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Zahra Arzhangi

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine)

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

**Collagen Biomaterials for the Delivery of FVIII-Producing Blood Outgrowth Endothelial Cells in the
Treatment of Haemophilia A**

TITRE DE LA THÈSE / TITLE OF THESIS

Mary Griffith

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

James Harden

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Catherine Tsilfidis

John Copeland

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

**COLLAGEN BIOMATERIALS FOR THE DELIVERY OF FVIII-PRODUCING
BLOOD OUTGROWTH ENDOTHELIAL CELLS IN THE TREATMENT OF
HAEMOPHILIA A**

**A Thesis Submitted to the
School of Graduate Studies
University of Ottawa**

In Partial Fulfillment of the Requirements for the Degree of
Master of Science
Department of Cellular and Molecular Medicine
Faculty of Medicine

By

Zahra S. Arzhangi, B.Sc.

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ABSTRACT

In this thesis, we describe the fundamental aspects to the development of molecularly-defined tailor-made scaffolds for the delivery of FVIII-expressing endothelial cells in the treatment of Haemophilia A. In particular, scaffolds prepared by chemical crosslinking of type I collagen, and growth factor incorporation is discussed. The general strategy was to prepare tailor-made biomaterials as a specific microenvironment to enable cells to produce FVIII and secrete this coagulation factor into the blood stream. As an initial step, pure materials of known concentrations were combined to develop two forms of collagen scaffolds: an injectable hydrogel that may be formed *in situ* in the presence of cells and a capsule into which the cell-housing hydrogel can be injected. Next, scaffolds were crosslinked using natural- genipin- and synthetic- carbodiimide (EDC)- chemicals. Crosslinking resulted in collagenase-resistant scaffolds. The tissue response to scaffolds was evaluated following subcutaneous implantations in mice. Crosslinked scaffolds maintained their integrity and supported the formation of new extracellular matrix and neovascularization during tissue remodeling. Collagen scaffolds loaded with fibroblast growth factor 2 significantly enhanced FVIII production during long-term encapsulation. Potential applications of these scaffolds for ischemic models are discussed in more detail.

TABLE OF CONTENTS

ABSTRACT.....	ii
TABLE OF CONTENTS	iii
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	viii
ACKNOWLEDGEMENTS	x
1 INTRODUCTION	1
1.1. Haemophilia	1
1.2. Current Treatments and Limitations.....	2
1.2.1. Intravenous FVIII Infusions.....	2
1.3. Gene Transfer for Alternate Therapy	3
1.3.1. Direct Transgene Delivery	3
1.3.2. <i>Ex vivo</i> Transgene Delivery	4
1.3.3. Delivery of Therapeutic Cells	5
1.4. Extracellular Matrix Macromolecules as Cell Delivery Scaffolds	6
1.4.1. Matrigel™	6
1.4.2. Collagen	7
1.5. Crosslinkers	8
1.5.1. Genipin	9
1.5.2. Water-Soluble Carbodiimides	10
1.6. Growth Factors	14

1.6.1. Fibroblast Growth Factor 2 (FGF-2) and Delivery	14
1.7. Objectives and Hypotheses	17
2 METHODS	18
2.1. Cell Culture	18
2.1.1. IHUVECs	18
2.1.2. BOECs	18
2.2. Animals	19
2.3. Stereo and Scanning Electron Microscopy (SM and SEM)	20
2.4. Collagen Capsules	20
2.5. Collagen Thermogel	21
2.6. FGF-2-loaded Alginate Microparticles	21
2.6.1. Loading Capsules	22
2.7. Collagenase Digestion Analysis	22
2.7.1. Collagen-coated EDC-crosslinked Capsule	22
2.7.2. Genipin-crosslinked Collagen Thermogel	23
2.8. Cell Encapsulation within Collagen Thermogel	24
2.8.1. BOEC Viability	24
2.8.2. BOEC Characterization	24
2.8.2.a. Uptake of Acetylated Low Density Lipoprotein	24
2.8.2.b. Expression of Endothelial Marker CD31	25
2.9. FGF-2 Release and Bioactivity	26
2.9.1. ELISA Analysis	26

2.9.2.	WST-1: FGF-2 Bioactivity	26
2.9.3.	CyQUANT Cell Proliferation Assay	27
2.9.4.	Western Blot	28
2.10.	FVIII Release	29
2.10.1.	Serum FVIII Functionality Assay	29
2.10.2.	Serum cFVIII Antigen Quantification	30
2.11.	Tissue Fixation and Processing	30
2.12.	Histology	31
2.12.1.	Haematoxylin and Eosin Staining	31
2.12.2.	Immunohistochemistry	31
2.13.	Statistical Analysis	32
3	RESULTS	33
3.1.	Collagen Capsule	33
3.2.	<i>In vitro</i> FVIII Production and Release	34
3.3.	FGF-2 Alginate Microparticles	38
3.3.1.	<i>In vitro</i> FGF-2 Release and Bioactivity	40
3.4.	BOEC-FVIII Encapsulation and Implantation	41
3.5.	Pre- and Post-Implantation: Gross and Histological Assessment	46
4	DISCUSSION	53
4.1.	Collagen Capsule	53
4.2.	Release and Functionality of FVIII	54

4.3. Genipin Crosslinking	57
4.4. FGF-2/ Alginate Microparticles	58
4.5. Future Directions	60
4.6. Alternate Sites for Implants	61
4.7. Versatility of the Collagen Capsule System	62
5 CONCLUSION	63
6 APPENDIX	65
6.1. Genipin-Crosslinnked Collagen Thermogel	65
6.2. Primary, Secondary and Tertiary FGF-2 Encapsulation	69
6.3. Western Blot	69
7 REFERENCES	72

LIST OF FIGURES

FIGURE 1.1. Structure of geniposide, and genipin. -----	12
FIGURE 1.2. Collagen crosslinking using genipin. -----	12
FIGURE 1.3. Collagen crosslinking using EDC and NHS. -----	13
FIGURE 1.4. Bioactivity of FGF-2 in the ERK1/2 pathway -----	16
FIGURE 3.1. SEM microscopy of capsules -----	35
FIGURE 3.2. Enzymatic collagenase degradation of capsules. -----	36
FIGURE 3.3. <i>In vitro</i> FVIII release and functionality -----	37
FIGURE 3.4. FGF-2-loaded alginate microparticles -----	39
FIGURE 3.5. Bioactivity of FGF-2 released from thermogels -----	42
FIGURE 3.6. Bioactivity maintenance profile of released FGF-2 -----	43
FIGURE 3.7. Serum canine FVIII antigen levels released from capsules -----	45
FIGURE 3.8. Collagen capsule pre- and post-implantation -----	47
FIGURE 3.9. Host response to collagen capsule implant -----	48
FIGURE 3.10.A. CD31-positive structures on the apical side of the implant -----	50
FIGURE 3.10.B. CD31-positive structures on the apical side of the implant -----	51
FIGURE 3.11. SMA-positive structures near capsule wall -----	52
FIGURE 6.1. Enzymatic collagenase degradation of thermogels. -----	66
FIGURE 6.2. BOEC morphology and viability within collagen thermogels -----	67
FIGURE 6.3. BOEC behaviour within genipin-crosslinked collagen thermogel -----	68
FIGURE 6.4. Primary, secondary and tertiary encapsulation of FGF-2 -----	70
FIGURE 6.5. Western blot of FGF-2 on ERK-1/2 phosphorylation -----	71

LIST OF ABBREVIATIONS

APPT	activated prothrombin partial time
BOEC-FVIII _s	factor VIII-expressing blood outgrowth endothelial cells
BOEC _s	blood outgrowth endothelial cells
CaCl ₂	calcium chloride
DAPI	4', 6'-Diamidino-2-phenylindole dihydrochloride
ddH ₂ O	double distilled water
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
EGM-2	endothelial growth medium-2 % fetal bovine serum
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FGF-2	fibroblast growth factor 2 (basic fibroblast growth factor)
FVIII	coagulation factor VIII
HEPES	effective buffering agent for maintaining enzyme structure & function at low temperatures
H & E	haematoxylin and eosin
HI-FBS	heat-inactivated fetal bovine serum
i.v.	intravenous
IHUEC _s	immortalized human umbilical vein endothelial cells
DiI-acetylated LDL	fluorescent-labeled acetylated low density lipoprotein

M199	Medium 199, endothelial cell base medium
NaHCO ₂	sodium hydrogen carbonate, or baking soda
NaOH	sodium hydroxide
pH	a measure of the acidity or basicity of a solution
PDGF	platelet-derived growth factor
p-ERK-1	phosphorylated ERK-1
p-ERK-2	phosphorylated ERK-2
PVDF	polyvinylidene fluoride
SD	standard deviation
SEM	scanning electron microscopy
SM	stereo microscopy
SMA	smooth muscle actin
TGF-beta	transforming growth factor beta
Trypsin-EDTA	Trypsin-ethylenediaminetetra acetic acid
v/v	volume by volume
w/v	weight by volume

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I am proud to self-declare a Griffith Lab Swimmer 2010.

1 INTRODUCTION

1.1 Haemophilia

Natural processes such as bleeding and proper blood clotting are often overlooked and taken for granted in healthy individuals. Blood clotting, also referred to as coagulation, is the process that controls bleeding. Procoagulation factors circulating in the plasma, such as factor VIII (FVIII), activate some of the components necessary for clot formation. The loss or deficiency of this factor often negatively affects an individual's ability to form clots following injury. The normal level of FVIII in blood is 200 ng/mL and normal FVIII activity is 1 IU/ mL. Haemophilia A (HA) is considered the classic form of blood clotting diseases. This X-linked recessive genetic disorder, affecting mainly males, is caused by mutations in the coagulation FVIII gene (F8) [1] which leads to either decreased production of the FVIII protein or production of dysfunctional protein with the same end result of function below a normal threshold [2]. HA is classified into three categories based on plasma FVIII activity levels: severe (<1 % of normal activity levels), moderate (1- 5 % of normal activity levels) or mild (5- 30 % of normal activity levels) [3]. Approximately 50% of severe HA cases are a result of large chromosomal inversions that disrupt the F8 gene [1]. The remaining severe cases as well as cases of mild or moderate HA are attributed to single base substitutions, small insertions, or deletions that result in missense, nonsense or frameshift mutations, respectively [1]. To date, more than 900 different mutations, affecting 1 in 5000 males [4] have been documented from all over the world [5]. Clinically, HA is characterized by frequent spontaneous bleedings into joints [6] and soft tissues, often leading to chronic and debilitating arthropathy [3],

vision loss from bleeding into the eye, chronic anemia and neurological or psychiatric problems [7].

1.2 Current Treatment and Limitations

1.2.1 Intravenous FVIII Infusions

Regular intravenous (i.v.) FVIII infusions of up to three times per week have been shown to control blood coagulation and reduce or prevent the progression of haemophilia-related complications [8, 9]. For example, in the 1960s, infusions of prophylactic FVIII concentrates from donated whole blood were deemed effective against haemophilic such spontaneous joint bleeds causing arthropathy [10]. However, 20 years later, it was discovered that plasma-derived FVIII concentrates were contaminated by human immunodeficiency and hepatitis viruses [11]. Fortunately, since the advent of recombinant technology, human FVIII has been successfully produced and purified in laboratories and is currently used as the main source of treatment [10].

Despite the availability of a treatment, there are several major problems. The cost, for example for the recombinant FVIII is fairly prohibitive, costing up to \$100,000 USD per year [3]. More importantly, the patient's immune system can recognize the exogenous FVIII as a non-self protein [12] and produces antibodies that neutralize and clear this procoagulant from the system. About 25% of patients with severe HA develop neutralizing antibodies against FVIII [13]. Patients with antibodies to FVIII often present with severe bleeding that is difficult and expensive to control. Management options for these unfortunate patients include heavy immune suppression, which has its own severe side effects and immune tolerance induction (ITI). In ITI, high doses of clotting factor are

given on a regular basis with the aim of saturating the inhibitors. Although immune response may be minimized with ITI, this strategy is extremely costly, approaching \$1 million USD for the average 5-year-old patient [14]. Therefore, the development of new and effective therapies for long-term management of bleeding in haemophilic patients is required [6]. With these aforementioned limitations, the paradigm in the development of any novel therapeutic is that avoiding immune responses is more successful and desirable than attempting to eradicate an already established response [15].

1.3 Gene Transfer for Alternative Therapy

Current therapies for haemophilias, although not perfect, are relatively safe and effective in preventing or treating bleeding episodes. Therefore, any alternative strategies must be able to provide a therapy that is at least as safe and effective as the protein infusion treatments that are now the standard of care [16]. As the pathology of this disease stems from the disruption of a single gene, HA is an ideal model of disease for gene therapy strategies.

1.3.1 Direct Transgene Delivery

Direct gene transfer strategies have been developed that use a range of virally-derived vectors, e.g. adenoviral vectors, adeno-associated viruses (AAVs) and recombinant retroviruses, that encode the functional F8 gene, have been injected directly into the host circulation [2, 17-19]. From the very nature of this approach, various cell types are transduced; this may further aggravate the immune response. For example, the transduction and expression of vector genes within Antigen-Presenting Cells (APCs) such

as dendritic cells will result in the processing and presentation of viral and/ or transgene products on MHC class I molecules that will recruit and activate cytotoxic T-lymphocytes (CTLs). In the case where there is coincident expression of co-stimulatory molecules by APCs, a profound CTL response will result in the cytotoxicity of any cell presenting the respective viral or transgene products [20]. In an effort to avoid immune responses resulting from pathogenic gene expression, these vectors have been designed to contain few or no viral coding genes [21]. Nonetheless, viral vector delivery methods have been shown to have their respective limitations. For example, due to their highly exposed surface proteins, adenoviral vectors activate the innate immune system, whereas, having the ability to bypass innate immunity, AAVs get caught by the adaptive immune system [22]. Furthermore, recent propositions of using recombinant retroviruses as a means of transgene delivery have been debated principally by concerns of secondary complications associated with insertional mutagenesis [23]. Therefore, systemic delivery of transgenes through the circulation will inevitably result in either the innate or adaptive immune systems, further contributing to the production of inhibitory antibodies or cell cytotoxicity and ultimate inactivation of FVIII products. This problem can be overcome to some extent by a more targeted delivery method.

