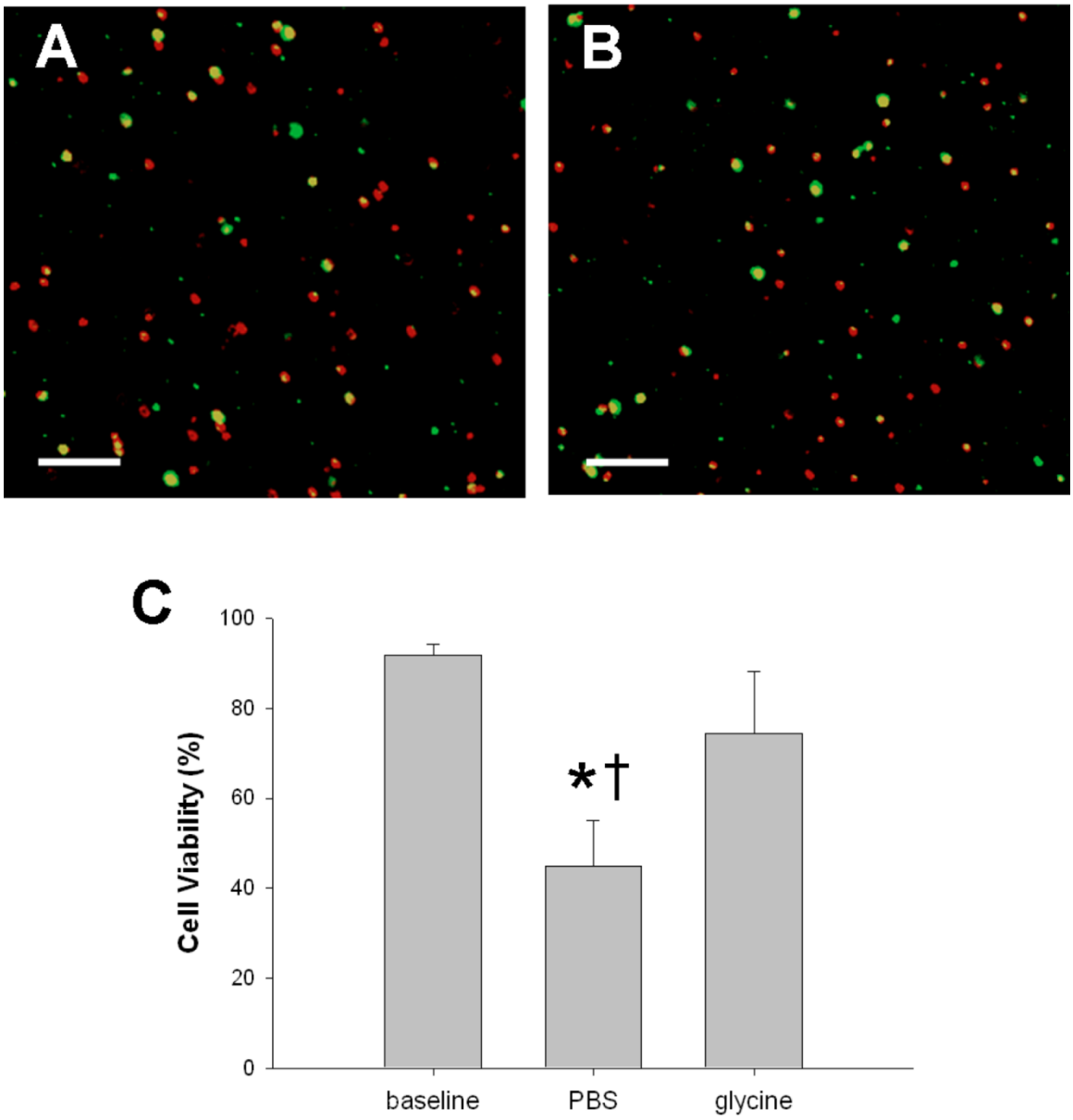

Figure 7: Effect of glycine on cell viability

*(A, B) Representative images of live/dead stain for CACs added to the collagen matrix in solution of (A) PBS or (B) glycine (red=dead cell; green=live cell; yellow=apoptotic). (C) CAC viability for cells added in glycine was greater than for CACs added in PBS (n=3). *p=0.003 and †p=0.03 versus baseline and glycine groups, respectively. Scale bar = 50 μm*

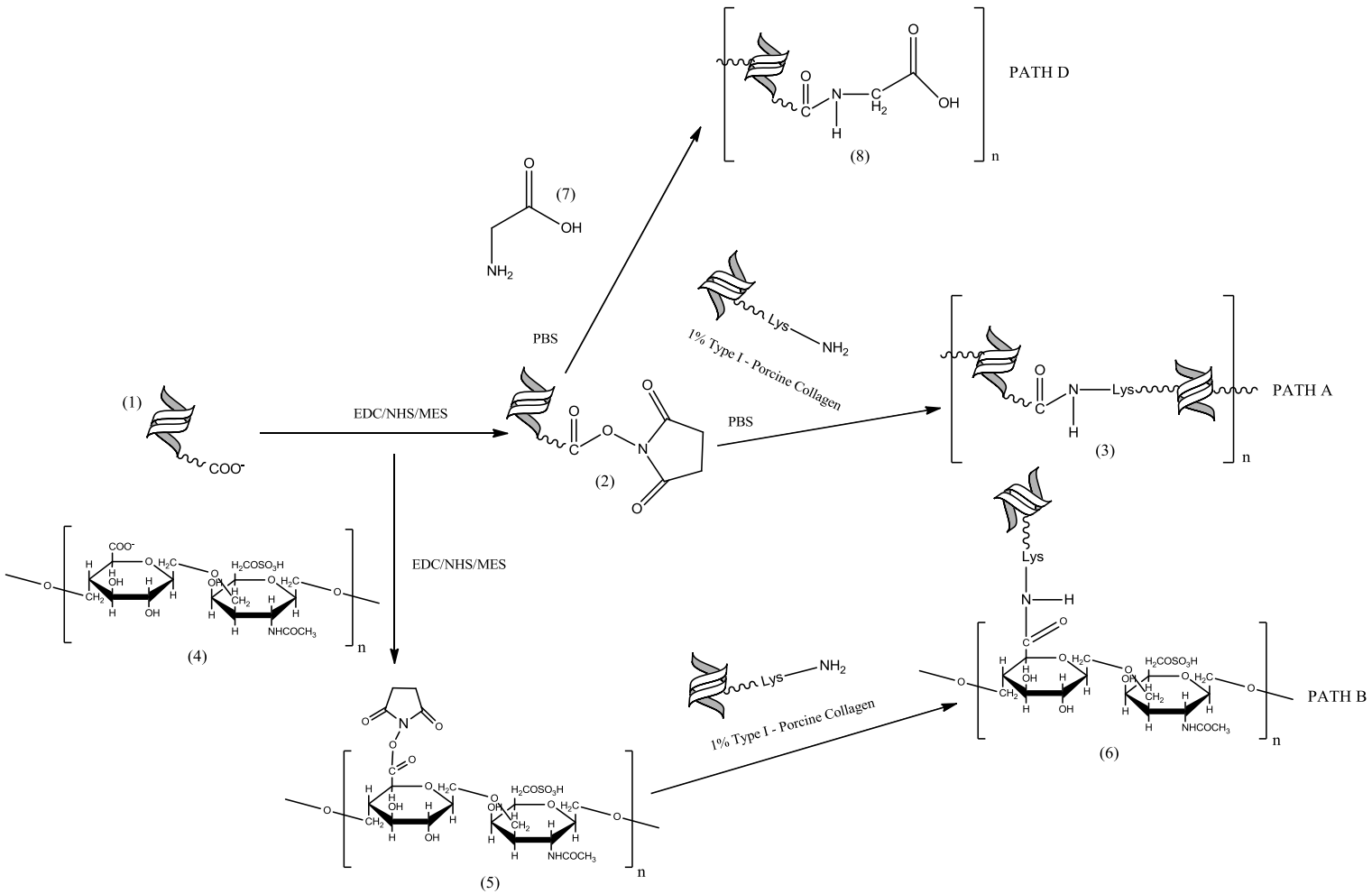

Figure 8: Reaction scheme of the pathways involved during gelation of the collagen matrix with chondroitin sulfate, glycine and cells PATH A (Collagen Hydrogel Pathway) = 1 → 2 → 3; PATH B (Collagen Chondroitin Pathway) = 1 → 4 → 5 → 6; and PATH D (Collagen–Glycine + Cells Pathway) = 1 → 2 → 7 → 8. PATH A dominates, and all other pathways (PATHS B and D), while they occur simultaneously with PATH A, they occur less frequently

It was demonstrated that the use of glycine was capable of improving the viability of CACs added to the hydrogel mixture. In fact, cell viability was not significantly different compared to baseline levels. The use of glycine to quench residual toxic aldehyde groups in glutaraldehyde cross-linked materials has been previously reported [93, 94]. Glycine can also undergo a coupling reaction with EDC, which is enhanced by the addition of NHS [95]. Therefore, resuspending cells in a glycine solution prior to their addition to the matrix is likely shielding the CACs from the cross-linker, through glycine-EDC reactions.

This study presents, for the first time, the possibility of using glycine as a cross-linker scavenger to protect cells from toxicity associated with the use of EDC/NHS, when the cells are added to the material mixture during the cross-linking reaction. The effect that improved viability of matrix-delivered cells (with the use of glycine) has on the regenerative potential of cell-matrix treatment constitutes an area for more targeted investigation in future evaluation of this therapy.

3.2.7 Phenotype of Circulating Angiogenic Cells in Collagen Matrix

The phenotype of viable CACs (added to the matrix mixture with glycine) was characterized 24 hours after gelation of the matrix. CACs within the hydrogel expressed the endothelial cell markers CD31 (30.6±2.3%) and VEGFR2 (8.0±0.9%), the adhesion molecule L-selectin (75.3±5.1%), and the progenitor cell markers CD34 (0.6±0.1%) and CD133 (0.5±0.1%).

Phenotypic analysis of the viable cells at 24 hours post-gelation revealed the ability of the matrix to support cells expressing endothelial (CD31 and VEGFR2) and progenitor (CD34 and CD133) markers, which have therapeutic value for vascularization of diseased or damaged tissue [96, 97]. Longer-term culture of CACs (4 days) on a similar collagen-

chondroitin sulfate matrix has been shown previously to enrich these endothelial and progenitor populations [98].

3.3 Study I: Summary

In this study, the successful synthesis of a collagen hydrogel containing chondroitin sulfate with the addition of alginate microspheres or glycine, and the interactions of cells with these materials is reported. Notably, the following were demonstrated: (1) that the addition of cells or microspheres to an injectable collagen matrix increases the viscosity of the matrix and reduces its time to gelation; and (2) that the use of glycine can protect cells from death associated with the EDC/NHS cross-linker in the matrix. This study therefore suggests that a better understanding of the interaction between materials and their additives is needed to help guide the optimization of cell-matrix therapy.