1.3.2 *Ex vivo* Transgene Delivery

Over the past decade, an *in vitro* gene transfer approach has been employed that allows for exclusive targeting of the viral vector to *ex vivo*-isolated cells, bypassing most complications related to nonspecific cellular gene transfer. Specifically, blood outgrowth endothelial cells (BOECs), isolated, purified and cultured from peripheral blood, were

transduced *ex vivo* with a lentiviral vector containing the canine FVIII (cFVIII) transgene [6]. Following *ex vivo* genetic modification, successfully-engineered FVIII-expressing BOECs (BOEC-FVIIIs) were introduced into the host to act as an effective supply of functional FVIII [6, 24, 25]. This method of gene transfer, although susceptible to CTL-based cytotoxicity, has been described to further limit innate responses to the gene transfer process [20].

1.3.3 Delivery of Therapeutic Cells

Successful delivery of BOEC-FVIIIs is also critical to the overall success of cell-based therapies. Various studies have suffered from the lack of effective delivery of cells into targeted sites or organs within the host, resulting in the survival and/or engraftment of only a small fraction of donor cells at desired locations [6, 26]. These problems can be mitigated by targeted delivery to the desired site [27]. Tissue engineering as a discipline is moving more towards the development of soft, flexible biodegradable polymers that would house and isolate genetically modified cells from the host, and can be assimilated into the body when new tissue is regenerated [28]. In many cell encapsulation strategies these scaffolds would ideally possess properties closely resembling those of the extracellular matrix (ECM). The ECM, custom-designed by resident cells of each tissue and organ, is in constant dynamic equilibrium with its surrounding microenvironment [29]. It provides pertinent structural and biochemical cues necessary for tissue-specific function [30]. Therefore, ideal tissue-engineered scaffolds or biomaterials would thus possess many of the beneficial characteristics of the ECM.

1.4. Extracellular Matrix Macromolecules as Cell Delivery Scaffolds

The extracellular matrix (ECM) is elaborated by cells during development, and the cells in turn respond to signals from bioactive components of the ECM. Because regeneration often recapitulates development, ECM components have been investigated as scaffold components for tissue engineering in regenerative medicine. Several ECM components and preparations that are commonly used in the form of hydrated gels or hydrogels are discussed below.

1.4.1 Matrigel™

Cells, particularly epithelial cells, rest upon a specialized ECM known as the basement membrane, which anchors them to the underlying connective tissues. ECM components of basement membranes include mostly collagen, proteoglycans and laminin. Basement membrane ECM components have also been shown to be essential for angiogenesis, accelerating endothelial cell differentiation [31]. Matrigel™, as a hydrated basement membrane gel, is produced by mouse sarcoma cells. It remains in a liquid form at low temperatures and solidifies as a gel at body temperature. Apart from laminin and type IV collagen, it also contains a range of various growth factors including transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), insulin-like growth factor 1, basic fibroblast growth factor (FGF-2), and platelet-derived growth factor (PDGF) [32]. Furthermore, possessing characteristics of a thermogel, this hydrogel makes local and direct delivery of cells possible by simple injections, circumventing the need for invasive surgery. Studies on treatments for haemophilia have made use of Matrigel™ as a cell encapsulation scaffold, where FVIII-expressing BOECs were mixed within the liquid

form of Matrigel™ and injected subcutaneously to act as a FVIII depot [6]. Other studies on myocardial repair using stem cells attest to the efficacy of Matrigel™ and its constituents as a scaffold [33]. Studies continuing to use Matrigel™ show effective delivery of encapsulated transgenic cells [6] and even *de novo* blood vessel formation around cell-containing gels [27]. However, for therapeutic translation of these laboratory-generated results, the use of Matrigel™ is not suitable. It is extracted from rodent cancer cells and contains unknown proteins with unknown functions. As an extracted animal protein, it also carries a risk of infection transmission and may also give rise to xenogeneic responses in haemophilia patients [34]. Therefore, the development of a fully defined and reproducible matrix is necessary [35].

1.4.2 Collagen

Collagen refers to a family of over 25 related proteins, although the main ones conferring a structural role are Types I through V. Collagen is the major structural ECM component of most tissues and organs. It is composed of repeats of glycine-X-Y and proline amino acids. Due to its reported ability to facilitate attachment, migration and proliferation of many different cell types [36], collagen has been widely used as cell culture substrates and in tissue engineering. Like Matrigel™, injectable collagen hydrogels have also been developed and used in many cell-delivery applications. Most notably, collagen hydrogels have been used to deliver platelets for ligament wound healing [37], autologous chondrocytes for cartilage repair [38, 39] and endothelial progenitor cells for revascularization of ischemic tissues [40] Type I collagen has been used for repair after myocardial infarction. Specifically, local injection of collagen into the infarct zone

yielded significantly higher levels of capillary formation, when compared with the saline control group [41]. However, unlike Matrigel, this is a single protein and recombinant sources of collagen that do not pose the same risks as animal extracted proteins, are now available and have been tested clinically in various applications [42, 43].

These findings not only support the use of collagen as a biomaterial for this study, but also suggest that collagen biomaterials may support local tissue vascularization important for our FVIII-expressing BOEC delivery system.

1.5 Crosslinkers

A drawback to using collagen as tissue engineering scaffold is that they are very susceptible to biodegradation. For use as a delivery system for delivery of FVIII producing BOECs, the collagen scaffolds will need to be rendered resistant to biodegradation long enough for the BOECs to produce FVIII for periods of 3 to 6 months. One method to overcome early biodegradation is to stabilize biomaterials with crosslinking agents. A variety of stabilization methods to enhance biological longevity include physical (e.g. heat), and/or radiation (e.g. UV, gamma) crosslinking in the presence of activators. However, for *in vivo* applications, chemical crosslinking has been shown to be most effective in preventing rapid degradation of collagen-based biomaterials [44, 45]. In order to slow the degradation of collagen-based biomaterials specifically for applications involving endothelial cell seeding and FVIII delivery in haemophilia studies, chemical crosslinking of collagen scaffolds must be employed [46]. The two main methods for collagen crosslinking is using aldehyde-based crosslinkers e.g.

glutaraldehyde [47] or amide-based crosslinkers that include water-soluble carbodiimides [69].

1.5.1 Genipin

Recent reports on glutaraldehyde cytotoxicity [48] however, have prompted use of aldehyde crosslinkers with lower cytotoxicity. Genipin, a chemical isolated from the *Gardenia jasminoides Ellis* fruit, is a natural aglycone crosslinker derived from geniposide (Figure 1.1, A). It contains a dihydropyran ring and possesses the molecular formula $C_{11}H_{14}O_5$ [49] (Figure 1.1, B). When used with collagen, genipin crosslinks free amino groups, including lysine, hydroxylysine, and arginine [50, 51] and forms intramolecular and intermolecular crosslinks (Figure 1.2). Genipin has been used in several studies to increase the resilience of biological tissues [52-57] as well as matrices comprised of fibrin [58-61] and collagen [54, 62, 63]. Though the mean adhesive strength (kPa) values of genipin-crosslinked type I collagen did not result in a suitably strong material as compared to glutaraldehyde crosslinking [64], genipin has been shown to be 10,000 times less cytotoxic than glutaraldehyde [48, 50]. Studies also suggest that genipin elicits a significantly reduced inflammatory reaction than glutaraldehyde-crosslinked collagen tissues [53, 65]. When comparing genipin to carbodiimide crosslinkers, Sung *et al.* found that the former crosslinking occurs comparatively slower [66]. Therefore, low cytotoxicity, less rigorous and slow crosslinking makes genipin a suitable candidate for the crosslinking of cell-containing collagen thermogels in this study. Previous work in our laboratory demonstrated that collagen thermogels, housing

BOECs, supported the formation of vessel-like structures with or without genipin crosslinking, and supported over 85% BOEC viability (Appendix- Figures 6.1- 6.3).

1.5.2 Water-Soluble Carbodiimides

Water-soluble carbodiimides (WSCs) belong to the carbodiimide class of chemicals ($\text{HN}=\text{C}=\text{NH}$) that are isomeric with cyanamide, $\text{NH}_2\text{C}\equiv\text{N}$ [67]. Carbodiimide crosslinkers have been shown to stabilize tissues equally as well as aldehyde crosslinkers. Collagen crosslinking with a carbodiimide is generally considered to take place as a single reaction of the following steps. The carbodiimide activates carboxylic acid residues of aspartic and glutamic acid resulting in the formation of intermediate groups. Possible side reactions such as the hydrolysis of the intermediate group occur rapidly during this process. Therefore, N-hydroxysuccinimide (NHS) is used in the crosslinking reaction to yield stable NHS-activated carboxylic acid groups, less susceptible to hydrolysis and rearrangement [68]. The combination of carbodiimide with NHS is generally considered to result in a more efficient crosslinking reaction. For instance, in a study investigating crosslink densities of gelatin at the optimum EDC: NHS ratio (5: 1) lower concentrations of free amine groups were detected, which means that most amine and carboxylic acid residues were consumed during crosslinking [68]. Nucleophilic attack of the free amine groups of lysine and hydroxylysine residues on the activated carboxylic acid residues, form crosslinks, with a urea derivative as a leaving group [69] (Figure 1.3). In this final step, because the water soluble by-products are easily eliminated during routine rinsing [70] the risk of releasing non-reacted or depolymerized cytotoxic reagents after implantation is significantly reduced [70]. One of the members of

the WSC family that has been used to significantly increase the strength of collagen tissues [71] and matrices [72-75] is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). As a zero-length crosslinker, EDC is only present during the crosslinking procedure [76]. In fact, for tissue-engineered scaffolds composed of collagen, EDC crosslinking has not been shown to cause any *in vivo* cytotoxic reactions [77]. In this study, EDC has been used as a robust crosslinker to fortify collagen in the development of a novel collagen capsule. In the previous cell-delivery study [6] early biodegradation of MatrigelTM was suspected to be due to the thermogel's susceptibility to enzymatic degradation. As a continuation to the recent study, in order to overcome premature biodegradation, the EDC-crosslinked collagen capsule was designed to encapsulate the cell-housing collagen thermogel. The objective was thus to protect the encapsulated FVIII-expressing BOECs without impeding their survival or their production of FVIII.

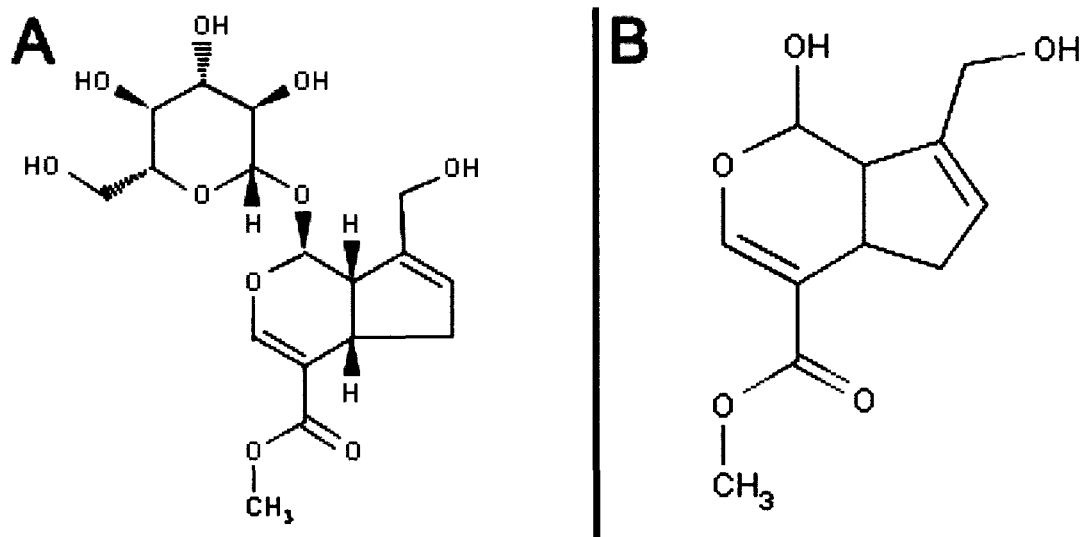


FIGURE 1.1. Structure of (A) geniposide, a natural chemical extract of the *Gardenia jasminoides Ellis* fruit and (B) its derivative, genipin.

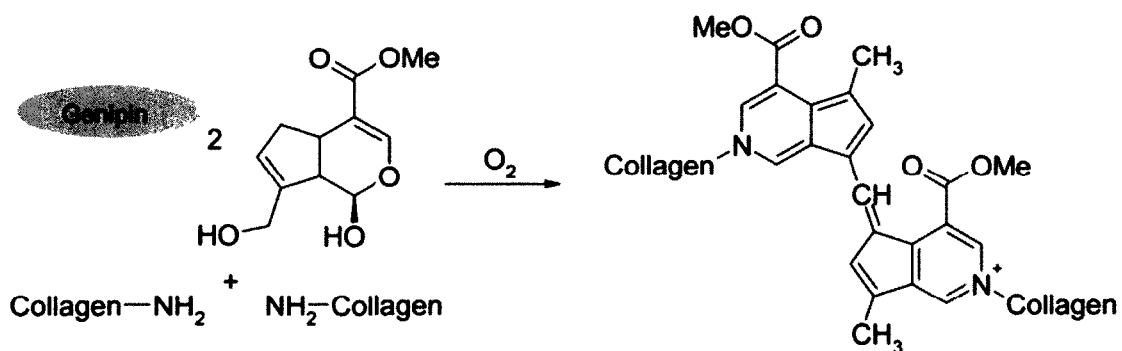


FIGURE 1.2. Schematic of collagen crosslinking using genipin. Genipin, similar to glutaraldehyde, covalently binds to amino groups of collagen, but can only bind to one other genipin molecule. Image modified from Englert *et al.* [78].

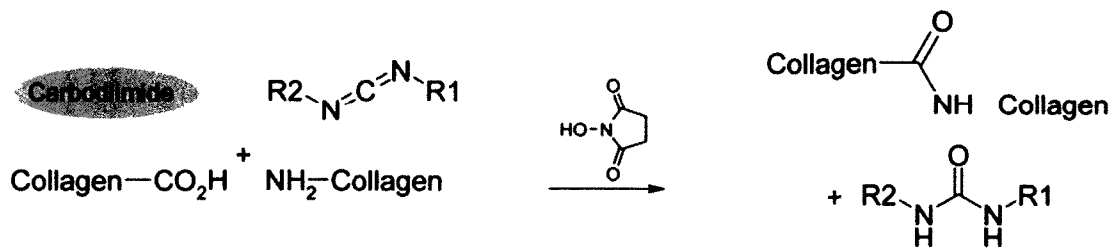


FIGURE 1.3. Schematic collagen crosslinking using 1-ethyl-3-diaminopropyl-carbodiimide (EDC) and N-hydroxysuccinimide (NHS). EDC/NHS catalyzes covalent bindings between carboxylic acid and amino groups of collagen structures. Water soluble by-products can easily be washed post-crosslinking. Image modified from Englert *et al.* [78].

1.6 Growth Factors

In order to provide an optimal microenvironment for encapsulated cells within a scaffold, growth factors, cytokines and/ or bioactive peptides should be provided in a controlled and timely manner [79, 80]. With the increased prevalence of cell-based therapeutic research in the emerging field of tissue engineering, such as in the case of haemophilia research, the reliance on growth factor-rich scaffolds is increasing [28]. In fact, it has been suggested that the use of growth factors in tissue engineering approaches would be a significant breakthrough, especially in reconstructive surgery, to not only improve patient-healing response but also to regenerate soft tissue of depressed regions occurring after surgery [81]. Incorporation of growth factors within our cell-delivery vehicle may accommodate the transition from growth factor-rich Matrigel™ to pure growth factor-deficient collagen.

1.6.1 Fibroblast Growth Factor 2 (FGF-2) and Delivery

Fibroblast growth factor 2 (FGF-2), also referred to as basic FGF (bFGF), is a single-chain polypeptide with a pleiotrophic role in various cell types and tissues. Delivery of FGF-2 to damaged tissues has been shown to stimulate regeneration of muscle, bone, cartilage, and nerves [82-87]. FGF-2 is also known to play an important role during development, and is also a key component of the wound healing response [88]. In addition, FGF-2 is also involved in angiogenesis [89]. Upon binding to its high affinity receptor (FGFR-2), FGF-2 induces tyrosine phosphorylation and activation of FGFR-2.

In turn, FGFR-2 induces activation of FRS2 (FGFR Stimulated2 Grb2 binding protein), which recruits GRB2 (Growth Factor Receptor Bound Protein-2), SOS and other signal transduction proteins. These events promote the sustained activation of Ras, which in turn activates the Raf1-MEK-MAPK (ERK1/2) pathway leading to changes in gene transcription (Figure 1.4). As seen from western blot analyses, FGF-2 in various media, increase the levels of phosphorylated ERK-1 and ERK-2, with respect to total ERK-1/2 proteins, within endothelial cells (Appendix- Figure 6.5). The increase in ERK-1 and ERK-2 phosphorylation in turn leads to changes in gene transcription that ultimately results in endothelial cell survival and proliferation [90]. Since the delivery of FGF-2 has been shown to significantly increase the formation of microvascular networks in pig models of myocardial infarction [91], the use of FGF-2 for our FVIII-expressing BOEC delivery system may provide support for cell survival and proliferation *in vivo*. Direct introduction of FGF-2 into a system may, however, lead to rapid diffusion and degradation [92]. Controlled delivery of FGF-2 on the other hand results in higher microvessel density [93] and enhances angiogenesis [94]. As such, FGF-2 encapsulation and incorporation into our collagen cell-housing microenvironment, is deemed necessary for a controlled FGF-2 release over a long-term implantation period. A natural polymer such as alginate, both biodegradable and biocompatible, has been widely used in the past to encapsulate growth factors such as FGF-2 [95]. The utility of encapsulated and transplanted BOEC-FVIIIs, for the treatment of haemophilia, was thus examined in the presence and absence of FVIII alginate microspheres.

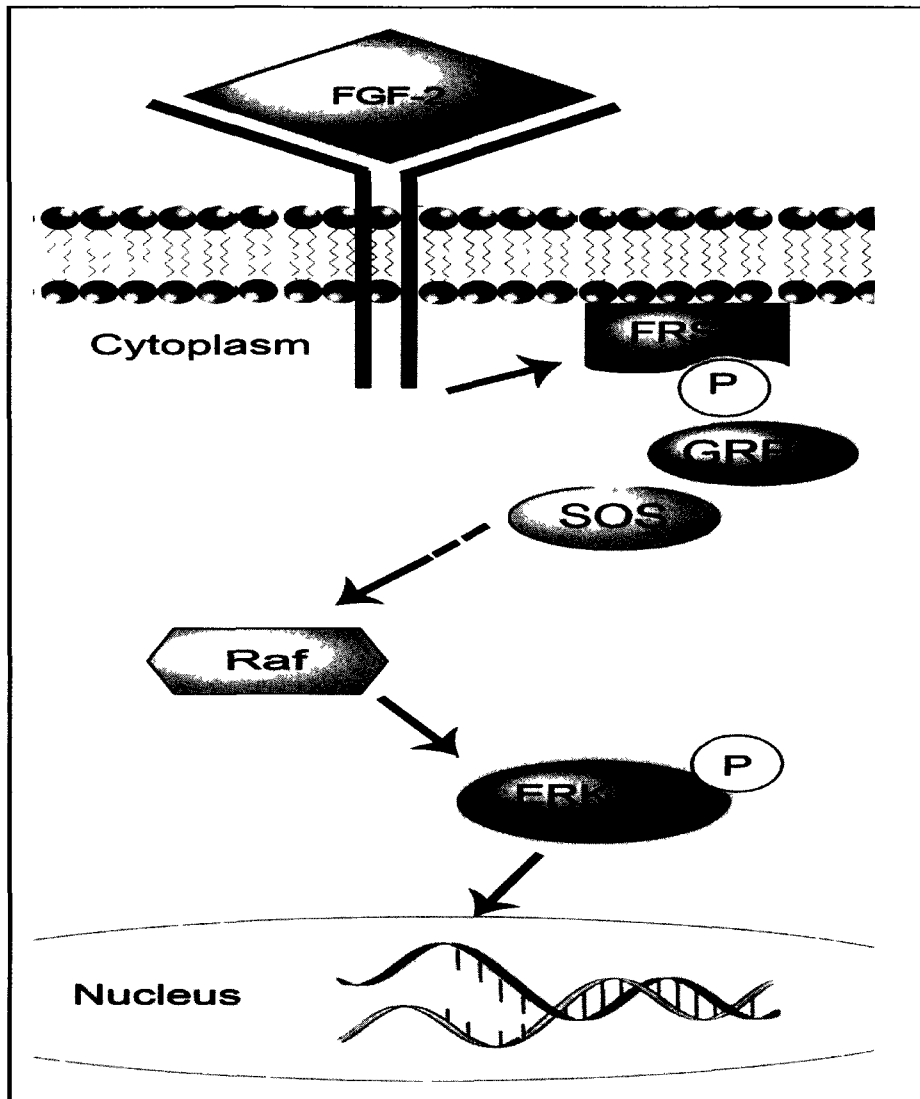


FIGURE 1.4. Bioactivity of FGF-2 in the ERK1/2 pathway. The formation of FGF-2/FGFR-2 complex leads to ERK 1/2 phosphorylation, changes in gene transcription and ultimately, endothelial cell survival and proliferation [90].

1.7 Objectives and Hypotheses

The opportunity of large scale expansion of postnatal endothelial progenitor cells in culture- *ex vivo*- has become an attractive feature for cell therapy applications.

Genetically modified BOECs could serve as an autologous cellular vehicle for the production and secretion of therapeutic doses of FVIII in the treatment of HA. However, the usefulness of BOEC-FVIII in transgenic cell therapy will rely upon the suitability of the cell-housing biomaterials used to deliver these cells. The objective of this study was therefore to develop a tailor-made delivery system for local implantation of genetically-modified BOECs that would produce Factor VIII for long-term HA treatment.

Specifically, the aim was to replace previously used Matrigel™ [6] with type I collagen-based scaffolds for encapsulation and *in vivo* implantation of FVIII-expressing BOECs. It was thus hypothesized that the combination of collagen scaffolds and crosslinkers, along with FGF-2, will support long-term FVIII release from encapsulated endothelial cells.

The ultimate goal of this delivery strategy would be timely degradation of our collagen capsule system and optimal integration of FVIII-expressing BOECs with host vasculature to give rise to transgenic FVIII-expressing blood vessels.

2 METHODS

2.1 Cell Culture

2.1.1 Immortalized Human Umbilical Vein Endothelial Cells (IHUVECs)

IHUVECs were grown in 25 cm² tissue culture flasks (Corning Inc., NY, USA) coated with 1 % gelatin and supplemented with growth medium: Medium199 (M199, Invitrogen, Ontario, Canada) containing 10 % heat-inactivated fetal bovine serum (HI-FBS), 90 mg/L heparin (Sigma, MO, USA), 50 µg/mL endothelial cell growth supplement (ECGS) (Sigma, MO, USA), and 5 µg/mL gentamicin (Invitrogen, Ontario, Canada). Cells were maintained in a humidified incubator at 37 °C and 5 % CO₂, until 80 % confluence. Trypsin-ethylenediaminetetra acetic acid (Trypsin-EDTA) (Invitrogen, Ontario, Canada) was used to detach the cells from the substrate, after rinsing with Ca²⁺- and Mg²⁺-free 0.1 M phosphate buffered saline (PBS). Cells were incubated for 2 minutes with 0.05 % Trypsin-EDTA, after which the reaction was terminated by adding serum-containing M199. Dislodged cells were then centrifuged at 1500 rpm for 5 minutes, resuspended in M199 containing 2 % HI-FBS without ECGS to obtain a concentration of 150, 000 cells/mL.

2.1.2 Progenitor Cell Derived-Blood Outgrowth Endothelial Cells (BOECs)

FVIII-expressing BOECs were kindly provided by Dr. David Lillicrap, Queen's University, Kingston, Ontario, and grown in 25 cm² tissue culture flasks coated with rat tail collagen I (50 µg/mL) (BD Biosciences). Cells were maintained in MCDB 131 growth medium (Invitrogen) supplemented with EGM-2 SingleQuots (Cambrex), HI-FBS (10 % v/v) (Invitrogen), penicillin (100 U/mL) (Sigma), streptomycin (100 µg/mL)

(Sigma), Glutamax (2 mM) (Invitrogen), and Fungizone (0.25 µg/mL) (Invitrogen). Cells were maintained in a humidified incubator at 37 °C with 5 % CO₂ until 80 % confluent, at which point, they were passaged using 0.25 % trypsin-EDTA (Invitrogen) to detach the cells during the passaging.

2.2 Animals

Cell Delivery Study- Animal surgeries investigating the efficacy of collagen capsules in BOEC-FVIII cell delivery were conducted at Queen's University (Kingston, Ontario) on three week old non-obese diabetic/ severe combined immune deficiency (NOD/ SCID) mice (Taconic Farms Inc., Hudson, NY, <http://taconic.com>). All animal procedures were reviewed and approved by the Queen's University Animal Care Committee. Each mouse was implanted with one capsule. The mice received either Matrigel-loaded capsules (N = 4) or collagen-loaded capsules (N = 2- 4). A small pocket was created in the upper back of each mouse by making an incision ~10 mm long and creating a pocket for insertion of the capsule. The incision was then closed with a skin clip. The capsules were implanted for a period of 12 to 18 weeks. To test serum levels for canine FVIII antigen, blood samples (0.1-0.2 ml per time point) were taken via saphenous bleed, using a 23 G needle, one week post-implantation and every week thereafter.

FGF-2 Delivery Study- Animal surgeries investigating the efficacy of collagen capsules in FGF-2 delivery were conducted at the Heart Institute, Ottawa Hospital Research Institute (Ottawa, Ontario) on three week old BALB/c Nude mice. Each mouse was implanted with two capsules containing FGF-2-loaded collagen thermogels (N= 5).

A small pocket was created at the right and left dorsal sides just above the hind limb. The incisions were closed with sutures. The capsules were implanted for a period of 3 weeks after which, retrieved capsules were prepared for histology and analysis of vascularization (see sections 2.11 and 2.12).

2.3 Stereo and Scanning Electron Microscopy (SM and SEM)

The appearance and morphology of alginate microparticles and collagen capsules were investigated using stereo (SM) (Zeiss, Stereo Discovery V12) and scanning electron microscopy (SEM) (Model S-2250N, HITACHI, Japan). For SM, the particles were mounted on metal holders using conductive double-sided tape. For SEM, samples were mounted on metal holders and sputter-coated with a gold layer for 60 seconds at 0.1 bar vacuum pressure (Cressington Sputter Coater, 108) prior to examination under vacuum.

2.4 Collagen Capsules

Porcine type I atelocollagen (10 % w/w) (Nippon Ham, Japan) was prepared in ddH₂O as per Liu et al. [1] and was loaded into a sterile syringe (BD Biosciences, MA, USA). Collagen solution (100 µL) was transferred onto a sterile plate and warmed at 37 °C in a humidified incubator to decrease viscosity. An empty gelatin capsule (No. 4; T.U.B. Enterprises, Ontario, Canada) was coated with the collagen and subsequently crosslinked with EDC / NHS (both from Sigma-Aldrich, Ontario, Canada) (2:1 w/w), within the necessary pH range 3-5. The capsule was then washed twice with ddH₂O and stored overnight at 4 °C in 1 % chloroform (in PBS) to maintain sterility. Subsequent washes with PBS were conducted, daily for 5 days, to remove any residual chloroform.