Although the physical properties of *in situ* gelation materials developed for delivery purposes are routinely characterized, as are their effects on cell behavior, the literature regarding the effects that cells or other additives have on materials is limited. Few studies report how the materials' properties (gelation in particular) are affected by the addition of the therapeutic agents they are designed to deliver. Also, a potential limitation to the use of *in situ* gelation materials for the delivery of cells is the toxicity resulting from the cells' direct exposure to chemical cross-linkers, such as EDC/NHS. This study demonstrated that the incorporation of either cells or microspheres to a collagen hydrogel changed its rheological characteristics. In addition, it was shown the use of glycine can protect cells from cross-linker toxicity, thus making EDC/NHS cross-linked injectable collagen gels a viable option for cell delivery. A better understanding of how materials and cells (and other additives)

respond to each other will help towards the goal of improving scaffolds being developed for regenerative therapy.

A version of this chapter has been published in 'Tunable Collagen Hydrogels are Modified by the Therapeutic Agents they are Designed to Deliver'. Journal of Biomaterials Science-Polymer Edition. 2012 (See Appendix A for copyright permission)

CHAPTER 4: STUDY II Synthesis and Characterization of a Collagen-Based Hydrogel for the Delivery of Insulin-Producing Tissue

4.1 Study II: Materials and Methods

4.1.1 Synthesis of Collagen Matrix

As described previously [17, 80] type-I porcine collagen (1% w/v; Nippon Ham), chitosan (1.5% w/v, 20:1 collagen:chitosan or 10:1 collagen:chitosan; Sigma) and chondroitin sulfate-C (40% w/v; Wako Chemicals) were chemically cross-linked. A cross-linking mixture with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide (EDC/NHS; Sigma), 10% w/v solution in 0.5M MES buffer (pH ~6.0; EMD Chemicals) was added to the matrix mixture in a glass centrifuge tube on ice. The pH was adjusted to 7.2±0.2 using sodium hydroxide, and matrices were thermogelled for 30 minutes at 37°C. Matrices with 20:1 collagen:chitosan and 10:1 collagen:chitosan ratios were prepared (herein referred to as 20:1 matrix and 10:1 matrix, respectively) with or without 40 μg laminin/ mL matrix added prior to cross-linking (herein referred to as 20:1 matrix+LM and 10:1 matrix+LM, respectively).

4.1.2 Material Characterization

Material characterization was conducted for matrices with 10:1 and 20:1 collagen:chitosan ratios +/- LM. Each matrix composition was tested with one of the following cell culture media: endothelial basal media (EBM) supplemented with 0.15 M glycine or Ham's media, to mimic conditions for the CACs and islets, respectively.

4.1.3 Mechanical Testing

Each matrix formulation was subjected to unconfined compressive loading using an Instron (MTS Bionix 858) servo-hydraulic material testing system with a 50 N load cell at a

crosshead speed of 50 mm/s to a maximum of 30 % strain; parameters were set after pre-conditioning samples thereby eliminating or minimizing the toe region. This ensures data collection on all samples starts at the same point and improves repeatability of the results. Cross-head position (mm) and load (gf) were monitored and recorded with Instron Wavemaker software. Samples were hydrated in PBS for 48 hours at 37°C and punched into 6mm diameter disks prior to testing. Sample disks were maintained at 37°C in a PBS bath for one hour prior to testing and for the duration of testing. Specimens were positioned between non-porous plates and six loading and unloading cycles were completed to precondition the sample. Six samples were tested for each matrix formulation.

Mechanical Testing Data Analysis:

Displacement and load data was used to calculate engineering stress- engineering strain, based on sample thickness, cross-sectional area and initial gauge length after preconditioning. A five-parameter double exponential growth model, Equation 1, was used to fit the engineering stress- engineering strain data.

$$\sigma = y_o + a \cdot \exp(b \cdot \varepsilon) + c \cdot \exp(d \cdot \varepsilon) \qquad \text{Equation (1)}$$

where σ is stress, ε is strain and y_o, a, b, c and d are curve fitting parameters obtained from SigmaPlot v.10.0. The elastic modulus, Equation 2, was calculated by differentiating Equation (1) as a function of strain.

$$\sigma' = a \cdot b \cdot \exp(b \cdot \varepsilon) + c \cdot d \cdot \exp(d \cdot \varepsilon) \qquad \text{Equation (2)}$$

where σ'is the elastic (tangential) modulus, ε is strain and y_o, a, b, c and d are curve fitting parameters [15, 99]. The elastic modulus (in kPa) was calculated at 15% strain where all materials were elastically deforming (linear region in stress-strain curve). A student t-test

was used to compare each material, with significance set at $p<0.05$. See Appendix B for sample raw data and calculations.

4.1.4 Degradation

Samples were hydrated in PBS overnight prior to commencing the degradation studies.

Specimens were cut into 6mm diameter disks using a biopsy punch and were submersed in water, PBS, collagenase (0.125 U/mL; Gibco) or α-amylase (220 U/mL; Sigma). Degradation was performed at 37°C in static conditions. Hydrolytic and enzymatic degradation of each matrix formulation was monitored via weight at time points of 0, 36, 48 and 72 hours (n=3 per fluid).

4.1.5 Scanning Electron Microscopy (SEM) with Critical Point Drying (CPD)

One day prior to imaging, samples of each matrix formulation, 6mm in diameter, were washed sequentially in 70, 80, and 90% ethanol solutions for 20 minutes each. Samples were immersed overnight in 95% ethanol, then in 100% ethanol the following day. Matrices with cells (islets or CACs) were fixed in 3% glutaraldehyde (Sigma) for 30 minutes then washed 3 times in PBS before the ethanol washes to preserve cell morphology. Prior to viewing, samples were dried using a critical point dryer (Emitech K850) at a temperature of 31.1°C and a pressure of 1072 psi using CO_2 as the transitional fluid. For cross-sectional viewing, samples were fractured after immersion in liquid nitrogen. To circumvent charging effects, samples were sputtered (Anatech, Hummer VII) with a palladium/gold alloy (60:40 palladium:gold) to form a thin coating of 3 nm. SEM images were obtained using an accelerating voltage of 5 kV in order to minimize sample damage. Cross-sectional images were obtained using backscattering and secondary electron detectors. Micrographs were

processed with VEGA software and were evaluated for porosity and pore diameter using Image-J 1.43u software.

4.1.6 Surface Contact Angle

The wettability of the matrices was evaluated using water surface contact angle methods. Matrix samples were hydrated overnight in PBS at 37°C and punched into 6mm diameter disks prior to testing (n= 3). Measurements were obtained on a VCA Optima contact angle apparatus, which operates on the theory of deformation of drops or bubbles by gravity. A water drop (3 μL) was suspended from a motorized syringe. Initially the drop was supported by surface tension, then deformed by gravitational forces and dropped to the sample surface. Contact angle images were analyzed with VCA Optima Image Analysis software.

4.1.7 Rheology

Rheological properties were measured following the same protocol described in section 3.1.7, except samples were subjected to a constant shear rate for 60 minutes as the addition of media altered the gelation properties.

4.1.8 Isolation and Culture of Human Circulating Angiogenic Cells

CACs were isolated following the same protocol described in section 3.1.4.

4.1.9 Isolation and Culture of Porcine Neonatal Islets

Porcine neonatal islet cells were isolated from one to three day old pigs. Pancreatic glands were dissected from euthanized piglets. Glands were chopped into fragments and washed with Hanks Buffered Salt Solution (HBSS) before digesting in 1 mg/mL collagenase. Pancreas fragments in collagenase were placed in a shaking water bath at 37°C, then gently

agitated until the tissue was sufficiently digested. The digest was filtered through a nylon screen (500 μm) and washed three to five times with HBSS. Isolated islets were cultured on non-tissue culture treated plates in Hams F-10 tissue culture medium (Sigma) supplemented with 14.3 mM sodium carbonate (BDH), 10 mM nicotinamide (Sigma), 10 mM D-glucose (Sigma), 2 mM L-glutamine (Sigma), 1.6 mM calcium chloride dehydrate (Sigma), 0.5% bovine serum albumin (BSA; Sigma), 50 μM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX; Sigma), and 0.5% penicillin/streptomycin (Sigma) at 37°C (5% CO_2, 95% air). Media and plates were changed one day after isolation and media was changed every other day thereafter. Islets were shipped from the Alberta Diabetes Institute to the University of Ottawa Heart Institute in Ham's F-10 media at room temperature. Once received, islets were washed twice with HBSS then resuspended in Ham's media and plated. The following day islets were embedded in matrices for survival and functional assays [100].

4.1.10 Collagen Matrix Preparation for Cell Viability Assessment

CACs were incorporated into the collagen matrices prior to thermogelation in a 0.15 M glycine (Sigma) solution in EBM, as previously described [14]. In a 24-well plate, matrices with CAC density of 5.0×10^5 cells per 500 μL matrix were added and thermogelled at 37°C for 30 minutes. Porcine islet cells were embedded in matrices prior to thermogelation in Ham's media. In a 24-well plate, matrices with islet cell density of ~300 IEs per 500 μL matrix were added and thermogelled at 37°C for 30 minutes. As islets are aggregates of many cells and they vary largely in size, a standard method of counting them is required. The number of IEs is calculated based on the size, morphology and number of cells in sample. After gelation, 1 mL of EBM or Ham's media (for matrices with CACs and porcine

islets, respectively) was added on top of each matrix and the plate was incubated for 24 and 48 hours at 37°C.

4.1.11 Circulating Angiogenic Cell Live/Dead Assay

To simultaneously assess for viable and non-viable cells embedded in matrices after 24 and 48 hours, a live/dead cell staining kit (Invitrogen) was used. Calcein-AM (green) and ethidium homodimer-1 (red) stained for viable and non-viable cells, respectively. To quantify CAC viability, the matrices with embedded cells were immersed in the staining solution for 30 minutes at room temperature. Five fields of view were imaged at 20× magnification using a fluorescent microscope (Olympus BX60). Green cells were considered viable, while red or yellow were considered dead. The viability of cells at the time of isolation (pre-treatment) was considered as baseline. CAC viability data reported is normalized to baseline viability.

4.1.12 Islet Live/Dead Assay

As islets cannot accurately be quantified by counting microscope images, viability was assessed using flow cytometry methods. Hydrogels with embedded islets were digested using collagenase (400 U/mL) and α-amylase (1100 U/mL). The digest was trypsinized for five minutes then pipetted rapidly using a 1000 μL pipette, then using a 200 μL pipette to break up the islets into individual cells [101]. The islet cells were stained following the same live/dead assay described for CACs. Islet cells were analyzed and quantified with a BD FACSAria cell sorter (BD Biosciences).