2.5 Collagen Thermogel

A collagen thermogel was produced using a procedure modified from Yang *et al.* [96]. Briefly, rat tail type I collagen (BD Biosciences, MA, USA) was mixed on ice with buffer (10 x M199, ddH₂O, 7.5 % NaHCO₂ and 1 M HEPES; 10: 8: 1: 1) at a ratio of 8:2 v/v. The pH of the collagen mixture was then adjusted to 7 by titration with 1 M NaOH. Genipin solution (Wako Pure Chemicals, Japan) (2 mg/mL), prepared by thorough suspension in ddH₂O and filtering (0.2 μm pore size), was then added to the buffered collagen solution (0.05 mg/mL). The thermogel was stirred slowly to allow for homogenous crosslinking on ice. For FGF-2 release and bioactivity assessments, particles suspended in ddH₂O were added to the thermogel (2:8 v/v). To assess *in vitro* cell viability and phenotypic behaviour, BOEC suspension (5 x 10⁵ cells/ mL) was mixed homogeneously into the gel (2.5: 7.5 v/v). For cell encapsulation and implantation, approximately 300 μL of BOEC-FVIII thermogel suspension (6.7 x 10⁶ cells/ mL) was injected into the capsule to obtain 2 x 10⁶ cells/ capsule.

2.6 FGF-2-loaded Alginate Microparticles

A 2 % (w/v) solution of alginic acid sodium salt solution, from *Macrocystis pyrifera*, medium viscosity (Sigma, MO, USA) was prepared in ddH₂O and autoclaved. Under sterile conditions, human recombinant FGF-2 (10 μg) (BD Bioscience, MA, USA) solution was prepared in 0.1 % BSA in PBS, and allowed to dissolve on ice with gentle agitation. Alginate solution was added to the FGF-2 solution (9:1) and allowed to thoroughly mix at room temperature. The FGF-2 / alginate solution was loaded into a 1 mL syringe (27 G needle) and sprayed (height = 14 cm) into a bath of 2.5 % (w/v) CaCl₂

by a spray gun connected to an air compressor. Growth factor-free alginate solution was used as a control. Alginate particles were harvested, rinsed twice with ddH₂O to remove loosely associated FGF-2 and extra CaCl₂ and resuspended in 1 mL ddH₂O. Images of hydrated particles were taken (Nikon Eclipse TE2000-E, Ontario, Canada) and particle diameters were measured. Mean particle diameter \pm SD for FGF-2-containing and alginate only particles were calculated.

2.6.1 Loading Capsules

Rat-tail collagen thermogels were prepared as above. Microparticles were mixed into the gel at a ratio of 1: 9 v/v and injected into the collagen capsule, using a 16 G 1 ½ needle.

The capsule delivery system was sealed inside a 12-well Costar® Transwell plate (Corning, NY, USA) and placed in a humidified incubator overnight at 37 °C to gel.

For the *in vivo* mouse studies, the same steps were taken as above with some additions.

Matrigel™ (BD Biosciences) without FGF-2 microparticles was used as a control since it was used previously in non-encapsulated experiments by Matsui *et al.*[6]. Collagen and Matrigel™ capsules containing BOEC-FVIII (2×10^6 cells per capsule) were implanted into NOD/ SCID mice. One capsule type was implanted per NOD/ SCID mouse.

2.7 Collagenase Digestion Analyses

2.7.1 Collagen-coated EDC-crosslinked Capsule

The effect of EDC crosslinking on the resilience of collagen was evaluated by collagenase digestion assay. Percent residual mass was used as a measure of collagenase digestion and protease resistance. Collagen capsules were prepared as described in

section 2.4, with and without EDC/NHS crosslinking. Each capsule was added into a 75 mm-diameter insert (0.4 μ m pore sized membrane bottom) (Millipore Corp, MA, USA) and placed in a 6-well plate. To fully immerse the capsule in type I collagenase solution (110 U/mL) (Invitrogen, MA, USA), a volume of 3 mL was added to the top and 3 mL to the bottom of each transwell insert. The capsules were placed in a 37 °C water bath and weighed at 20- to 30-minute intervals, after blotting off excess fluid, until they were completely digested. Weights of remaining gels were recorded at each time interval and converted to a percentage of the initial weight. Fold difference was calculated at each time point using the following equation.

$$\text{Fold difference} = \frac{\% \text{ residual mass (collagen-coated EDC-crosslinked capsules)}}{\% \text{ residual mass (non-coated, non-crosslinked capsules)}}$$

Mean fold difference \pm SD was calculated from fold difference of all time points.

2.7.2 Genipin-crosslinked Collagen Thermogel

A collagen thermogel was prepared as described in section 2.5, without FGF-2 particles, with and without genipin crosslinking. Each sample was added into a Millicell-CM 30 mm-diameter insert (0.4 μ m membrane bottom) (Millipore Corp, MA, USA) and placed in a six well plate. The plate was placed overnight in a humidified incubator at 37 °C. Gels were removed from the incubator and weighed. Type I collagenase (Invitrogen, MA, USA) solution (25 U/ mL) was added on top of the gel and under each insert. The gels were then placed back in the incubator for 15 minutes, after which time they were removed and weighed. Weights of gels were recorded at each time interval and converted to a percentage of the initial weight. This process was repeated every 15

minutes until the gels were completely digested. Fold difference was calculated at each time point using the following equation.

$$\text{Fold Difference} = \frac{\% \text{ residual mass (collagen-coated genipin-crosslinked thermogel)}}{\% \text{ residual mass (non-coated, non-crosslinked thermogel)}}$$

Mean fold difference \pm SD was calculated from fold difference of all time points.

2.8 Cell Encapsulation within Collagen Thermogel

2.8.1 BOEC Viability

Genipin-crosslinked rat-tail collagen thermogels mixed with BOECs were prepared as above. Cells were grown inside the matrix for seven days, and then stained with the LIVE/DEAD Viability/ Cytotoxicity Kit (Invitrogen) for 30 minutes at room temperature. The gels were washed twice with PBS for 10 minutes and examined by fluorescence microscopy (Nikon Eclipse TE2000-E) within one hour of staining. A semi-quantitative assessment of the viability of BOECs, either crosslinked with genipin or not, was achieved by cell counts. Four different fields were photographed within each gel, and the total number of nuclei and dead cells were counted in each field. The mean percentage with respective standard deviation of live and dead cells was calculated from N = 3 separate trials.

2.8.2 BOEC Characterization

2.8.2a Uptake of Acetylated Low Density Lipoprotein

Genipin-crosslinked rat-tail collagen thermogels containing BOECs were prepared as above and were stained for their ability to uptake 3,3'-dioctadecylindocarbocyanine

acetylated low density lipoprotein (DiI-acetylated LDL). Gels were incubated for 5 hours at 37 °C in BOEC medium containing DiI- acetylated LDL (0.91 µg/ mL) (Molecular Probes). The gels were then washed at 37 °C with medium and incubated overnight in BOEC medium. Gels were examined by fluorescence microscopy.

2.8.2b Expression of Endothelial Marker CD31

Genipin-crosslinked rat-tail collagen thermogels were prepared as described in section 2.5- with a BOEC suspension of 5×10^5 cells/ mL but without FGF-2 microparticles- and were stained with a monoclonal antibody against CD31. Gels were washed three times with PBS (Invitrogen) to remove any traces of serum, and then fixed with a solution of 1:1 acetone: methanol for 30 minutes at -20 °C. Gels were again washed three times with PBS. The PBS was aspirated and the gel was blocked with 1 % bovine serum albumin (BSA; MP Biomedicals Inc.). The gel was then incubated at 37 °C for 30 minutes. The solution was replaced with mouse anti-human CD31 (10 µg/mL) (BD Biosciences) in PBS containing 1 % BSA. The gel was incubated for 1 hour at room temperature then washed three times with PBS (5 minutes per wash). PBS was replaced with goat anti-mouse IgG (1: 400) (Molecular Probes) in PBS/BSA for 1 hour at room temperature, and then washed three times with PBS (5 minutes per wash). Gels were examined by fluorescence microscopy.

2.9 FGF-2 Release and Bioactivity

2.9.1 ELISA

Enzyme-linked immunosorbent assays (ELISA) were used to quantify the amount of FGF-2 released from the following three scaffolds: 1) FGF-2 alginate microparticles only, 2) collagen thermogels containing FGF-2 alginate microparticles and 3) capsules loaded with collagen thermogel containing FGF-2 alginate microparticles. For all three test samples, controls were comprised of FGF-2-free alginate microparticles. All samples were incubated in growth factor-free M199 medium (Invitrogen, Ontario, Canada) containing 2 % HI-FBS (Invitrogen, Ontario, Canada) at 37 °C. Media containing released FGF-2 were collected and replaced with fresh media for 29 days. Samples were collected daily and stored at -20 °C. The concentration of released FGF-2 was quantified by ELISA (R & D Systems Inc., MN, USA), as instructed by manufacturer. Values from control gels were subtracted from test gels. Daily FGF-2 released (pg/ mL) was calculated from gels (N = 3), pooled together for each encapsulation group. ELISA quantifications of each encapsulation group were performed in triplicates. Average FGF-2 secreted over 24 hours, for 0-9 days, 10-19 days, and 20-29 days were calculated from the above ELISA values.

2.9.2 WST-1: FGF-2 Bioactivity

The mitogenic activity of the released growth factor was determined by a colorimetric assay based on formazan dye formation (WST-1, Roche, Indianapolis, IN), which directly correlates with the number of metabolically active cells in the culture. An increase in formazan dye formation indicates an increase in the overall concentration of

mitochondrial dehydrogenases within the sample, suggesting an increase in cellular metabolic activity. Daily-released FGF-2 supernatant samples (50 μ L) were added to HUVECs pre-seeded (7,500 cells/ well) in 2 % FBS M199 media (50 μ L) in a 96-well plate. For the normalization assay, daily-released FGF-2 supernatant samples were diluted to a normalized concentration (0.3 ng/mL) using 2 % FBS M199 media. Non-encapsulated, exogenously added FGF-2 (0.3 ng/mL) in 2 % FBS M199 and 2 % HI-FBS M199 media alone were used as positive and negative controls, respectively. After incubation of the cells for a period of 72 h, 10 μ l/well of the reagent WST-1 were added and incubated for 1 h at 37 $^{\circ}$ C. The formazan dye was quantified by measuring the optical density at 450 nm with a scanning multiwell spectrophotometer (Bio-Tek Instruments, Inc, Burlington, VT). Media and formazan dye were aspirated, quickly and carefully, after microplate reading and plates were stored at -20 $^{\circ}$ C for further CyQUANT analysis. Fold increase was calculated at each time point using the following equation. Mean fold \pm SD was calculated from value of all time points. Values from FGF-2 test samples were compared to respective no FGF-2 controls.

$$\text{Fold Difference} = \frac{\text{Optical Density at 450 nm (FGF-2 alginate particles in thermogel)}}{\text{Optical Density at 450 nm (alginate only particles in thermogel)}}$$

2.9.3 CyQUANT Cell Proliferation Assay

To confirm bioactivity of FGF-2 released from collagen thermo-gels, IHUVEC proliferation in 96-well tissue culture plates was determined with a fluorescence-based cellular DNA quantifier, the CyQUANT Cell Proliferation Assay (Molecular Probes, Eugene, OR). Treatment of HUVECs with supernatants was conducted as in the WST-1

assay. The CyQUANT assay was performed according to the manufacturer's instructions. Fluorescence was measured directly on a microplate fluorescence reader (Bio-Tek Instruments, Inc, Burlington, VT) with excitation at 485 nm and emission detection at 530 nm. Fold increase was calculated at each time point using the following equation. Mean fold \pm SD was calculated from values of all time points. Values from FGF-2 test samples were compared to respective no FGF-2 controls.

$$\text{Fold Difference} = \frac{\text{Fluorescence (FGF-2 alginate particles in thermogel)}}{\text{Fluorescence (alginate only particles in thermogel)}}$$

2.9.4 Western Blot

Western blot analysis was conducted to further test bioactivity of FGF-2, specifically to investigate intracellular signaling. BOECs were plated in 6-well plates with 7.5×10^4 cells/ well in EGM-2 containing 10 % FBS for 48 h with media changes conducted the following day. Serum and growth factor starvation of cells was conducted using M199 medium for 24 h. Serum-starved BOECs were next subjected to the following three treatment groups for 30 min at 37 °C: fully supplemented EGM-2, M199 containing 20 ng/ml FGF-2, and M199-only media. Cells were washed once with ice-cold PBS and were harvested in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA) containing NaF (10 mM), Na deoxycholate (1 mM), Na_3VO_4 (1 mM), 1x Halt phosphatase and protease inhibitor cocktail (Thermo Scientific, IL, USA). Protein determination was performed by BCA protein assay (Thermo Scientific, IL, USA). Equal amounts of protein (50 μg) for each sample were electrophoresed in acrylamide gels and blotted onto a PVDF membrane. Blot was probed with primary antibodies for

phosphorylated ERK1/2 (1:100) and reprobbed for total ERK1/2 (1:100) (Cell Signaling Technology, MA, USA) and for total GAPDH (1:500) (Abcam, MA, USA) to verify loading evenness. After incubation with peroxidase-conjugated anti-rabbit IgG (1: 1000) (Santa Cruz Biotechnology, Santa Cruz, CA), specific reactions were revealed with a chemiluminescent detection method (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL). Before reprobing with another antibody, membranes were stripped by incubation in Re-Blot 1x antibody stripping solution (Chemicon International, Temecula, CA) for three 5 min washes. Densitometry values were expressed in arbitrary units and estimated by AlphaEaseFC Software (Alpha Innotech Corporation, San Leandro, CA).

2.10 FVIII Release

2.10.1 Serum FVIII Functionality Assay

BOEC-FVIIs were mixed into collagen thermogels as described above. A portion of the mixture was used to inject collagen-coated EDC-crosslinked capsules while the other portion was loaded onto 12-well transwells, to be used as thermogel plugs. FVIII functionality released from collagen-coated EDC-crosslinked capsules and control thermogels was assessed using TriniCLOT Automated activated prothrombin partial time -APPT - assay (Trinity Biotech), as per manufacturer's instruction. Briefly, the APPT test uses clot formation with respect to time as measurement of FVIII functionality [97-99]. That is shorter times for clot formation was correlated to high FVIII activity. The COAG-A-MATE MAX (Behnk Elektronik, SN4080) was used to quantify coagulation time with respect to a human serum standard curve.

2.10.2 Serum cFVIII Antigen Quantification

Serum canine-FVIII: Ag (ng/mL) levels of mice implanted with capsules were quantified using Asserachrom FVIII: Ag ELISA kit as per manufacturer's instructions, using a microplate colorimetric reader (Bio-Tek Instruments, Inc, Burlington, VT).

2.11 Tissue Fixation and Processing

BOEC-FVIII Delivery Study- Implants removed from euthanized mice in the BOEC-FVIII delivery study were fixed overnight in 4 % PFA in 0.1 M PBS at 4 °C. Implants were washed three times in 0.1 M PBS and were placed in increasing concentrations of sucrose (10 %, 20 % and 30 %) and left at 4 °C until saturated (between 24 h and 48 h). Once saturated, the implants were transferred into a 1:1 mixture of 30 % sucrose and optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, CA, USA) and allowed to equilibrate for 24 h at 4 °C. Implants from each mouse were placed in a plastic mould filled with 1:1 OCT: sucrose. The mold was lowered onto a metal rack bathing in liquid nitrogen, and once frozen, transferred to a -80 °C freezer for storage. A Shandon Cryotome FSE (Thermo Electron Corporation, MA, USA) was used to prepare 11 µm cryosections, air dried for 2 hours and stored at -20 °C with desiccant for histological use.

FGF-2 Delivery Study- Implants removed from euthanized mice in the FGF-2 delivery study were formalin-fixed and paraffin-embedded following commonly used procedures. Formalin-fixed, paraffin-embedded tissue samples were cut in 5-µm-thick sections on a microtome with a disposable blade.

2.12 Histology

2.12.1 Haematoxylin and Eosin Staining

Haematoxylin & eosin (H & E) staining was conducted according to standard protocols, with 15 and 30 seconds incubation in each stain, respectively. A representative sample of slides were chosen from each implant, stained with H & E, mounted in Permount (Fisher Scientific) and cover-slipped. Images of the capsules were captured on a Zeiss Axioskop light microscope with a Zeiss AxioCam camera.

2.12.2 Immunohistochemistry

Smooth Muscle Actin Staining- Immunohistochemistry was used to identify the presence and location of smooth muscle actin (SMA)-positive vessels, with respect to the collagen capsule. Sections showing vessel-like structures were first identified based on the H & E staining, and subsequent slides were chosen for immunofluorescence. A mouse anti-SMA antibody (1:50; Cedar Lane, Ontario, Canada) was used as a primary antibody to detect SMA, and a goat anti-mouse IgG secondary antibody (Alexafluor 488, 1:400, Molecular Probes, Oregon USA) was then applied. Slides were also stained with 4', 6'-Diamidino-2-phenylindole dihydrochloride (DAPI) (1 in 10000, D9542, Sigma-Aldrich), as a nuclear stain. Fluorescence images were obtained using an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation).

CD31 Staining- Paraffin-embedded sections were deparaffinized in xylene, rehydrated in ethanol, and then blocked with 10% hydrogen peroxide in water for 5 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwave treatment of the sections in citrate buffer for 10 min, followed by blocking of with normal

rabbit serum (1:5). CD31 staining was performed using rabbit polyclonal anti-CD31 (1:100) (Abcam; MA, USA) followed by biotinylated secondary anti-rabbit antibody (1:1000) for 45 min. After incubation in avidin-biotin peroxidase complex for 30 min, 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as chromogen. Between steps, the slides were rinsed for 5 min in PBS three times. Sections were then counterstained with haematoxylin, dehydrated, and mounted.

2.13 Statistical Analysis

All *in vitro* values are given as mean \pm standard deviation (SD) from at least three independent experiments. A two-tailed Student's *t*-test was used when comparing the mean values of each conducted experiment unless otherwise indicated.

3 RESULTS

3.1 Collagen Capsule

The initial step to achieving the overall objective of this study was to develop a more protease-resistant collagen scaffold for cell delivery. Commercially available, unfilled gelatin capsules were coated with type I Nippon ham collagen and then crosslinked in a bath of EDC to stabilize the collagen coating. During preparation, no obvious disintegration of capsules was observed. Even after one week of washing in PBS, collagen-coated EDC-crosslinked capsules were robust and pliable, and were easy to handle during cell encapsulation and surgical implantations (Lillicrap, personal communications). Non-crosslinked collagen-coated gelatin capsules on the other hand, swelled in PBS and disintegrated into small pieces on touch. SEM analysis was performed to compare the surface architecture of capsules before and after crosslinking. Uncoated, uncrosslinked gelatin capsules had no distinguishable surface features (Figure 3.1, A) whereas coating with collagen and crosslinking with EDC resulted in a new surface architecture, which appeared as aligned fibrils (Figure 3.1, B). The next step in the development of the collagen capsule was to quantify the extent of resistance EDC-crosslinking conferred. Collagenase digestion on capsules was thus performed *in vitro* to assess relative resistance to enzymatic degradation. With respect to control non-coated non-crosslinked gelatin capsules, percent residual mass of collagen-coated EDC-crosslinked capsules were significantly higher ($p < 0.01$) at all time points after 30 minutes as seen from the collagenase degradation profile (Figure 3.2, A). Overall, in comparison to control non-coated, non-crosslinked capsules, the collagen-coated EDC-

crosslinked capsules displayed an averaged 9-fold (p-value < 0.001) resistance to collagenase digestion across all time points post-30 minutes (Figure 3.2, B).

3.2 *In vitro* FVIII Production and Release

It was also important to confirm that the capsule did not interfere with production and release of FVIII with clotting activity, i.e. functional protein. APPT coagulation assays- measuring clot formation time of *in vitro*-collected media samples with respect to clot formation time of FVIII standards- demonstrated that both non-encapsulated and encapsulated collagen thermogels supported FVIII production and release from FVIII-expressing BOECs. More specifically, encapsulated thermogels supported consistent release of FVIII though the activity levels were significantly lower (p < 0.01 to p < 0.05) than FVIII released from non-encapsulated thermogels (Figure 3.3). This suggests that the additional encapsulation step may have interfered with higher amounts of released FVIII. Since the objective of this study was to provide a more collagenase-resistant collagen capsule for the encapsulation of cells, confirmation of release and functionality of FVIII- even at low levels- was sufficient for the purpose of this study. In the case where higher levels of FVIII activity is desired, further *in vitro* coagulation experiments, using extra control groups and variations in cell density and crosslinking degrees, would be necessary.



FIGURE 3.1. Representative SEM images of (A) a gelatin capsule with no distinguishable surface features and (B) a collagen-coated EDC-crosslinked gelatin capsule showing the organized fibril structure of the crosslinked collagen-coating. Scale bars, 10 μm . Images courtesy of Jin Zhang, PhD.

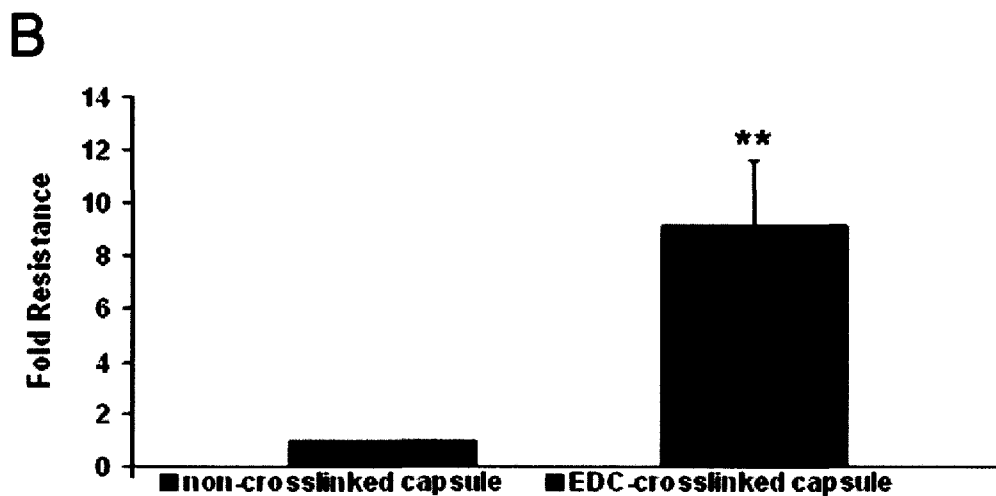
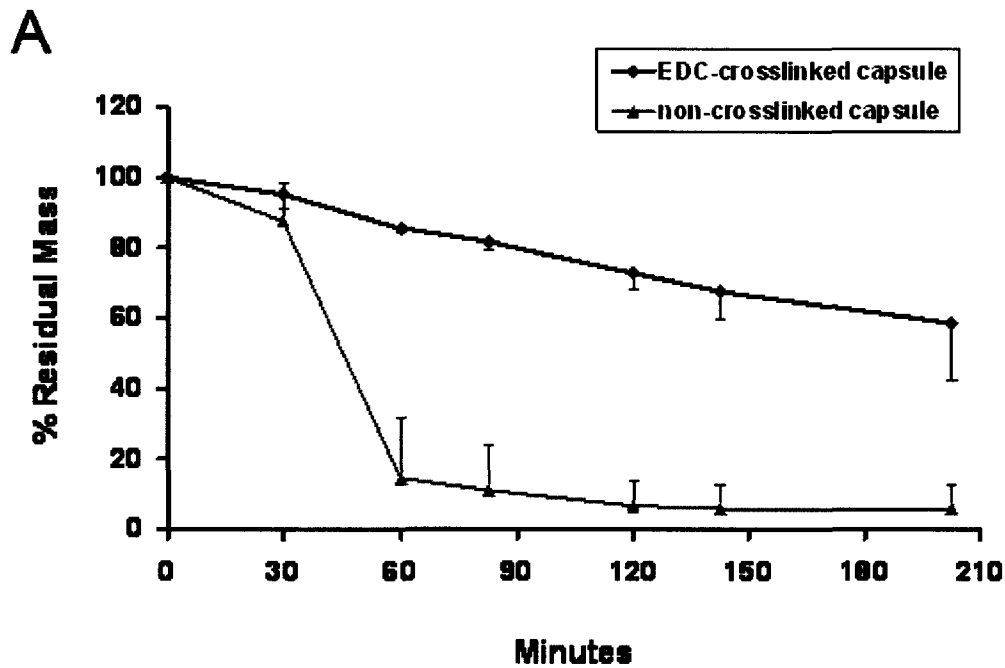


FIGURE 3.2. Collagenase degradation profile and averaged fold resistance of capsules.

(A) Percent residual mass of collagen-coated EDC-crosslinked capsules were significantly higher at all time points after 30 minutes and (B) averaged a 9-fold resistance over all time points with respect to non-coated non-crosslinked capsules (N = 4). Double asterisks (**) represent significant difference ($p < 0.0001$) by a two-tailed Student *t*-test.

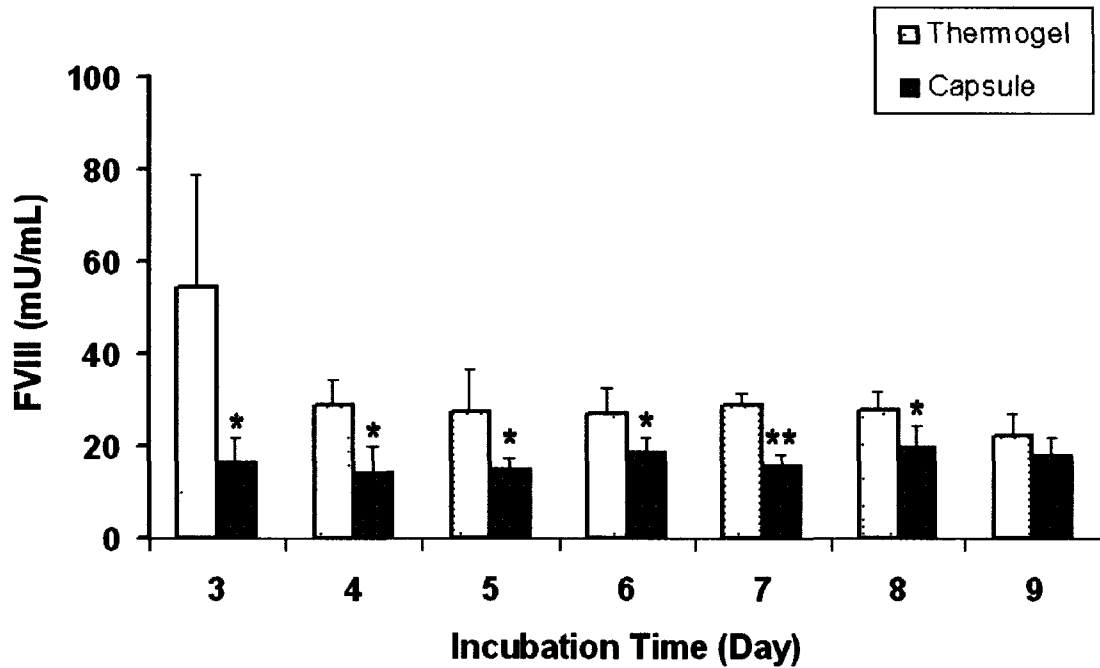


FIGURE 3.3. *In vitro* FVIII release and functionality. Both non-encapsulated and encapsulated collagen thermogels supported release and functionality of FVIII from FVIII-expressing BOECs housed within collagen thermogels. Specifically, non-encapsulated thermogels yielded significantly higher FVIII activity compared to encapsulated thermogels. The additional encapsulation step in the latter test group demonstrated a more consistent release and functionality of FVIII. Values are represented as mean \pm SD. N= 4 independent trials. Single (*) and double asterisks (**) represent significant differences ($p < 0.01$ and $p < 0.05$, respectively) by a two-tailed Student *t*-test.