4.1.13 Islet Functionality

4.1.13.1 Glucose Stimulated Insulin Secretion

Similar to a previously described protocol [102, 103], a glucose stimulation assay was performed to assess islet function. Islets were embedded in hydrogels (described above) and cultured overnight in Ham's media. The media was removed from each well and replaced with Kreb's buffer (pH 7.4) containing 3.3mM glucose (basal working solution; Sigma) for 30 minutes at 37°C. The basal working solution was removed and then replaced with either fresh basal working solution or Kreb's with 16.7mM glucose (high glucose working solution) for 1 hour at 37°C with gentle shaking. These supernatants were stored at -80°C prior to insulin analysis by ELISA and the islets in hydrogels were lysed for protein quantification by bicinchoninic acid (BCA) assay (see below).

4.1.13.2 Insulin ELISA

The supernatants from the glucose stimulation assay were analyzed for insulin content using a porcine insulin ELISA (Mercodia Porcine Insulin ELISA, Cat# 10-1200-01) according to manufacturer's protocol. Briefly 25 μL of sample (diluted if necessary) or standard were mixed with 100 μL of the enzyme solution and incubated for two hours with gentle shaking. Wells were washed six times with provided buffer before incubating with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 15 minutes at room temperature. At this point, the stop solution was added to the plates, gently mixed, and then the plates were read at 450 nm within 30 minutes on a microplate reader (PowerWave XS microplate reader; Bio-Tek Instruments Inc.).

4.1.13.3 Islet Lysis

After the glucose stimulation assay supernatants were collected, the islets embedded in the hydrogels were incubated in collagenase (400 U/mL) at 37°C for one hour (to digest hydrogel). The cell solution was then centrifuged for five minutes to pellet the cells. Ice cold RIPA buffer with protease inhibitor cocktail tablet (Roche 04693124001) was added and the cells were incubated on ice prior to sonication using a probe sonicator (Branson Sonifier 450) to break up and lyse the cells (output 1, 7 pulses, twice). Protein content was quantified by BCA assay.

4.1.13.4 Bicinchoninic Acid (BCA) Protein Assay

As islet cells vary largely in size, the results of the insulin ELISA were normalized to the amount of protein from the corresponding cells. BCA assay was performed on 20 μL aliquots of the cell suspension post-lysis according to the manufacturer's protocols (Pierce BCA Protein Assay Kit, Cat#23225). Working reagent (50 parts reagent A to 1 part reagent B) was incubated with samples or standards in a 96 well plate for 30 minutes at 37°C. Plates were then read at 562 nm (PowerWave XS microplate reader; Bio-Tek Instruments Inc.).

4.1.14 Statistical Analysis

Values are reported as mean ± SD. Statistical analyses were performed in SigmaStat v.3.5. Comparisons of continuous data between groups were analyzed by a one-way ANOVA. A probability value of $p<0.05$ was considered significant.

4.2 Study II: Results and Discussion

Chemically cross-linked collagen and chitosan based hydrogels have contributed enormously as scaffold platforms for 3D cell culture. We have shown that these biomaterials can improve the retention, survival, and function of transplanted and/or recruited cells [27, 80] because of their structure and compositional similarities to the extracellular matrix.

Chitosan and collagen are suitable for use as scaffold materials because they are both naturally derived materials that are non-toxic, biodegradable, biocompatible and have the ability to mimic the extracellular environment and promote physiological interactions with cells. Modifying the bioactive characteristics of the derived hydrogels is expected to yield a material able to support cell adhesion, proliferation, migration, viability and other functional properties. For islet graft design a number of factors must be considered to create a suitable milieu for survival and function of the cells. In this study, matrix formulations with varying components were characterized *in vitro* to determine which best established the physical and chemical properties needed to promote the survival and function of islets and vascular cells. The different matrix formulations tested were: 1) 20:1 matrix, 2) 20:1 matrix + LM, 3) 10:1 matrix and 4) 10:1 matrix + LM. These results will help towards the goal of developing a hydrogel for use as a highly vascularized ectopic islet transplant site.

4.2.1 Mechanical Testing

The compression testing stress versus strain curves for matrices synthesized with EBM (for CACs) and Ham's media (for islets) are shown in Figure 9A and B, respectively. The elastic modulus was calculated in the linear region at 15% strain. The elastic moduli of matrices with EBM and Ham's media are outlined in Figure 9C and D, respectively. For matrices

with EBM, the 10:1 matrices +/- LM were significantly stiffer than the 20:1 matrices +/-LM ($p \leq 0.005$). Similar results were seen by Deng *et al.* [17] whereby increasing chitosan content in a collagen-based hydrogel increased stiffness of the material. Higher chitosan content increases the number of amino groups available to partake in the chemical cross-linking reaction with EDC/NHS; thus increasing the cross-linking density and reinforcing the matrix [17]**.** Furthermore, the GAG-like properties of chitosan improves structural integrity of the material when subjected to compressive forces [24, 25].

The elastic moduli of matrices with Ham's media are significantly greater than the same matrix formulation with EBM ($p \leq 0.038$). As the EBM used to deliver CACs to the matrix is supplemented with 0.15 M glycine, the amine groups of glycine compete in the cross-linking reaction to couple with EDC, which is augmented in the presence of NHS [95]. With EDC molecules coupling with glycine, less is available to establish cross-links between the collagen, chitosan and chondroitin sulfate-C molecules thus altering the elasticity of the matrix.

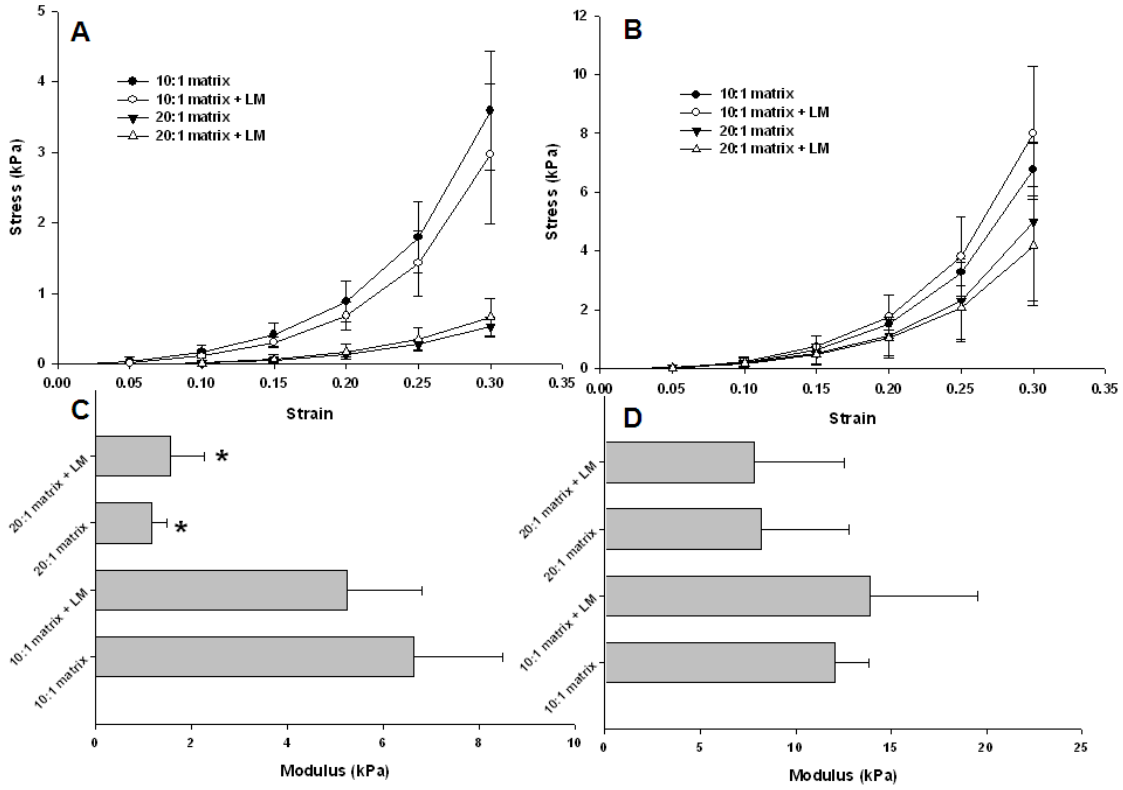

Figure 9: Effect of matrix composition on compression modulus

*(A and B) Stress-strain curves from compressive testing for matrices with EBM and Ham's media, respectively (n=4). (C) Elastic moduli of matrices with EBM. The modulus of 10:1 matrices (+/- LM) with Ham's medium is significantly higher than 20:1 matrices (+/- LM) with EBM (*p≤0.005). (D) Elastic modulus for matrices with Ham's media. The elastic moduli of matrices (+/- LM) with Ham's medium were significantly higher than the same matrix formulation with EBM (p≤0.038).*

4.2.2 Degradation

Enzymatic and hydrolytic weight degradation time course plots for matrices with EBM and Ham's media are shown in Figures 10 and 11, respectively. Overall, the 10:1 matrices were more resistant to enzymatic degradation than the 20:1 matrices. Specifically, the 10:1 matrices with EBM degraded by approximately 5-40% in collagenase, while the 20:1 matrices with EBM experienced a weight loss of 50-80% (Figure 10A). Collagenase had a negligible effect on the weight of hydrogels with Ham's media (Figure 11A). Matrices with EBM media were more susceptible to enzymatic degradation, most drastically observed in α-amylase (Figure 10B). Matrices with EBM were completely degraded by 48 hours in α-amylase, while matrices with Ham's media exhibited minimal degradation (Figure 11B). Degradation trends match those of the mechanical testing as the stronger matrices with 10:1 collagen:chitosan and/or Ham's media experienced less enzymatic mass loss over the 72 hour time period. All of the samples swelled in water, increasing in mass 20-40% after 72 hour (Figures 10C and 11C), which is characteristic of hydrogels [104]. In PBS, 5-10% weight loss was observed for the matrices with EBM (Figure 10D), while matrices with Ham's media lost 1-5% of their original mass via hydrolysis mechanisms (Figure 11D).

Matrices synthesized with Ham's media did not display significant differences in degradation behaviour. This trend is in agreement with the mechanical testing studies, as the Ham's matrices did not differ significantly in terms of elastic moduli. In addition, Ham's media is well buffered and aids to prevent material degradation *in vitro*.