3.3 FGF-2 Alginate Microparticles

Before proceeding to *in vivo* characterization of our cell delivery vehicle, optimization of the collagen microenvironment with FGF-2 was necessary for the housing of FVIII-expressing BOECs. Since long-term cell encapsulation and implantation was projected, slow and timely release and exposure of FGF-2 for encapsulated BOEC-FVIII was important. FGF-2 was therefore encapsulated, separately, within alginate particles, before incorporation into the cell-housing collagen thermogel. Upon contact with CaCl₂ solution, sodium alginate droplets, containing an FGF-2/ PBS solution, formed soft gel beads. Representative stereo micrograph image revealed alginate particles of various sizes with a glossy surface appearance (Figure 3.4, A). Higher magnification of a larger particle revealed a highly textured surface (Figure 3.4, B). On average, 98% of all particles measured were within the micrometer range (data not shown). As such, alginate particles were referred to as microparticles. In general, loading FGF-2 within alginate microparticles decreased the overall diameter length from $602.0 \pm 45.5 \mu\text{m}$ to $577.0 \pm 21.3 \mu\text{m}$, however, the difference between the means was not statistically significant (data not shown). For potential large scale FGF-2 encapsulation within alginate microparticles, freeze-drying would be essential for preservation of microparticles post-production [100]. SEM analysis was therefore performed to compare the surface structures of alginate particles post freeze-drying at -80 °C. Representative SEM image revealed preservation of particle shape and further confirmed the presence of surface-protruding networks of alginate fibres (Figure 3.4, C).



FIGURE 3.4. FGF-2-loaded alginate particles. (A) Representative stereo micrograph image reveals spherical particles of various sizes with a glossy appearance. Scale bar, 1000 μm . Higher magnification (x100) of the largest particle reveals (B) a highly textured surface. Scale bar, 500 μm . (C) SEM micrograph of the largest freeze-dried particle showing surface folds and protrusions. Scale bar, 200 μm . Images courtesy of Mehrdad Rafat, PhD.

3.3.1 *In vitro* FGF-2 Release and Bioactivity

Prior to incorporating FGF-2/alginate microparticles into a cell-containing collagen thermogel, the biofunctionality of FGF-2 released from alginate microparticles within cell-free thermogels into minimal media was assessed. In this case, the effect of FGF-2 on cell proliferation was the bioactivity that was examined. It should be noted that the 2% HI-FBS within the minimal media likely contained FGF-2, so the media served as a baseline. Results reported were on the biological activity of released FGF-2 over baseline FGF-2 present in FBS. Media samples were tested for bioactivity on pre-seeded FGF-2-sensitive human umbilical vein endothelial cells (IHUVECs)- in place of BOECs, because the former cell line was able to proliferate under serum-deprived conditions [101]. WST-1 and CyQUANT proliferation assays conferred a significant 30 % increase in cell metabolic activity and cell proliferation, respectively. Specifically, the mitogenic activity (WST-1) of FGF-2, in media samples collected at various time points for up to 24 days, conferred an average of 1.35 ± 0.16 fold increase in optical density (O.D.) with respect to control thermogels (Figure 3.5, A); O.D. values correlated to colorimetric quantification of formazan dye formed only in the presence of metabolically active cells in culture. An increase in formazan dye formation indicated an increase in the overall concentration of mitochondrial dehydrogenases within the sample, suggesting an increase in cellular metabolic activity. FGF-2 supernatant samples released from test thermogels were compared to control thermogels containing FGF-2-free alginate microparticles to discriminate the biological activity of released FGF-2 over potentially-released thermogel components. To further confirm bioactivity of released FGF-2, values from the fluorescence-based cellular DNA quantifier assay (CyQUANT) conferred on average, a

1.37 ± 0.23 fold increase with respect to control thermogels (Figure 3.5, B). Similar to the WST-1 assay, an increase in fluorescence values correlated to an increase in total DNA content of cells in culture. Overall, results from WST-1 and CyQUANT assays suggest that released FGF-2 elicited bioactivity on pre-seeded IHUVECs. The next step in assessing biofunctionality was comparing cell metabolic effects of released FGF-2 with fresh, non-encapsulated FGF-2, on pre-seeded IHUVECs. First, enzyme-linked immunosorbent assay (ELISA) values of FGF-2, released from alginate microparticle-containing thermogels, were as low as 0.3 ng/mL (data not shown). In order to assess whether FGF-2 encapsulation resulted in a loss of bioactivity, all media samples were normalized (diluted) to the lowest sample concentration (0.3 ng/ mL) and were compared to minimal media with and without freshly added, non-encapsulated FGF-2 (neFGF-2) (0.3 ng/ mL). Pre-seeded cells incubated with normalized thermogel-released FGF-2 samples, generated higher O.D. values with respect to the negative control (0 ng/ mL neFGF-2) ($0.05 < p < 0.01$) but were not statistically higher than the positive control (0.3 ng/mL neFGF-2). This suggests that FGF-2 released from alginate microparticles within collagen thermogels neither gained nor lost bioactivity post-encapsulation and release. Once bioactivity of released FGF-2 was confirmed on cell monoculture, it was important to assess the effect of FGF-2 on encapsulated cells, and thus FVIII production.

3.4 BOEC-FVIII Encapsulation and Implantation

The next step of this study was combining all of the cell-delivery vehicle components and assessing the effect of each individual component on FVIII production *in vivo*. First, ELISA values confirmed that for up to 4 weeks, no detectable canine FVIII (cFVIII)

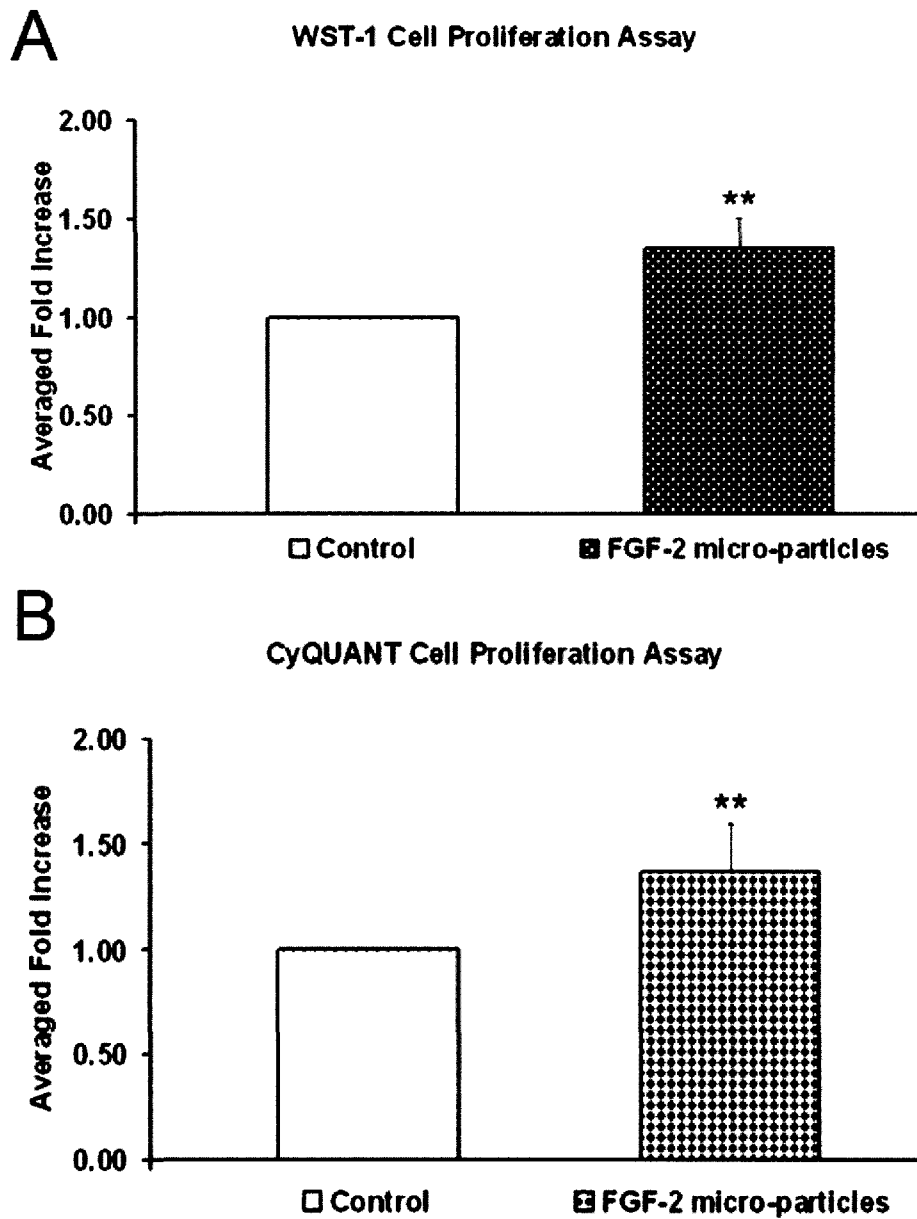


FIGURE 3.5. Bioactivity of FGF-2 released from thermogels. Pre-seeded IHUVECs incubated with released FGF-2 conferred on average (A) a 1.35 ± 0.16 fold increase (WST-1) and (B) a 1.37 ± 0.23 fold increase (CyQUANT), in O.D. values, with respect to control thermogels. Values are mean \pm SD. Double asterisks (**) represents significant difference ($p < 0.01$) by a two-tailed Student *t*-test.

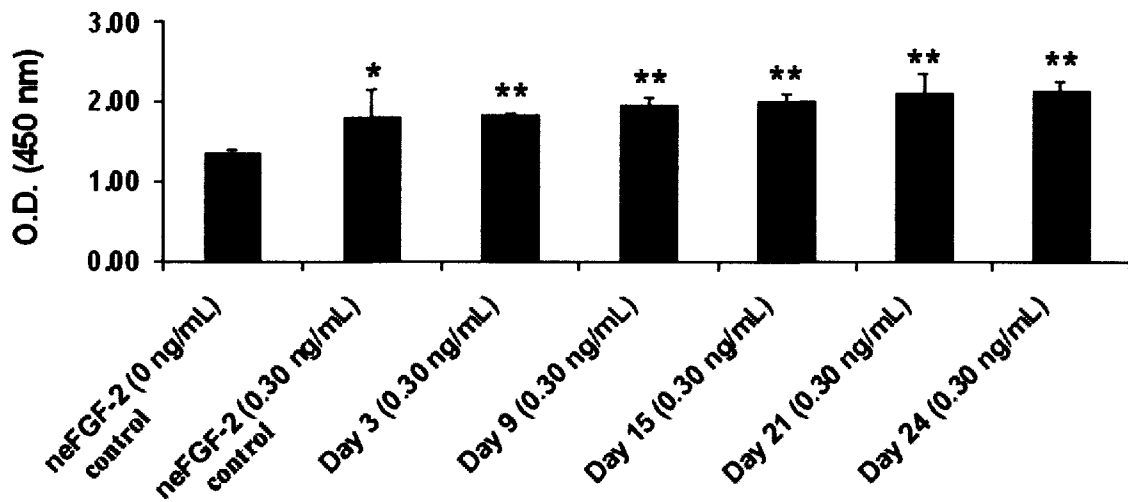


FIGURE 3.6. Normalization of FGF-2, released from alginate microparticle-containing thermogels, to assess maintenance of bioactivity post-encapsulation. Pre-seeded IHUVECs incubated with normalized (diluted) released FGF-2 (0.30 ng/mL) media samples, collected at various time points, demonstrate that bioactivities of released FGF-2 were not significantly different compared to the freshly added, non-encapsulated FGF-2 (neFGF-2) control. Values are mean \pm SD. Single (*) and double asterisks (**) represent significant differences ($p < 0.05$ and $p < 0.01$) by a one-way ANOVA, with Dunnett's correction, on $N= 3$ independent trials.

antigen levels were present in serum of mice implanted with collagen capsules containing non-transduced BOECs, non FVIII-producing BOECs (data not shown). Next, FVIII-producing BOECs were embedded within various thermogel test groups: Matrigel™ only, non-crosslinked collagen thermogel only, non-crosslinked collagen thermogel with FGF-2 microparticles and genipin-crosslinked collagen thermogel with FGF-2 microparticles. Thermogel groups were then further encapsulated within collagen-coated EDC-crosslinked capsules. An equal number of cells (2×10^6 BOEC-FVIII/capsule) was incorporated in each group and implanted subcutaneously in NOD/SCID mice (N= 2- 5 mice per group). Mice were monitored and blood samples were collected for up to 18 weeks. Canine FVIII (cFVIII) antigen levels were detected in mice serum as early as 4 weeks. At 5 weeks post-implantation, mice of all treatment groups (N = 13) had detectable levels (>10 ng/mL) of cFVIII in their sera. The highest cFVIII antigen levels were detected in mice implanted with capsules containing genipin-crosslinked FGF-2 microparticle-containing collagen thermogels. These levels (20- 30 ng/mL) persisted in at least 2 mice for up to 12 weeks and up to 14 weeks in one mouse. Similar findings were observed in sera of mice implanted with capsules filled with Matrigel™. All mice from the Matrigel™ and genipin-crosslinked FGF-2-containing collagen thermogel test groups died at various time points before 15 weeks post-implantation (Figure 3.7). Most notable in this experiment were the persistent long-term (18 weeks post-implantation) levels of cFVIII antigen in all mice implanted with collagen thermogel only and FGF-2 microparticle-containing collagen thermogel groups. Between 12 to 17 weeks post-implantation, higher cFVIII antigen levels were detected in mice sera of the

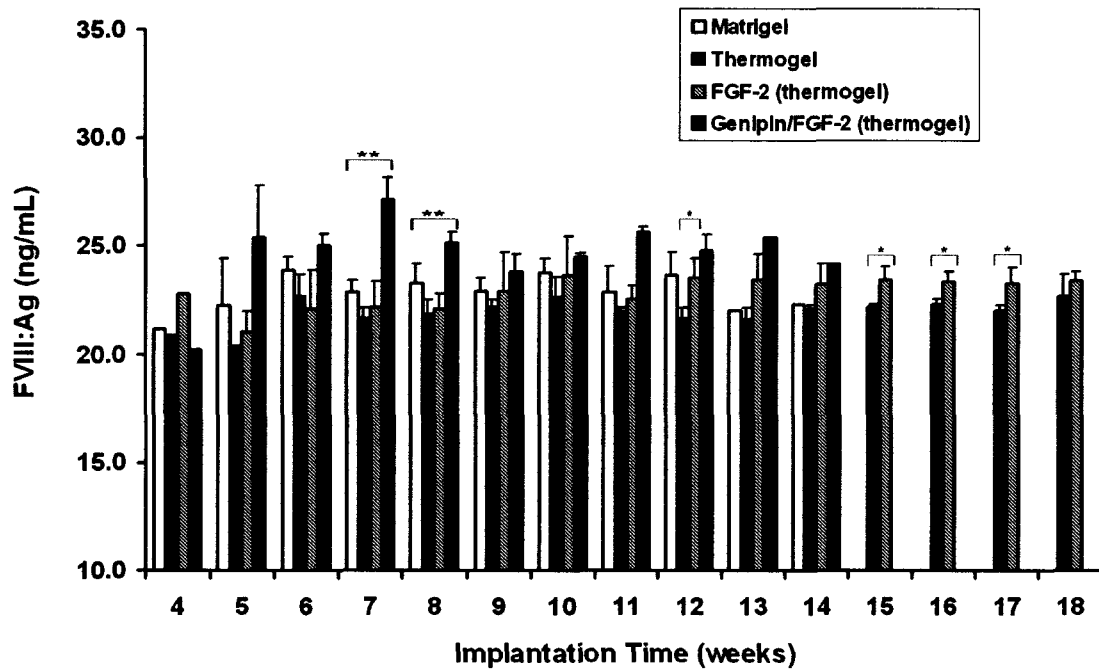


FIGURE 3.7. Serum canine FVIII antigen levels released from capsules containing various thermogels containing BOEC-FVIII (2×10^6 cells/ capsule). Mice implanted with capsules containing genipin-crosslinked thermogels loaded with FGF-2 particles resulted in significantly higher cFVIII antigen level over weeks 8 and 9 with respect to Matrigel-filled capsules. All mice in genipin and Matrigel™ groups were dead at 15 weeks post-implantation. Significantly higher cFVIII antigen levels were detected, at 12, 15, 16 and 17 weeks post-implantation, in mice implanted with FGF-2-containing collagen thermogels compared to the thermogel-only group ($p < 0.01$). All mice implanted with the latter two thermogel groups were alive throughout the 18 weeks implantation study. Values are represented as mean \pm SD. $N = 2$ to 5 mice per treatment group. Single (*) and double asterisks (**) represent significant differences ($p < 0.05$ and $p < 0.01$) by a one-way ANOVA.

FGF-2 group ($p < 0.01$) (Figure 3.7). Mice from these two groups were euthanized at 18 weeks post-implantation for ethical reasons. Overall, the non-crosslinked collagen thermogel groups supported FVIII production and release by encapsulated BOEC-FVIIIs.

3.5 Pre- and Post-implantation: Gross and Histological Assessment

The ultimate cure to HA would be optimal integration of FVIII-expressing BOECs with the host endothelium for continuous production and secretion of FVIII into the blood stream. Therefore, host integration with the implant was qualitatively assessed to evaluate effects of implantation on capsule integrity. As compared to pre-implanted capsules with a bulky glossy appearance (Figure 3.8, A), representative subcutaneously implanted capsule appeared flattened and possibly ruptured post-implantation (Figure 3.8, B); capsules were attached to either the apical or basal side of the implantation site and appeared to be engulfed by a dense network of vascularised fibrous tissue. Histological staining of capsules surrounded by vascularized tissue was further investigated with H & E histology. Representative images of fibrous tissue peeled off of a 2-week implant, revealed the formation of distinct blood vessel-like (BV) structures adjacent to where the capsule surface would be (Figure 3.9, A-left). At a higher magnification (Figure 3.9, A-right), infiltration of mainly fibroblasts (FB) was observed within dense collagen tissues (DCT) (Dr. Hamid Faraji, personal communication). Representative images of a 12-week implant reveal the presence of the capsule shell (Figure 3.9, B-left). Higher magnification reveals an abundance of newly-formed capillary-like structures (C) near the capsule surface (Figure 3.9, B-right), each containing at least three individual erythrocytes. Histological staining of capsules for identification of surrounding



Figure 3.8. Collagen capsules pre- and post-implantation. (A) Digital image of smooth, translucent collagen capsules prior to implantation. Scale bar, 5000 μm . (B) Capsule recovered at 2 weeks post-implantations, showing deformation of the capsular structure. The implanted capsule was surrounded by a dense network of highly vascularized fibrous tissue. Scale bar, 5000 μm

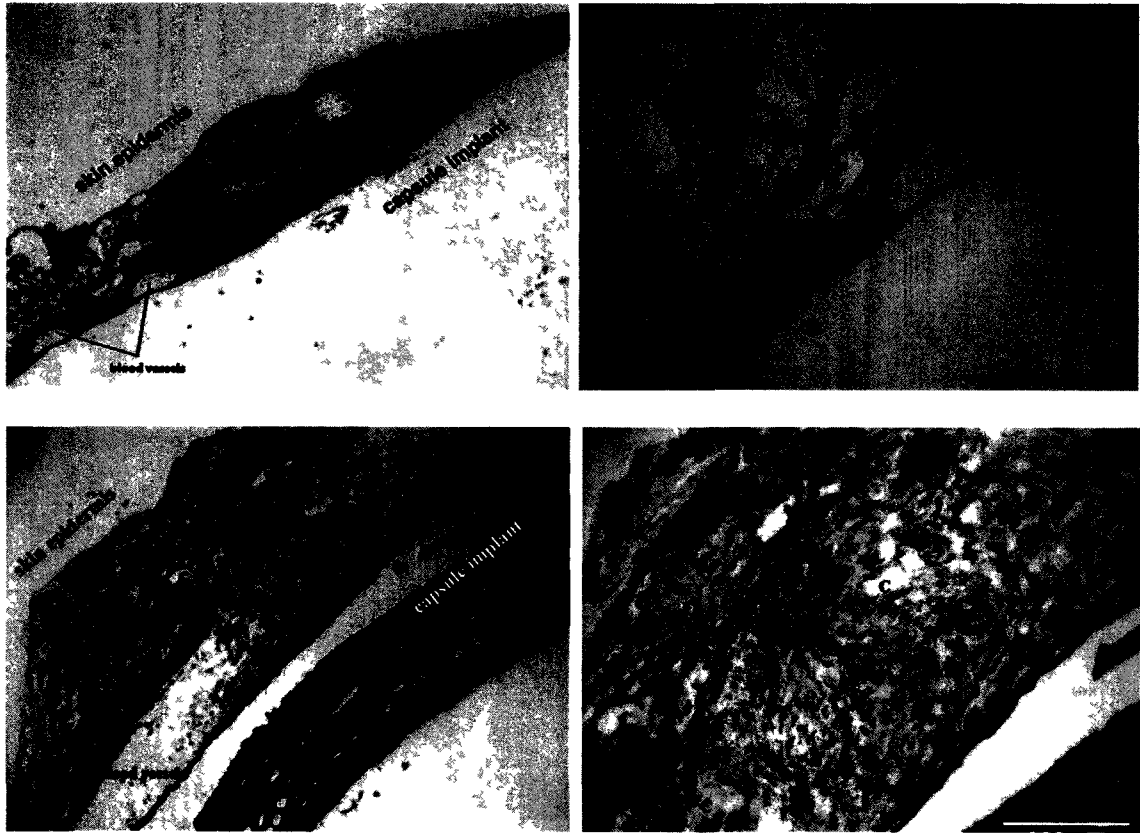


FIGURE 3.9. Host response to collagen capsule implant. Host response to EDC-crosslinked capsule implants was assessed at 2 and 12 weeks following subcutaneous implantation in NOD/ SCID mice. Histology (H & E) analyses of sections from the crosslinked collagen implants demonstrate signs of healthy remodeling (A) 2 weeks and (B) 12 weeks post-implantation. Healthy fibrous tissue formation is evident from the formation of dense collagen tissue networks as early as 2 weeks post-implantation (A-right) and continue to be present even at 12 weeks post-implantation (B-right). Vascularization of the implant is suggested from the presence of vessel-like structures (A-left & right; Scale bar = 500 μm .) including erythrocyte-filled capillaries identified close to the capsule surface (B-left & right; Scale bar = 200 μm .). C- capillary; DCT- dense collagen tissue; FB- fibroblast cells.

vasculature was further confirmed with immunostaining against CD31. The monoclonal CD31 antibody analysis showed that CD31-positive endothelial cells, defining capillary structures, were visibly crowded around the implant. CD31-positive structures were identified both on the apical side of the implant, between the skin dermis and the capsule (Figure 3.10, A- arrows) as well as on the basal side of the implant, between the capsule and the muscle/lipid pads (Figure 3.10, B- arrows). Further immunostaining against smooth muscle actin (SMA) identified enclosed structures near the capsule shell under green fluorescence (Figure 3.11). Overall signs of host vascularization of the collagen capsule implant were apparent.



FIGURE 3.10.A. Immunostaining of CD31-positive structures on the apical side of the implant. A representative section from the crosslinked bioimplant showing CD31-positively stained endothelial cells defining capillary structures (arrows) adjacent to the implant. Scale bar, 500 μm .



FIGURE 3.10.B. Immunostaining of CD31-positive structures on the basal side of the implant. FGF-2 containing capsules without cells were implanted subcutaneously in mice for 3 weeks. Retrieved implants were stained with hematoxylin and CD31, a marker for endothelial cells. CD31-positive cells were visualized using DAB (arrows), a 3,3-diaminobenzidinetetrahydrochloride substrate. Scale bar, 500 μm .

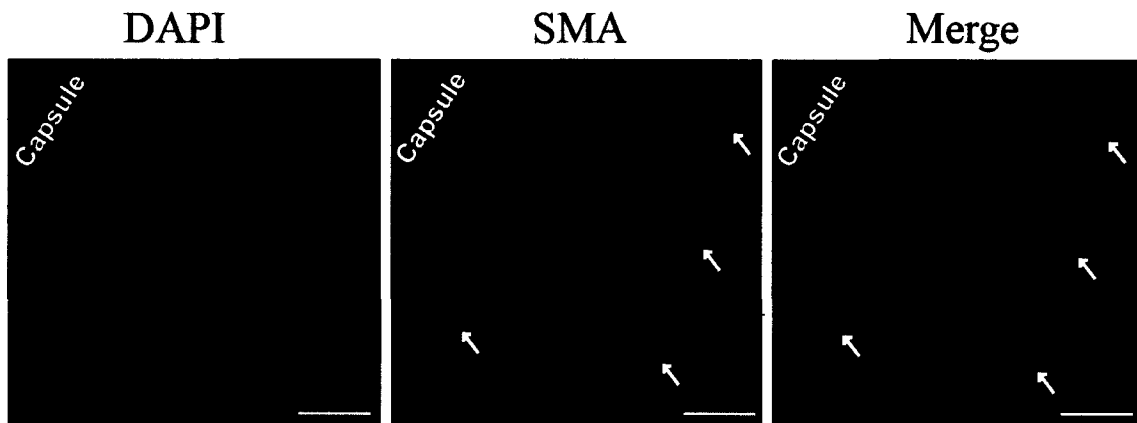


FIGURE 3.11. Immunostaining of SMA-positive structures near capsule wall. Vessel-like structures near the capsule wall are identified (arrows) as enclosed structures positive (green fluorescence) for smooth muscle actin (SMA). In blue are nuclei stained with DAPI and merged as a final image. Scale bar, 1000 μm .

4 DISCUSSION

4.1 Collagen Capsule

In the present study, a collagen-based vehicle for the delivery of FVIII-expressing endothelial cells (BOECs) was developed and tested as an alternative to a previously used Matrigel™ scaffold. Since Matrigel™ is produced and secreted by sarcoma cells, there is a lack of product consistency and a risk of pathogen cross-transfer that may compromise clinical translational studies. Furthermore, the use of soft hydrogels as cell-delivery biomaterials resulted in premature degradation. The results of this study show that collagen can be used as an alternative to Matrigel™ for supporting BOEC survival and long-term FVIII production for potential treatment of HA. Type I collagen, the major ECM protein, has proven to be biocompatible and enhance tissue regeneration in various experimental and clinical applications [102]. In a previous *in vitro* study conducted by Alex Hyatt, M.Sc., encapsulating BOECs within collagen type I did not adversely affect cell viability (Appendix- Figure 6.1– Figure 6.3). Furthermore, various crosslinkers were used to further increase collagen's resistance to protease degradation. Literature reports that carbodiimide crosslinking of collagen scaffolds delays *in vivo* degradation to at least 20 weeks [103] while non-crosslinked collagen scaffolds were gradually resorbed and replaced by host connective tissue [104]. To stabilize the collagen capsule from premature degradation, EDC was selected as a collagen crosslinker in this study. EDC is a zero-length crosslinker that does not become incorporated into the final matrix, hence, there is no concern over toxic breakdown products from the crosslinker. In our subcutaneous implantation study, capsules were easily identified and removed even at 18 weeks post-implantation. Histological assessment of retrieved capsules revealed the

presence of a thick hematoxylin-positive layer- collagen capsule- with minimal degradation. In fact, complete breakdown of any one capsule was not observed in any of the mice used in this study. Tightly woven collagen fibres formed on the surface of EDC-crosslinked capsules, as seen from representative SEM micrographs, may be the attributing cause of *in vivo* resistance as well as a 9-fold *in vitro* resistance to collagenase digestion. In addition to variations in crosslinker concentrations, different buffer solutions also seem to have an effect on the efficiency of collagen crosslinking [105]. In our study, water was used as the base solvent for EDC crosslinking. Other studies have used buffer systems, such as MES, for more controlled crosslinking [106]. Modifications in any or all of the aforementioned parameters should be investigated to obtain superior collagen biomaterials for the delivery of FVIII-expressing cells.