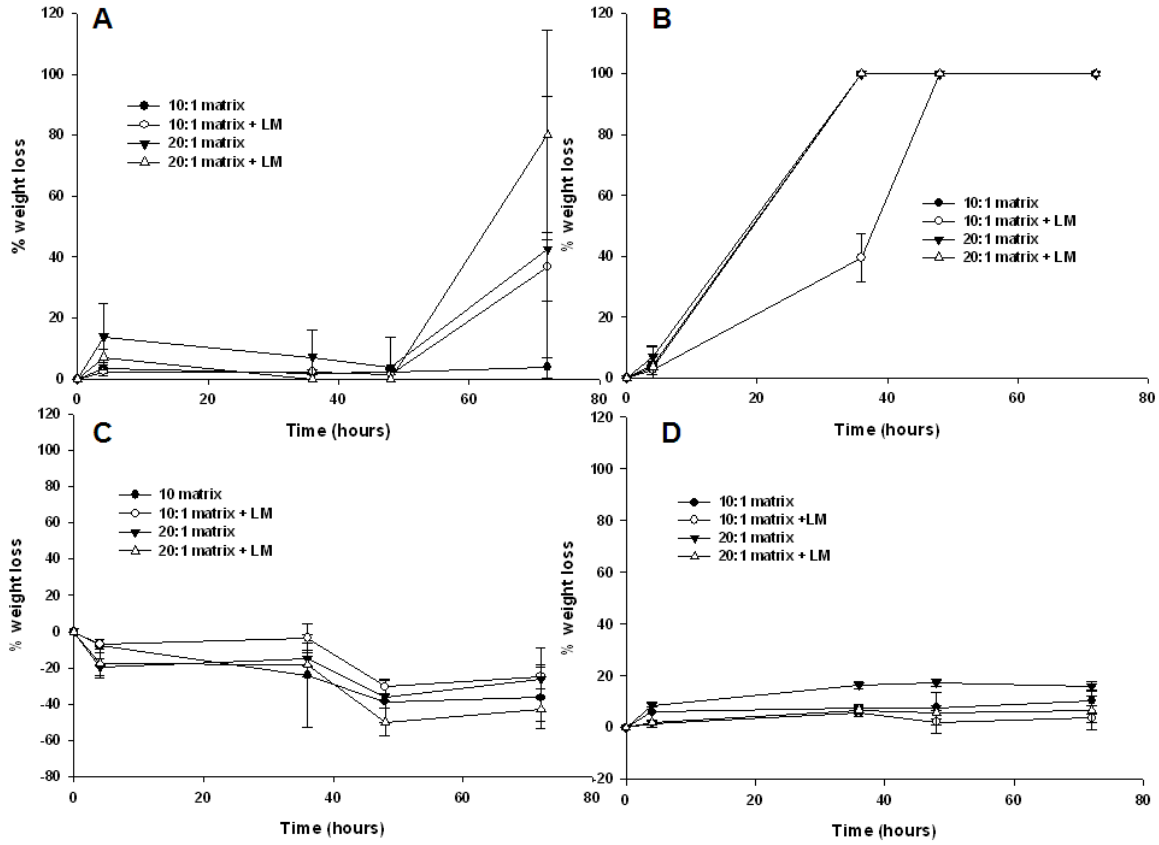

Figure 10: Degradation time course plots for matrices with EBM

Degradation was reported as % weight loss over a 72 hour period. (A) Weight loss of matrices in collagenase (0.125 U/mL). (B) Weight loss of matrices in α-amylase (220 U/mL). (C) Weight loss of matrices in water. (D) Weight loss of matrices in PBS.

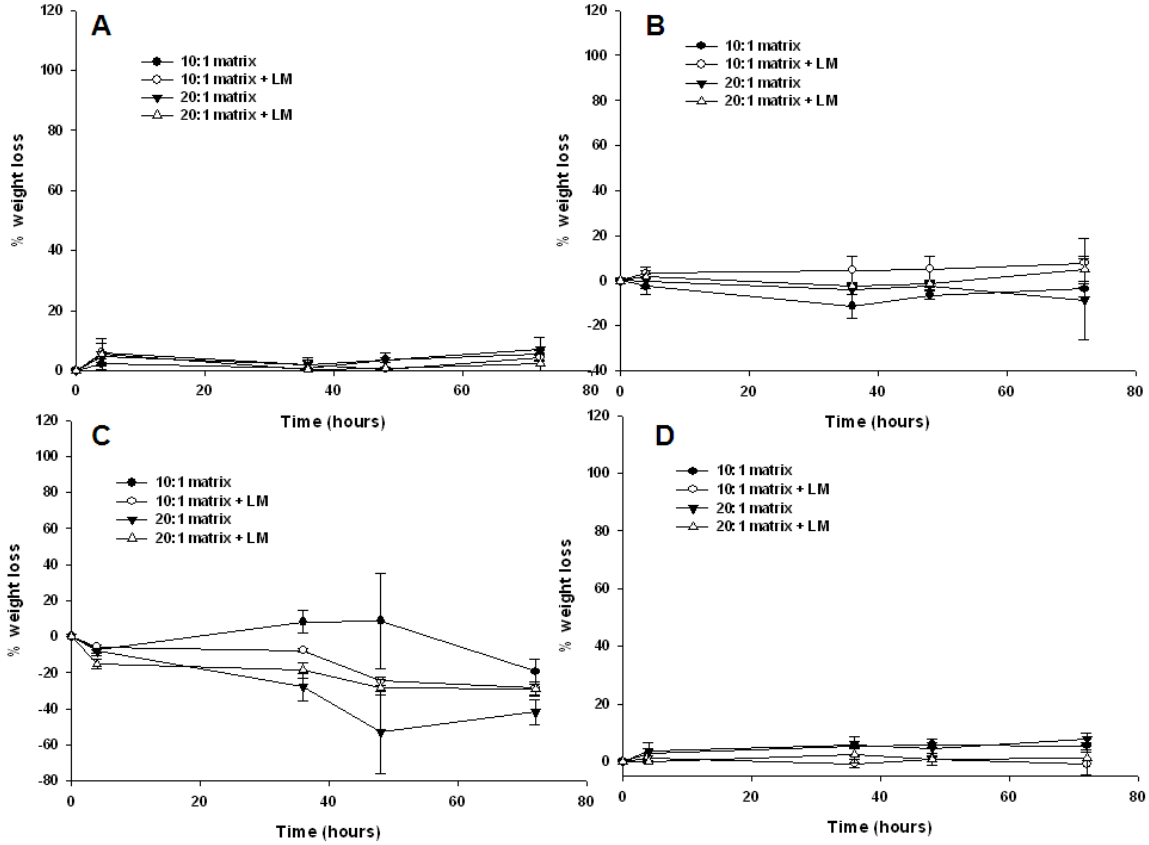

Figure 11: Degradation time course plots for matrices with Ham's media

Degradation was reported as % weight loss over a 72 hour period. (A) Weight loss of matrices in collagenase (0.125 U/mL). (B) Weight loss of matrices in α-amylase (220 U/mL). (C) Weight loss of matrices in water. (D) Weight loss of matrices in PBS.

Matrices that were mechanically stronger were less vulnerable to enzymatic degradation. The stronger materials, with higher chitosan content and Ham's media, have greater cross-linking density as chitosan participates in the cross-linking reaction and Ham's media does not contain glycine, which hinders the cross-linking. Collagenase degradation executes cleavage specifically after the 775th residue in the glycine amino acid of collagen [105]. α-Amylase readily degrades polysaccharides, and is responsible for cleaving chitosan chains in the matrices. α-Amylase acts as a catalyst to hydrolyze α-(1,4)-glycosidic linkages in the chitosan molecules [106]. The greater extent of cross-linking, which can protect these cleavage sites, aids in the preservation of the materials structure.

4.2.3 Scanning Electron Microscopy (SEM)

Fracture electron micrographs were obtained to determine the morphology of the matrices. Porosity ranged from 16-19% for the 10:1 matrices and 22-28% for the 20:1 matrices, as depicted in Figure 12A and B. Pore size was larger in the 20:1 matrices, ranging from 0.20-0.33 μm, compared to 0.16-0.23 μm for the 10:1 matrices. It has previously been shown that increasing chitosan content in a collagen-chitosan matrix results in a denser material, supporting the porosity trends observed in this study [17, 21]. Media type did not have as great of an effect on porosity as it did on the other physical properties. Islets and CACs embedded in matrices were also imaged and are shown in Figure 12C and D. Islets and CACs maintained the same morphology as seen in control standard culture conditions (i.e. islets plated in Ham's media and CACs on fibronectin coated plates in EBM). The maintained morphology indicates that the matrices are supporting the survival and function of the cells.

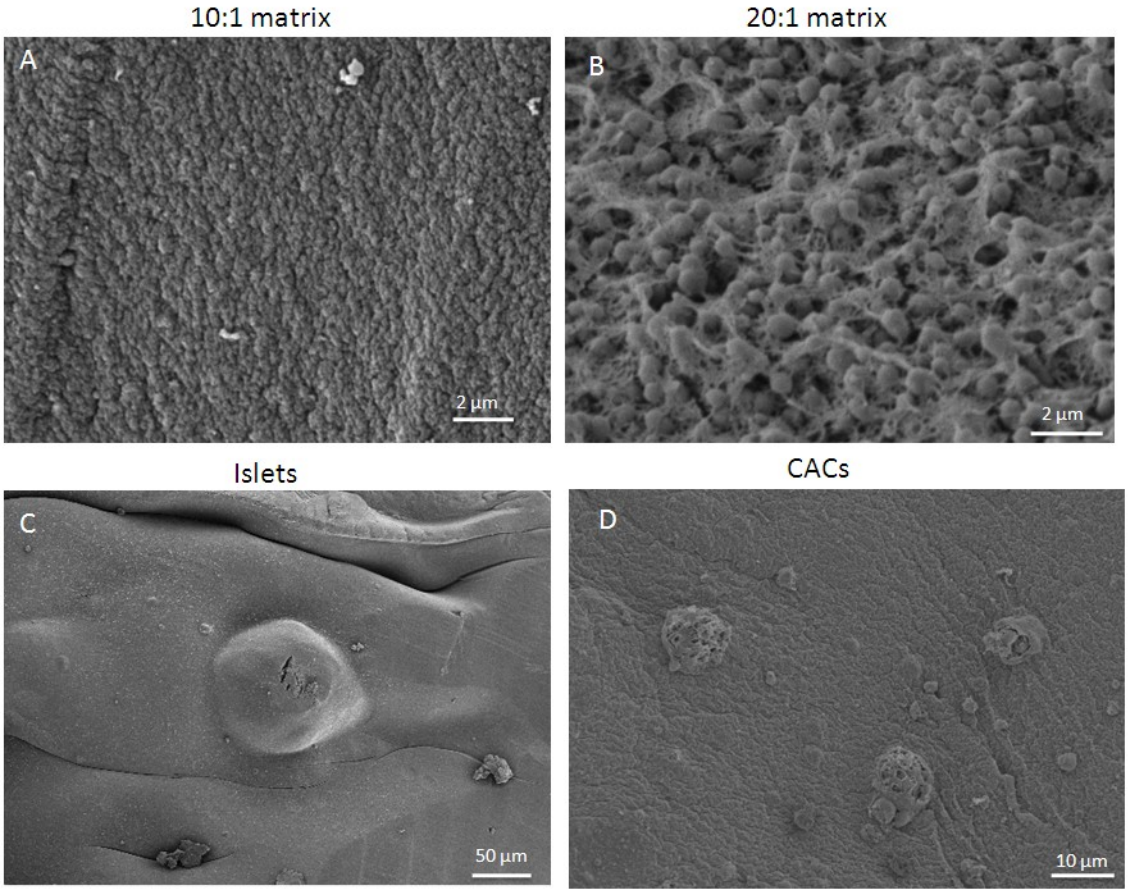

Figure 12: Fracture scanning electron micrographs of matrices

SEMs of the 10:1 matrix (A) and the 20:1 matrix (B) depicts greater porosity and pore size in the 20:1 matrix. Islets (C) and CACs (D) were embedded in matrices.

4.2.4 Surface Contact Angle

Matrices with stronger mechanical integrity demonstrated greater water contact angles, hence greater extent of hydrophobicity, as outlined in Table 1. The 10:1 matrix with Ham's media (115.67 ± 5.59°) had a significantly higher water contact angle than the 20:1 matrix with EBM (83.83 ± 11.44°, p=0.017), 10:1 matrix with EBM (97.10 ± 2.63°, p=0.008) and 20:1 matrix with Ham's (87.92 ± 11.44°, p= 0.008).

Table 1: Matrix composition effects on water contact angle

Collagen: Chitosan	Media Type	Contact Angle (°)	Standard Deviation
20:1	EBM	83.83	11.44
20:1	Ham's media	87.02	7.58
10:1	EBM	97.10	2.63
10:1	Ham's media	115.67	5.59

Smaller pore size and greater mechanical integrity of the 10:1 matrices and matrices with Ham's media contribute to greater surface tension, making them less hydrophilic biomaterials.

Proteins adsorb readily to less hydrophilic (more hydrophobic) materials. The adsorbed proteins may promote cell adhesion [6], which is advantageous for CACs as they are an adherent population of cells and their survival and their function is likely to be enhanced in this environment.

4.2.5 Rheology

Rheological properties of the matrices are reported in terms of gelation time (Figure 13). In accordance with mechanical testing data, matrices with Ham's media gelled significantly faster than matrices with EBM. The 10:1 matrix with Ham's media (352.00 ± 29.46 s) had a significantly lower gelation time compared to the 20:1 matrix with EBM (0.20-fold change; $p<0.001$), 20:1 matrix + LM with Ham's media (0.7-fold change; $p=0.012$) and the 10:1 matrix + LM with Hams media (0.6-fold change; $p=0.001$). The 20:1 matrix with EBM (1797.33 ± 120.97 s) had a significantly greater gelation time compared to the 10:1 matrix with EBM (2.1- fold change; $p=0.010$), 10:1 matrix + LM with Ham's media (3.2-fold change; $p<0.001$), 20:1 matrix +/- LM with Ham's media (3.6 and 4.5- fold change, respectively; $p<0.001$).

The trends observed in the rheological analysis solidify the contribution that chitosan content and media type play during thermogelation of the matrices. Matrices with higher chitosan content gelled faster as more amino groups on chitosan were readily available for cross-linking [17]. Furthermore, matrices with EBM supplemented with glycine displayed considerably longer gelation times compared to matrices with solely Ham's media as the glycine participates in the cross-linking reaction, hindering the thermogelation process between the matrix components [95].

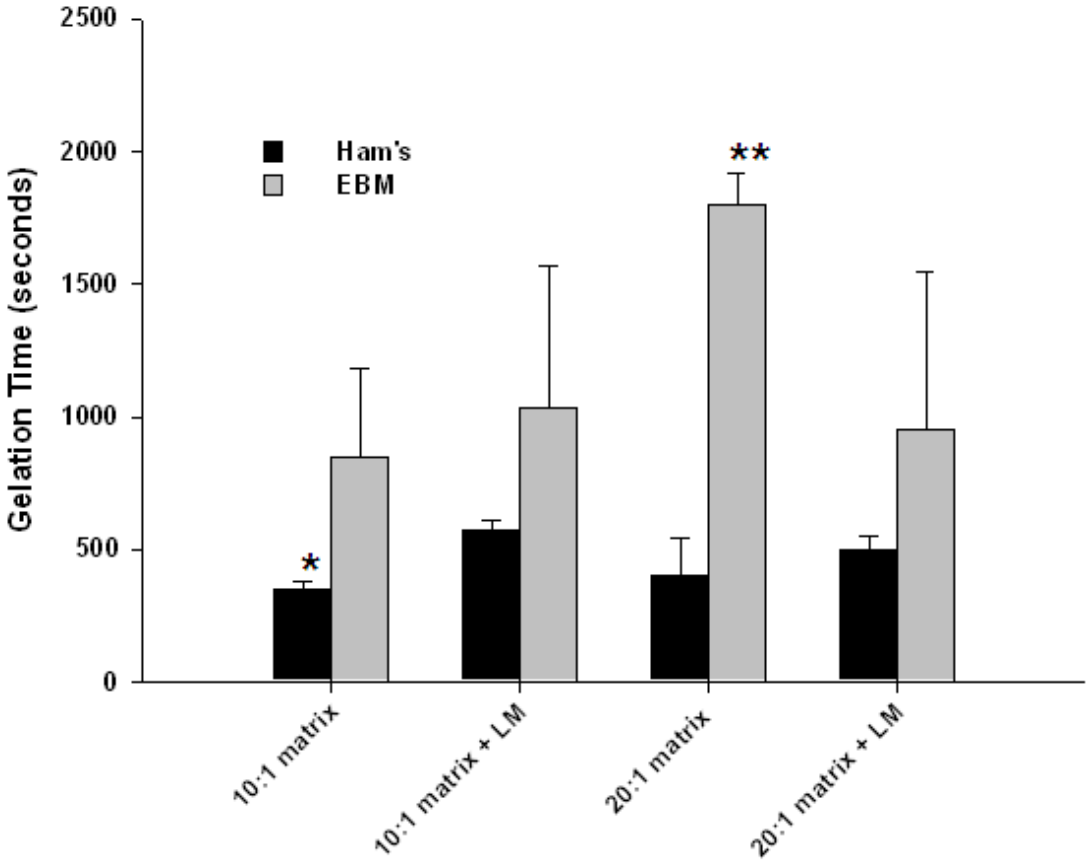

Figure 13: Matrix composition effects on gelation time

*Gelation time for each matrix composition (n=3). The gelation time for the 10:1 matrix with Ham's media matrix is significantly lower than the 10:1 matrix + LM with Ham's media, the 20:1 matrix with EBM, and the 20:1 matrix + LM with Ham's media (*p=0.001, p<0.001 and p=0.010, respectively). The gelation time for the 20:1 matrix with EBM is significantly higher than the 10:1 matrix with EBM, the 10:1 matrix + LM with Ham's media, the 20:1 matrix + LM with Ham's media, and the 20:1 matrix with Ham's media (**p=0.010, (p<0.001, p<0.001, p<0.001, respectively).*

4.2.6 Circulating Angiogenic Cell Viability

The viability of CACs embedded in matrices is illustrated in Figure 14. Viability after 24 hours was significantly higher in the 20:1 matrix + LM (90.5±3.1%) than in the 20:1 matrix (68.3±12.1%; p=0.012), and the 10:1 matrix +/- LM (74.0±11.2% and 52.3±6.3%, respectively; $p \leq 0.029$). CAC viability at 24 hours in the 20:1 matrix + LM was also superior to the that of CACs after 48 hours in the 20:1 matrix +/- LM (72.3±9.0 and 58.5±8.9%, respectively; $p \leq 0.009$). The CAC viability in the 20:1 matrix + LM at 48 hours was significantly higher than the 10:1 matrix +/- LM at this time point (40.4±7.3%; p=0.001 and 36.3±11.4%; p=0.003, respectively). The 10:1 matrix + LM 24 hour viability was significantly higher than the 10:1 matrix at 24 and 48 hours (p=0.015 and p=0.003, respectively) and the 10:1 matrix + LM at 48 hours (p=0.002). At 24 hours, the 10:1 matrix supported CAC survival to a greater extent than the 10:1 matrix +/- LM at 48 hours (p=0.048 and p=0.049 respectively). At 48 hours, the 10:1 matrix viability was significantly less than the 20:1 matrix (p=0.022).

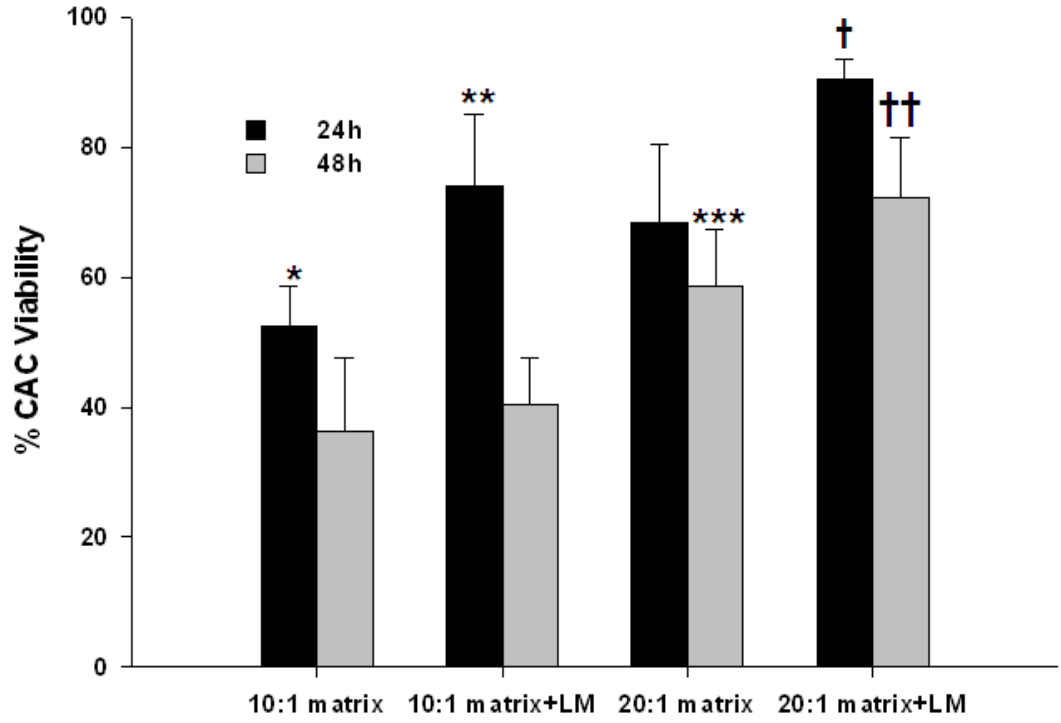

Figure 14: Matrix composition effects on CAC viability

The effect of laminin and chitosan content on CAC viability embedded in matrices at 24 hours and 48 hours (n=4). Viability was significantly higher in the 20:1 matrix + LM at 24 hours compared to 20:1 matrix, 20:1 matrix and 10:1 matrix + LM at 24 hours, as well as the 20:1 matrix, 20:1 matrix + LM at 48 hours ($^{\dagger}p \leq 0.029$). CAC viability in the 20:1 matrix + LM at 48 hours is significantly higher than the 10:1 matrix +/- LM at 48 hours ($^{\dagger\dagger}p=0.001$ and $p=0.003$, respectively). 24 hour viability in the 10:1 matrix is significantly higher than 10:1 matrix +/- LM at 48 hours ($p=0.048$ and $p=0.049$, respectively). 10:1 matrix + LM 24 hour viability is significantly higher than the 10:1 matrix at 24 hours and the 10:1 matrix +/- LM at 48 hours (**$p=0.015$, $p=0.002$, and $p=0.003$, respectively). Viability in the 20:1 matrix at 48 hours is significantly higher than the 10:1 matrix at 48 hours ($p=0.022$).*

In the first study, CAC viability in a similar collagen-based matrix without chitosan or laminin was reported. When cells were introduced into the matrix in EBM with glycine, 24 hour viability was 74.4±10.1% [14]. In the present study, CAC viability in the 20:1 matrix + LM was 90.5±3.1% and 72.3±9.0% at 24 and 48 hours, respectively. A comparison of these results demonstrates that CAC viability is enhanced in the matrix with chitosan and laminin additives, at least at early time points. Viability was lower in the high chitosan concentration hydrogel (10:1 matrix) compared to the low chitosan concentration matrix (20:1), which may be explained by the material's porosity. As seen in SEM micrographs of the matrices, pore size decreases with increasing chitosan content. The smaller pores of the 10:1 matrices may limit diffusion of nutrients to the CACs and hinder removal of waste within the 3D environment, ultimately leading to increased cell death [25].

The addition of laminin enhanced the capacity of the hydrogels to support CAC survival. The RGD-peptide sequence of laminin provides a site for cell attachment to enhance cell-material interactions, thus increasing CAC survival [72].

Despite reduced CAC viability in matrices compared to baseline controls (89.8 ± 2.3%), collagen matrices can enhance the viability and ability of the transplanted CACs to revascularize and restore perfusion to ischemic tissue (rat model) compared to transplanted cells alone [27, 85].

4.2.7 Islet Viability and Functionality

For all matrix conditions viability remained above 69% over the 48 hour period. The ability of the matrices to support islet survival is presented in Figure 15A. Islet viability in the 20:1 matrix + LM was significantly greater compared to the 10:1 matrix at 24 hours (94.9±5.9%

and 70.9±0.03%, respectively; p=0.002). Viability at 48 hours significantly decreased in the 20:1 matrices +/- LM compared to their corresponding 24 hour viability (94.9±5.9% and 80.4±4.0%, 89.7±10.0% and 69.3±6.4%, respectively; p=0.04 and p=0.03). Representative flow cytometry scatterplots are shown in Figure 15B.

Islets embedded in matrices maintain their insulin secretion capabilities at 24 hours, as shown in Figure 16. The 20:1 matrices +/- LM displayed superior stimulation indices (1.86±0.64 and 1.65±0.50, respectively) compared to 10:1 matrix (0.90±0.20, p<0.038). This is in accordance with the viability data, confirming islets are better supported by the 20:1 matrix +/- LM compared to the 10:1 matrix.

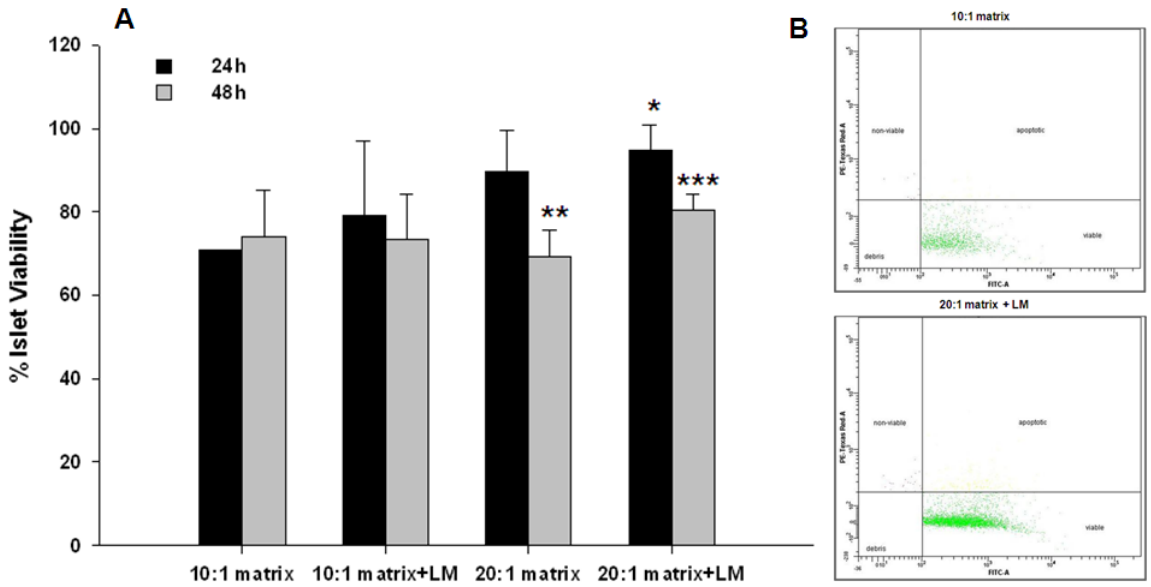

Figure 15: Matrix composition effects on islet viability

*(A) Viability of islets embedded in each matrix formulation for 24 and 48h (n=3), measured by flow cytometry. Viability in 20:1 matrix+LM was significantly increased compared to 10:1 matrix at 24h (*p=0.002). Islet viability at 48hr in 20:1 matrix and 20:1 matrix+LM decreased significantly compared to their 24h viability (**p=0.04 and ***p=0.03, respectively).(B) Representative flow cytometry scatterplots.*

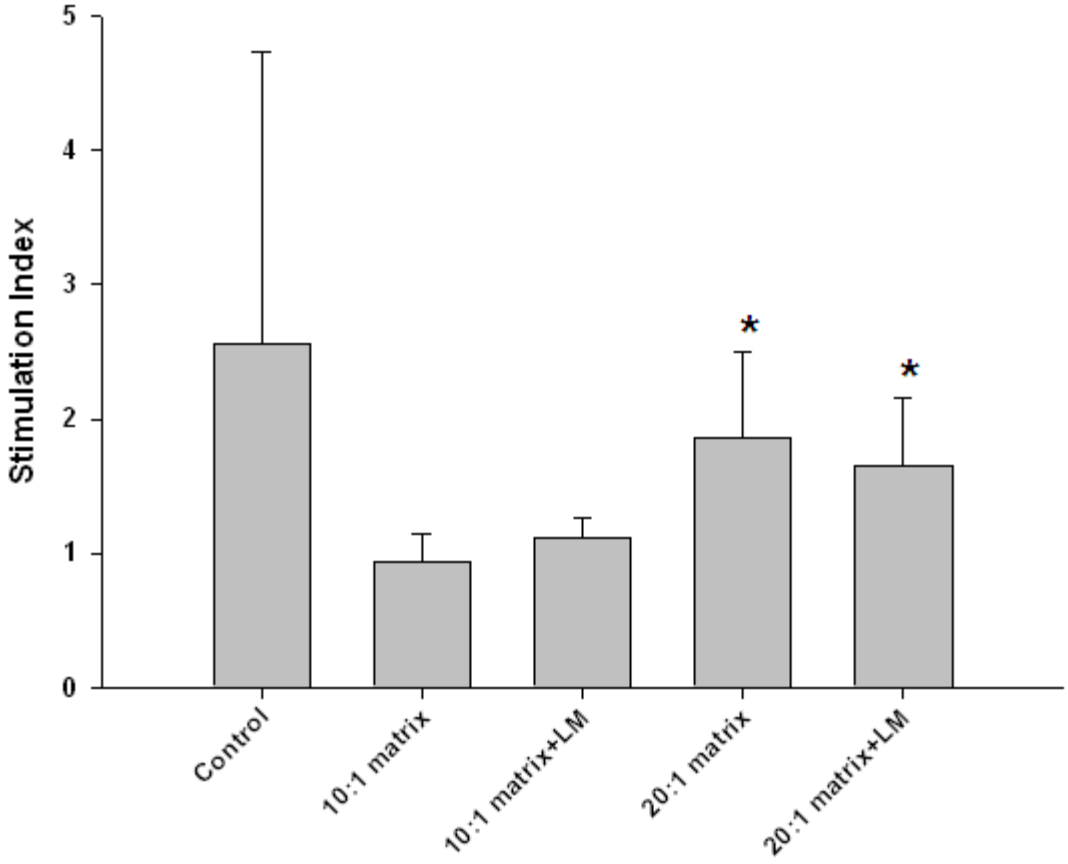

Figure 16: Matrix composition effects on islet functionality

*Islet functionality based on stimulation index following a glucose challenge at 24h (n=4). *p≤0.038 compared to the 10:1 matrix.*

Islet survival and functionality was superior in the 20:1 matrix + LM, which may be attributed to several factors including mechanical environment and cell-matrix interactions. Mechanical properties of the scaffold are strongly associated to cell survival, function, motility and morphology [78]. The goal was to provide a 3D environment similar to the native tissue for *in vitro* islet culture, and future *in vivo* implantation. Based on the analysis of the materials' mechanical properties, the elastic moduli of the 20:1 matrix + LM with Ham's media is 7.8±4.7 kPa. One group reported the elastic modulus of the pancreas to be 8.25 kPa, via atomic force microscopy methods [107]. While this value cannot be directly compared to the compression elastic modulus determined in the current study, it can be appreciated that the values are within the same range and closer to that of the 20:1 matrices compared to the 10:1 matrices. The 20:1 matrices may physically mimic the local environment of the pancreas more so than the 10:1 matrices, contributing to enhanced islet support capabilities. Furthermore, the addition of laminin improved islet performance at 24 hours which may be attributed to improved cell-material interactions. Weber *et al.* demonstrated PEG encapsulation of murine islets supplemented with 100 μg/mL laminin had a 4-fold increase in glucose-stimulated insulin secretion compared to encapsulation without laminin. Encapsulated islets pre-treated with α_6 antibody no longer exhibited augmented insulin secretion, thus confirming that the mechanism involved islet-laminin interactions, specifically through the laminin receptor $\alpha_6\beta_1$ [74]. Another study reported stimulation indices of human islets of ~1 and ~1.5 when plated on collagen type-I and laminin, respectively [70]. The stimulation indices of all the collagen-chitosan hydrogels fall within this range. In addition, human islets cultured on either collagen type-I, collagen type-I supplemented with additional ECM components, or in a 3D poly(DL-lactide-co-glycolide)

acid scaffold with collagen type-I and additional ECM components displayed increasingly higher stimulation indices (~0.9, ~1.2 and ~1.5, respectively for each culture condition) [108], thus confirming the importance of mimicking the native islet environment by providing ECM components in a 3D structure.

4.3 Study II: Summary

Collagen-chitosan matrices can be tailored to encompass a range of physical properties in order to optimize cell support capabilities. Matrices with lower chitosan content, and therefore decreased mechanical integrity, displayed superior support for islet and CAC survival and function *in vitro*. Incorporating laminin into the matrices further enhanced these cell support capabilities. A collagen-chitosan matrix, supplemented with laminin, is a promising scaffold for the delivery of vascular cells and islets for the treatment of type-I diabetes.

CHAPTER 5: CONCLUSIONS, FUTURE WORK AND RECOMMENDATIONS

5.1 Conclusions

Hydrogels are attractive biomaterials for the delivery of therapeutic cells for soft tissue regeneration. Among their many applications, they can be tailored for use as an injectable scaffold for delivery of angiogenic cells for cardiac regeneration and as a scaffold for islet cell transplantation for the treatment of type-I diabetes.

The first study depicted the necessity of ascertaining interactive effects between biomaterials and therapeutic additives. The collagen-based matrix used for this study best supported cell viability when cells were introduced into the matrix in a glycine solution, which protected the cells from the negative effects of the chemical cross-linker. Additives such as alginate microspheres or CACs increase the mechanical integrity of the material by participating in the cross-linking reaction. These factors are important to consider in order to provide the best cell support capabilities whilst maintaining mechanical property harmony between biomaterial and native tissue.

The second study demonstrated the importance of designing islet transplantation scaffolds to match the environmental properties of the native tissue of the islets. Providing a highly porous material (20:1 matrix) that displayed lower compression modulus resulted in increased cell survival for both CACs and islets. The addition of laminin further increased cell survival.

5.2 Future Work and Recommendations

Hydrogels can be fine-tuned to meet the needs of various soft tissue applications. Further investigation into ideal concentrations of each matrix component and the effect of additional ECM additives would further aid to refine biomaterials for specific applications. The next

steps for further characterizing these matrices include *in vivo* experiments to give insight into *in situ* degradation and remodeling, and to elucidate mechanisms of cell support within the tissue they are designed to regenerate. The use of physical cross-linkers instead of chemical cross-linkers warrants exploration as the chemical cross-linking reaction has negative effects on cell viability, therefore potentially reducing the therapeutic potential of the cells prior to delivery.

Hydrogels offer an alternative technique to transplant therapeutic cells for cardiovascular disease and type-I diabetes treatments. With the prevalence of these ailments worldwide, such novel treatment techniques may be necessary to improve upon the moderate success of cell therapies that have been reported to-date.

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Appendix A

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Appendix B

Sample Instron Mechanical Tester Compression Raw Data for 20:1 collagen:chitosan matrix with Ham's media

Parameters	Sample 1	Sample 2	Sample 3	Sample 4
0mm position (mm)	-51.54	-51.54	-51.54	-51.54
Diameter (cm)	0.6	0.6	0.6	0.6
Area (m^2)	2.82743E-05	2.82743E-05	2.82743E-05	2.82743E-05
Thickness (mm)	2.185627	2.125153	2.126903	2.009262

Sample 1		Sample 2		Sample 3		Sample 4	
Position (mm)	Load (gf)	Position (mm)	Load (gf)	Position (mm)	Load (gf)	Position (mm)	Load (gf)
-49.354373	0.044977069	-49.414847	0.071112392	-49.413097	0.066857805	-49.530738	0.11548166
-49.35105	0.40783261	-49.415708	0.19449543	-49.413322	-0.18142777	-49.529098	0.01701835
-49.354938	-0.24372709	-49.412659	-0.011548166	-49.412949	-0.36802183	-49.530666	0.70626154
-49.352339	0.19935782	-49.414298	0.1039335	-49.414836	0.088738541	-49.53122	-0.004254588
-49.352651	-0.032821104	-49.416278	-0.2057397	-49.410707	0.15377295	-49.53122	0.036467894
-49.351966	0.079621568	-49.415126	-0.23521791	-49.413273	0.18902525	-49.531352	0.31727067
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-49.883713	-1.9115254	-49.956596	-6.1111073	-49.949341	-7.4130111	-50.035445	-11.024244
-49.88759	-1.4866745	-49.954183	-6.4374949	-49.950559	-7.6473173	-50.038335	-11.023029
-49.890891	-1.858647	-49.956794	-6.2931428	-49.950542	-7.801698	-50.038593	-11.127874
-49.889356	-1.8112387	-49.957512	-6.5432518	-49.95454	-7.7545937	-50.039032	-11.405638
-49.892619	-2.0306539	-49.95794	-6.3818814	-49.953953	-7.9873804	-50.039328	-11.236974
-49.891237	-1.9875002	-49.958049	-6.4137908	-49.955105	-8.0016637	-50.040024	-11.329055
-49.891018	-1.9932743	-49.959108	-6.8432002	-49.954688	-8.0031831	-50.040413	-11.276784
-49.890294	-1.8452754	-49.961866	-6.5323114	-49.956267	-7.7664457	-50.040567	-11.290156
-49.89433	-1.8562158	-49.960517	-7.0544101	-49.957128	-8.2639286	-50.042431	-11.645414
-49.893853	-1.8449715	-49.962946	-6.9507805	-49.959782	-8.0591006	-50.044428	-11.867868
-49.897033	-1.9331023	-49.964054	-6.798831	-49.958159	-8.2912795	-50.044104	-11.901905
-49.895218	-1.8212674	-49.964388	-6.889089	-49.959689	-7.9791751	-50.046429	-11.918012
-49.897165	-1.888733	-49.964613	-6.7699605	-49.962551	-8.1110673	-50.046116	-12.068138
-49.899451	-2.0020874	-49.966472	-6.9896796	-49.962508	-8.235666	-50.047438	-12.156876
-49.898536	-2.2661757	-49.968298	-6.8043011	-49.962957	-8.0542382	-50.048288	-12.421572
-49.899928	-1.9103098	-49.967931	-6.7073573	-49.9656	-8.2064917	-50.048557	-12.503929
-49.902884	-2.3874314	-49.970645	-7.01855	-49.965502	-8.542908	-50.049505	-12.493293
-49.901617	-2.0741114	-49.967893	-7.2166922	-49.965781	-8.6757119	-50.050635	-12.666515
-49.904162	-2.4746505	-49.970448	-7.1653333	-49.968885	-8.2192554	-50.051622	-12.428562
-49.903339	-2.0792777	-49.970316	-7.3412909	-49.968161	-8.6830055	-50.054177	-12.448012
-49.906783	-2.3318179	-49.972597	-7.4592037	-49.968112	-8.9790032	-50.055384	-13.009313
-49.9063	-2.5491058	-49.972247	-7.2021051	-49.969675	-8.9115376	-50.054375	-12.835787
-49.906964	-2.1956711	-49.975153	-7.3935615	-49.971128	-8.7173461	-50.053914	-13.074348
-49.908028	-1.7404302	-49.974747	-7.2783838	-49.971852	-8.8413369	-50.055801	-13.13452
-49.908532	-2.4919727	-49.975718	-7.3254881	-49.97308	-8.7522945	-50.057402	-12.873166
-49.910545	-2.2482456	-49.975783	-7.3063425	-49.972641	-9.0027073	-50.058778	-12.955523
-49.909289	-2.1920243	-49.978778	-7.4175696	-49.974703	-9.157392	-50.058822	-13.172507
-49.911225	-2.3518752	-49.976491	-7.6047714	-49.972762	-9.1801844	-50.059414	-13.030282
-49.912557	-2.0023913	-49.978531	-7.5327473	-49.975082	-9.0002761	-50.058784	-12.901125
-49.914394	-2.2670874	-49.979211	-7.6011246	-49.976595	-8.8787165	-50.060149	-13.307742
-49.914372	-2.195975	-49.979315	-7.5418643	-49.975981	-9.109072	-50.062973	-13.325368
-49.917734	-2.1747021	-49.983609	-8.0317497	-49.979902	-9.3676902	-50.060549	-13.610122
-49.916856	-2.2242376	-49.981355	-8.0764228	-49.978717	-9.3603966	-50.063214	-13.931951
-49.918831	-2.3573454	-49.982473	-7.9609412	-49.981267	-9.452478	-50.064388	-13.799147
-49.916961	-2.5381654	-49.984124	-7.8020019	-49.981377	-8.9118415	-50.064547	-13.968419
-49.918732	-2.2926149	-49.985034	-7.9174836	-49.98215	-9.6232693	-50.065655	-14.403906
-49.91962	-2.6092778	-49.984612	-8.1499664	-49.982029	-9.4500468	-50.065485	-14.055942

-49.921578	-2.5867893	-49.989125	-8.1457118	-49.984404	-9.5667441	-50.066115	-13.955655
-49.921846	-2.9040599	-49.988451	-8.2131774	-49.984766	-9.5928794	-50.066965	-14.520907
-49.922165	-2.5901321	-49.988588	-8.0864515	-49.985248	-9.2783438	-50.070222	-14.261378
-49.923716	-2.5509292	-49.989849	-8.3459813	-49.986657	-9.437587	-50.067486	-14.519084
-49.924144	-2.3588649	-49.990962	-8.5380456	-49.988495	-9.8265778	-50.071133	-14.831796
-49.92444	-2.4087044	-49.990902	-8.4003793	-49.989706	-9.9198749	-50.072295	-14.731813
-49.924852	-2.6420989	-49.992393	-8.3985559	-49.987842	-9.8542326	-50.071966	-14.696257
-49.925729	-2.7730794	-49.995552	-8.6052073	-49.991543	-10.269663	-50.07195	-14.735156
-49.928339	-2.7475519	-49.993452	-8.6738885	-49.988028	-10.376635	-50.07439	-15.207416
-49.930368	-2.4865025	-49.997608	-8.6389401	-49.990896	-9.8949551	-50.074796	-15.010489
-49.929222	-2.6600289	-49.997866	-8.9470938	-49.99304	-10.06605	-50.077954	-15.464818
-49.930818	-2.7840198	-49.998985	-8.7911935	-49.995947	-9.9721455	-50.076485	-15.107433
-49.929491	-2.7624429	-49.998886	-8.6778392	-49.99457	-10.348069	-50.076973	-15.118069
-49.931733	-2.8697193	-49.997252	-8.9410158	-49.996797	-10.573258	-50.07983	-15.041183
-49.932485	-2.9924946	-49.999835	-8.9592498	-49.996967	-10.337128	-50.079501	-15.503109
-49.933675	-2.6961929	-50.000498	-8.9741408	-49.99797	-10.431337	-50.081245	-15.436252
-49.936373	-2.5171964	-50.001387	-9.0756431	-49.997175	-10.485431	-50.080976	-16.014572
-49.937371	-2.5579188	-50.003443	-9.1230514	-49.998497	-10.708797	-50.083098	-15.620414
-49.935616	-2.539381	-50.006349	-9.3348691	-50.000728	-10.644066	-50.084354	-16.126103
-49.938555	-2.9225978	-50.003893	-9.3196741	-50.001145	-11.002971	-50.083981	-16.133396
-49.937842	-2.7815886	-50.007397	-9.7578966	-50.001584	-10.870775	-50.084787	-16.403866
-49.938254	-2.4576321	-50.005724	-9.4236076	-50.002023	-11.04187	-50.085648	-16.520867
-49.941571	-2.5205392	-50.008164	-9.3558381	-50.003388	-11.139422	-50.087205	-16.268935
-49.940052	-2.7587961	-50.006651	-9.4509585	-50.003448	-11.114198	-50.087562	-16.947542
-49.942942	-2.671881	-50.01004	-9.633298	-50.006349	-11.427518	-50.087907	-16.788299
-49.941609	-2.698928	-50.010254	-9.9174437	-50.007281	-11.501974	-50.089393	-16.788907
-49.943825	-3.0265313	-50.011877	-9.7186936	-50.006196	-11.137599	-50.088708	-16.716275
-49.945278	-3.021365	-50.010851	-9.521767	-50.007183	-11.291979	-50.092168	-16.895271
-49.946457	-3.2450347	-50.013971	-9.6597372	-50.009957	-11.605299	-50.091735	-17.215885
-49.947927	-3.0241001	-50.014388	-10.018642	-50.008778	-11.341819	-50.09338	-17.454749
-49.946254	-2.9855049	-50.015414	-9.9912911	-50.011937	-11.749044	-50.09491	-17.537714
-49.948705	-3.1374544	-50.015441	-9.7746111	-50.013516	-11.651492	-50.09678	-17.502766
-49.950559	-3.265396	-50.017898	-10.316159	-50.013149	-11.668814	-50.095529	-17.793293
-49.952001	-3.0082973	-50.015567	-10.152358	-50.015638	-11.612593	-50.097679	-17.783872
-49.951529	-3.3237446	-50.019537	-10.334089	-50.013253	-11.927432	-50.094553	-18.188058
-49.954375	-3.0307859	-50.019164	-10.228029	-50.015501	-11.527197	-50.094817	-18.226349
-49.953904	-3.1894212	-50.020201	-10.341079	-50.016889	-11.989124	-50.101748	-18.340008
-49.954414	-3.2319671	-50.020217	-10.283946	-50.018271	-11.787335	-50.101731	-18.059813
-49.956371	-2.8472308	-50.023019	-10.620058	-50.016971	-11.997025	-50.100048	-18.179853
-49.957079	-3.2444269	-50.021511	-10.817289	-50.017174	-12.090626	-50.10081	-18.525386
-49.957852	-3.334685	-50.025997	-10.992943	-50.018523	-12.29667	-50.103799	-18.300501
-49.959245	-3.2951781	-50.025054	-10.510047	-50.019373	-12.176326	-50.103355	-18.209027
-49.959141	-3.4775176	-50.027434	-11.115414	-50.022948	-12.501194	-50.103207	-18.660925
-49.961394	-3.491193	-50.027395	-10.909066	-50.023184	-12.396349	-50.103678	-18.742978
-49.962085	-3.753154	-50.02796	-10.717914	-50.02172	-12.711492	-50.104424	-19.015271
-49.962617	-3.6018123	-50.03119	-11.379198	-50.026183	-12.957954	-50.104983	-19.286349
-49.964844	-3.2155565	-50.02819	-11.42296	-50.024566	-12.711492	-50.106288	-19.225266
-49.963385	-3.4589797	-50.031069	-11.638424	-50.025575	-12.980139	-50.106228	-19.353511
-49.963769	-3.6859923	-50.032961	-11.656658	-50.02716	-12.509703	-50.107835	-19.409732

-49.967377	-3.4212962	-50.033477	-11.660609	-50.027308	-12.888665	-50.109074	-19.665008
-49.965743	-3.4343639	-50.033164	-11.453046	-50.029567	-12.603608	-50.111695	-19.530684
-49.968381	-3.2453386	-50.035193	-11.701635	-50.028646	-12.939113	-50.11062	-19.857984
-49.969428	-3.4474315	-50.036345	-11.797971	-50.032627	-13.292243	-50.111931	-19.598454
-49.969812	-3.3720646	-50.036202	-12.00128	-50.033537	-13.152146	-50.113055	-19.739159
-49.970371	-3.5127698	-50.03675	-12.01374	-50.034365	-13.434772	-50.114804	-19.914813
-49.973047	-3.6574258	-50.03709	-12.333746	-50.034442	-13.784864	-50.115397	-20.223878
-49.97149	-3.3620359	-50.038566	-12.078774	-50.035297	-13.904904	-50.117787	-20.540237
-49.972164	-3.5282687	-50.038149	-11.924697	-50.034881	-13.864181	-50.114892	-20.58491
-49.973425	-3.5039568	-50.039755	-12.123143	-50.037036	-14.015523	-50.117157	-20.635054
-49.975602	-3.6835612	-50.041675	-12.664388	-50.038116	-13.734113	-50.118945	-20.906739
-49.976655	-3.7476839	-50.043369	-12.322805	-50.039679	-14.234634	-50.119657	-20.872703
-49.976173	-4.0169385	-50.043419	-12.708757	-50.041132	-14.058069	-50.119186	-20.97694
-49.978246	-3.9206025	-50.043726	-12.556807	-50.040902	-14.113987	-50.120118	-21.381734
-49.978761	-3.6580336	-50.046034	-12.984697	-50.042706	-14.399652	-50.121648	-21.359853
-49.978799	-3.9832057	-50.048529	-13.048212	-50.042289	-14.706286	-50.124593	-21.450111
-49.981152	-3.8084637	-50.04587	-12.813906	-50.045031	-14.466206	-50.122103	-21.733649
-49.983104	-3.7735153	-50.049429	-12.837306	-50.044614	-14.666171	-50.125985	-21.702955
-49.981892	-3.3067263	-50.050778	-13.408029	-50.04616	-14.751871	-50.123288	-22.031166
-49.983806	-3.8893009	-50.049094	-13.139382	-50.04667	-14.782261	-50.125454	-22.008678
-49.984382	-3.8817034	-50.050465	-13.14546	-50.046687	-14.845168	-50.127439	-21.929056
-49.985095	-3.8133261	-50.052779	-13.2187	-50.047454	-14.72452	-50.127614	-22.106533
-49.985396	-3.8820073	-50.053223	-13.346337	-50.049198	-14.998637	-50.128716	-22.583959
-49.987946	-4.2241977	-50.054002	-13.757209	-50.050235	-14.975844	-50.130054	-22.473643
-49.988401	-3.9184752	-50.055044	-13.896395	-50.051079	-15.559635	-50.132094	-22.836803
-49.988061	-3.9850291	-50.055828	-13.746572	-50.053717	-15.235982	-50.131787	-22.630759
-49.989257	-4.2339224	-50.058608	-13.799755	-50.051408	-16.04952	-50.133844	-22.454498
-49.992229	-3.7495073	-50.056645	-14.431865	-50.056426	-15.735592	-50.131327	-23.218804
-49.990715	-3.9166518			-50.056256	-15.358149	-50.132456	-22.899102
-49.994291	-4.0540142						
-49.99281	-4.292575						
-49.995398	-4.2284523						
-49.993156	-3.8452355						
-49.996572	-4.0151151						
-49.997394	-4.1166174						
-49.998491	-4.3846564						
-49.999462	-5.0207172						
-50.000509	-4.6004248						
-50.000361	-4.3491002						
-50.00216	-4.4265945						
-50.004392	-4.3645991						
-50.004682	-4.497403						
-50.003969	-4.6186587						
-50.005417	-4.7599718						
-50.008702	-4.310505						
-50.005039	-4.7168181						
-50.009223	-5.0413824						
-50.009661	-4.5597023						
-50.009146	-4.7380911						

-50.009404	-4.6767035
-50.010029	-4.7690888
-50.010703	-4.8848743
-50.011805	-4.9644959
-50.015052	-5.4999662
-50.016647	-4.8566117
-50.015063	-4.6475291

To convert load (gf) to load (N). The following equation was used:

$$Load(N) = Load(gf) \cdot \frac{1kg}{1000g} \cdot 9.81N$$

Load(N) was converted to stress (MPa) using the following equation:

$$Stress(MPa) = \frac{Load(N)}{Cross - \text{sec}tionalArea\,(mm^2)}$$

Displacement data was converted to strain using the equation:

$$Strain = \frac{(l - l_o)}{l_o}$$

where l= crosshead position and l_o= crosshead position when load is zero.

Stress (MPa) and strain data for each sample was plotted and fit to a five parameter double exponential growth model using Sigma Plot v10.0, as explained in section 4.1.3. Fitting parameters and R-squared values are summarized below. Stress-strain curves shown in Figure 9 are averaged over the 4 samples. Stress and modulus data in Figure 9 are presented in kPa for easier readability.

	Sample 1	Sample 2	Sample 3	Sample 4		
a	6.56E-06	7.83E-05	8.53E-05	0.0004		
b	16.2253	13.0728	13.745	5.865		
c	6.80E-06	2.52E-08	9.79E-11	3.11E-05		
d	16.2257	34.7643	47.2609	17.7768		
y0	-5.90E-05	-0.0001	-0.0001	-0.0005		
R-squared	0.9797	0.9973	0.9978	0.9987		
y = y0+a*exp(b*x)+c*exp(d*x)						
Strain	**Stress (MPa)**				**Average**	**SD**
0	-4.56369E-05	-2.17218E-05	-1.47409E-05	-0.0000689	**-3.8E-05**	**2.46E-05**
0.05	-2.89239E-05	5.05869E-05	6.95164E-05	0.000111871	**5.08E-05**	**5.9E-05**
0.1	8.69332E-06	0.000190047	0.000237048	0.000402858	**0.00021**	**0.000162**
0.15	9.33613E-05	0.000460695	0.000570229	0.000911138	**0.000509**	**0.000337**
0.2	0.00028393	0.000995417	0.001233589	0.001879957	**0.001098**	**0.000659**
0.25	0.000712857	0.002105305	0.002562263	0.0038778	**0.002315**	**0.001306**
0.3	0.001678277	0.004704703	0.005307603	0.008256334	**0.004987**	**0.002697**
0.35	0.00385122	0.012350126	0.011866753	0.018261579	**0.011582**	**0.005918**
0.4	0.008742029	0.042108379	0.036601491	0.041733018	**0.032296**	**0.015902**
0.45	0.019750146	0.184966814	0.210003837	0.097663802	**0.128096**	**0.08681**
y' = a*b*exp(b*x)+c*d*exp(d*x)						
Strain	**Modulus (MPa)**				**Average**	**SD**
0	0.000216807	0.001023863	0.00117189	0.002898236	**0.001328**	**0.001128**
0.05	0.000487984	0.001971704	0.002330038	0.004488673	**0.00232**	**0.001651**
0.1	0.001098342	0.003809426	0.0046331	0.007484464	**0.004256**	**0.002629**
0.15	0.002472122	0.007430491	0.009216226	0.01360113	**0.00818**	**0.004604**
0.2	0.005564194	0.014892534	0.018371975	0.026910006	**0.016435**	**0.008832**
0.25	0.012523756	0.032084665	0.037036778	0.057177954	**0.034706**	**0.018343**
0.3	0.028188173	0.081323608	0.079043941	0.127978798	**0.079134**	**0.040768**
0.35	0.063445271	0.268043036	0.214586846	0.296407048	**0.210621**	**0.103815**
0.4	0.142801113	1.15055687	1.036727467	0.701006557	**0.757773**	**0.45222**
0.45	0.321413363	5.824685298	8.542266472	1.678319348	**4.091671**	**3.779341**