4.2 Release and Functionality of FVIII

Not only was the development of a more protease resistant collagen scaffold a major goal of the present study, but another important factor to assess was FVIII production and release, by encapsulated BOEC-FVIIIs, to the surrounding environment. Studies conducted by Wissink *et al.* [46] demonstrated that the crosslink density of EDC-crosslinked collagen did not affect the secretion of endothelial cell-specific factors such as prostacyclin (PGI₂), von Willebrand factor (vWF), tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1). In the present study, though highly resistant to collagenase digestion, the presence of the additional capsule barrier resulted in significantly lower FVIII activity levels compared to non-encapsulated collagen thermogels. Therefore, there is a clear trade-off between increased resistance and FVIII

release. Since the primary objective of this study was to provide a more collagenase-resistant collagen capsule for the isolation and potential protection of encapsulated cells, confirmation of release and functionality of even low levels of FVIII was sufficient for the purpose of this study. Our next step was thus to investigate FVIII permeability through our crosslinked scaffolds *in vivo*. Since NOD/ SCID mice have normal FVIII levels, detection of cFVIII antigen levels was used to assess cFVIII production and release. In all NOD/SCIDs treatment groups, cFVIII antigen was detected at 4 weeks post-implantation. In previous studies, our collaborators reported cFVIII antigen detection at 3 weeks post subcutaneous injection [6]. The delay in cFVIII antigen level detection in the present study may be attributed to increased penetration time of cFVIII through the collagen capsule and in turn, lower detectable levels of cFVIII antigen; the discrepancy between 3-week versus 4-week however, was not deemed detrimental to the overall goal of the study. Therefore, based on cFVIII antigen levels in mouse serum, it is safe to assume that the collagen capsule FVIII-expressing BOEC-delivery system did not impede cFVIII release and antigen detection in the host circulation. Though the use of immune-compromised NOD/SCID mice in our tests did not permit for accurate biocompatibility testing of our delivery vehicle, previous studies from our laboratory (Merrett et al. 2008) have shown that unglycosylated, recombinant human collagen did not cause cross-species reactivity. Furthermore, in the current study, the reinforcement of collagen with EDC also allowed for organized remodeling, as opposed to resorption [79]. In our subcutaneous implantation study, histological assessment of retrieved capsules revealed the presence of normal fibrous tissue detected as early as 2 weeks post-operative, suggesting that our EDC-crosslinked capsule supports the interstitial

deposition and organization of a healthy extracellular matrix. In other studies using formaldehyde or glutaraldehyde for graft crosslinking, interference in host-tissue integration has been attributed to the release of toxic residues during *in vivo* degradation of the graft [46]. The use of EDC as a collagen crosslinker in this study did not seem to interfere with host-implant integration. Erythrocyte-filled, CD31-positive vessel-like structures within the host fibrous tissue were located adjacent to the capsule surface. SMA-positive structures were also present near the collagen wall of 8-week post-implanted capsules. These results, not only suggest early capsule vascularization by host vessels, but further suggest a potential route through which transgenically-produced cFVIII antigens were detectable in mice sera. Other than the assumption that FVIII reaches host circulation via simple diffusion through the ECM, more advanced experiments investigating host vascularization with FVIII-producing BOECs would further our current understanding. Since the ultimate goal of this delivery strategy is timely degradation of the collagen capsule system and optimal integration of FVIII-expressing BOECs with host vasculature, one way of ensuring a high angiogenic response in future studies is the application of vascular endothelial growth factor (VEGF) to our capsule system. In other studies, functionalization of collagen matrices has been achieved by heparin crosslinking, using EDC, and subsequent immobilization of VEGF [107]. A range of different heparin and EDC/NHS concentrations in combination with FGF-2 and VEGF may be suitable to induce further implant vascularization and eventual angiogenesis with host vasculature. Overall, long-term cFVIII antigen levels detected from mice implanted with type I collagen thermogels were in good agreement with respect to cFVIII antigen levels detected from mice implanted with Matrigel™. While

substitution of Matrigel™ with collagen type I has resulted in lower cellular performance [81, 108, 109], type I collagen used in this study resulted in a longer mice survival and thus FVIII production over Matrigel™. These results further demonstrate that encapsulation of FVIII-producing BOECs within collagen capsules, for protection from premature degradation, did not interfere with *in vivo* FVIII production and release.

4.3 Genipin Crosslinking

Of interest, the highest cFVIII antigen levels were detected in mice implanted with capsules filled with genipin-crosslinked collagen thermogels containing FGF-2 microparticles. As mentioned, the biggest challenge with most *in vivo* biomaterial delivery studies is premature degradation. Therefore, to prolong *in vivo* degradation, the cell-housing thermogel was also crosslinked. Using only a low concentration of genipin, our collagen thermogel was only 2-fold more resistant to collagenase digestion than its non-crosslinked counterpart ($p < 0.01$) (Appendix- Figure 6.1). Studies conducted by Avila *et al.*, demonstrated that up to a 5-fold increase in resistance to collagenase degradation could be obtained by crosslinking porcine corneas with genipin [110]. Crosslinking collagen hydrogels with concomitant cell encapsulation is a difficult task to accomplish; the crosslinker must be non-cytotoxic and slow, to allow for the addition of cells before completion of the crosslinking step. In this study, genipin was used to crosslink type I collagen thermogels since studies on various cell types have shown that genipin, at concentrations of 50- 100 μM , shows no significant cytotoxic effects on cell viability [111]. In fact genipin has been shown to inhibit hepatocyte apoptosis. Genipin was found to suppress *in vitro* Fas-mediated apoptosis in primary-cultured murine

hepatocytes [112]. Exposure of hepatoma cells to higher genipin concentrations (200 μ M) for 24 h, however, resulted in the appearance of apoptotic bodies [113]. Previous work in the lab supported these findings by demonstrating that genipin-crosslinking of collagen thermogels supported over 85 % BOEC viability. Encapsulated BOECs within genipin-crosslinked collagen thermogels were also able to express CD31 and uptake DiI-acetylated LDL. In genipin-crosslinked collagen thermogels containing FGF-2 microparticles, detectable cFVIII antigen levels in mouse serum persisted in at least 2 mice for up to 12 weeks, and 14 weeks in one mouse. However, all mice with genipin-crosslinked implants died before the 18-week termination point; this was also the case for mice implanted with Matrigel-filled capsules. Further studies using healthy mice models are necessary to investigate whether genipin in conjunction with our delivery system was responsible for mouse death.

4.4 FGF-2/ Alginate Microparticles

Representing one of the most widely used materials for the immobilization of enzymes and proteins, calcium-alginate particles are used most often for release of growth factors [114]. To ameliorate the cellular microenvironment and provide long-term trophic support to encapsulated cells *in vivo*, fibroblast growth factor 2 (FGF-2) was loaded within alginate particles. FGF-2-loaded alginate particles were then incorporated into collagen thermogels to initiate controlled release [92, 115, 116] to the surrounding growth factor-poor cell micro-environment. Alginate is advantageous because of its biocompatibility, biodegradability, and lack of systemic toxicity upon administration [53, 117-119]. In this study, FGF-2 was mixed with an alginate solution and subsequently

dripped into a bath of calcium solution resulting in the instantaneous formation of microparticles. In this study, only one milliliter of an alginate/ FGF-2 solution was used to manually expel alginate droplets at a given time. The use of alginate as a material for delivering FGF-2 is amenable to scale-up preparations [95] deemed useful for clinical applications. For large-scale production of microparticles, automatic syringe pumps [120] could be used to expel pre-set larger volumes of drug-alginate solution [121]. To investigate bioactivity of FGF-2 released from alginate microparticles from within collagen thermogels, cell metabolic activity and proliferation were assessed. Whereas cell number, quantified from total DNA content, is an absolute measure of cell proliferation, cell metabolic activity, quantified from intracellular mitochondrial reductant NADH or extracellular trans-plasma membrane electron transport, is more a measure of cell health [122]. Combining these assays can yield a more detailed view of cell activity with respect to bioactivity of released FGF-2. FGF-2 functionality upon release was confirmed from the increase in optical density- tetrazolium-formazan signals produced after reactions with cellular dehydrogenase- measuring cell metabolic activity (1.35 fold) and nuclear content (1.37). Further WST-1 assays comparing released FGF-2 with respect to non encapsulated FGF-2 confirmed that encapsulation of FGF-2 within alginate microspheres and collagen thermogels does not result in a reduction of bioactivity. In fact, incorporation of FGF-2/ alginate microparticles in collagen thermogels resulted in significantly higher serum cFVIII antigen levels in of mice 12 to 17 weeks post-implantation (p-value < 0.01) compared to control collagen thermogel (no FGF-2/ alginate microparticles). These findings suggest that FGF-2 bioactivity may confer bioactivity for encapsulated BOEC-FVIIIs for up to 17 weeks post-implantation.

However, further studies on the effects of FGF-2 microparticle concentrations and release kinetics, as well as FVIII production would be needed to optimize conditions for maximum FVIII production.

4.5 Future Directions

We have shown feasibility of transfecting attachment-dependent endothelial cells to produce and release coagulation factor FVIII. For long-term production of FVIII, long-term survival of FVIII-producing BOECs is required. Preliminary results demonstrating long-term (18 week) release of FVIII from encapsulated FVIII-expressing BOECs should thus be repeated in haemophilic mice or dogs not only for a longer implantation period but also to assess functionality of *in vivo*-secreted FVIII. However, the production of inhibitory antibodies against FVIII, rendering exogenous FVIII inactive [123] is one of the major limitations in the investigation of haemophilia treatments. As such, haemophilic mice and dogs must go through protocols to generate immunological tolerance to FVIII [124] before FVIII-expressing BOECs can be implanted. Collaborators at Queen's University, Kingston, Ontario are optimizing protocols to generate immunological tolerance to FVIII. Haemophilic mice (c57 BL6) are currently undergoing transient immune suppression with cyclophosphamide with pre-infusion of human recombinant FVIII (Lillicrap, personal communications). Once immunomodulatory management of FVIII inhibitors is controlled, functionality of FVIII released from the capsule delivery system may be assessed *in vivo*. Furthermore, since adequate investigations on long-term effects of transduced endothelial cell integration with host vasculature have not been conducted, future experiments merit the investigation of

BOEC-FVIII incorporation into neovasculature; gaining further insight on transgenic cell integration with host vasculature will be an important step toward developing a comprehensive strategy aimed at delivering genetically engineered FVIII-expressing cells for the treatment of haemophilia. The objective of future *in vivo* studies will thus not only be to sustain FVIII expression past 27 weeks in haemophilic models, but also to assess long-term biodegradability and cell-housing characteristics of our capsule system as well as FVIII functionality.

4.6 Alternate Sites for Implants

The optimal transplantation site for FVIII-expressing BOECs continues to be an important issue. A variety of sites have been proposed in other studies of *ex vivo* cell delivery including the peritoneal cavity, the kidney capsule and various subcutaneous sites [125]. If the subcutaneous site could be used in humans, it would be highly advantageous primarily due to easy accessibility of the capsule. Furthermore, studies conducted in the vascularization of implants containing β -cells for the treatment of type I diabetes mellitus (T1DM), have suggested that the subcutaneous site seems advantageous because of the potentially less invasive procedure that could be performed under local anesthesia with minimal risks of bleeding and thrombosis [126]. In the case of haemophilia patients, minimizing bleeding during surgeries is vital to the overall success of the treatment. Studies have also reported some inherent limitations with subcutaneous implantation of cell-containing biomaterials such as lack of early vascularization, induction of local inflammation, and mechanical stress on the graft, resulting in primary non-function [126, 127]. With our relative large capsule system (1 cm x 1 cm x 1.5 cm),

subcutaneous implantation in animal studies may result in the rupture of the cell-containing system due to itching, scratching and rolling around of the animal. Numerous studies have thus attempted to identify the most suitable site of implant while current pre-clinical studies of cell transplantation are being performed in the omnium of haemophilic dogs (Lillicrap, personal communications). The trade-off between transplant effectiveness over initial surgical invasiveness at various sites of implantation for alternative haemophilia A treatments, should thus be taken into consideration.

4.7 Versatility of the Collagen Capsule System

Depending on the location of implant and required degradation time, our collagen capsule system may be tailored to deliver various different cells or growth factors, including FGF-2 for the treatment of ischemia. Therapeutic angiogenesis is a novel approach to the treatment of ischemia based on the use of proangiogenic growth factors to induce the growth of new blood vessels to supply the area at risk [128]. Two of the biggest challenges faced in drug delivery initiatives are rapid release and *in vivo* protease digestion and degradation [129-132]. To circumvent the need for repeated injections, a delivery vehicle is necessary to both protect the growth factor from degradation as well as release it in a controlled manner. For example, controlled delivery of FGF-2 to target sites, via micro- or nanoparticles yielded higher microvessel density [93] and enhanced angiogenesis [94]. Nevertheless, many delivery vehicles suffer from what is known as an “initial burst”, in which case a high percent of the drug is released within the first 24-48 hours [133-135]. Employing a multiple encapsulation strategy, by incorporating FGF-2-loaded alginate microparticles within genipin-crosslinked collagen thermogels, the

release of FGF-2 within the first 9 days, was reduced from an average of 700 pg/ mL daily release to 500 pg/ mL daily average release. Further encapsulation of the FGF-2 microparticle-containing collagen thermogel within our reinforced collagen capsule further reduced FGF-2 release to 300 pg/ mL average daily release (Appendix- Figure 6.4). With the control over the design and shape of this novel collagen-based system and its ability to support capillary formation, our collagen capsule may thus play a pivotal role in FGF-2 delivery for ischemia treatment applications.

5 CONCLUSION

The results of this study demonstrated that the collagen biomaterials can be used in place of clinically-sub-optimal Matrigel™ as scaffolds for delivery of BOECs. We obtained a higher degree of control as a result of combining pure components, at exact concentrations, to obtain a functional and reproducible cell delivery system. Displaying higher resistance to protease digestion, our reinforced collagen system supported BOEC viability and phenotypic behaviour, and demonstrated healthy host integration as early as 2 weeks and as long as 12 weeks post-implantation. Most importantly, this collagen-based cell delivery system allowed for persistent cFVIII antigen detection for up to 18 weeks suggesting effective FVIII production and release from encapsulated BOEC-FVIII. It is important to note, however, that while these results suggest feasibility, long-term *in vivo* studies will further our knowledge on the suitability and efficacy of such a system. Specifically, following implantation, tracking and close monitoring of FVIII release and functionality within haemophilic animal models are required. Before this task can be accomplished, the major complication in haemophilia treatment - the induction of

neutralizing antibodies directed against the FVIII protein - must be controlled in order to take advantage of FVIII released from a delivery system such as the one we developed. Finally, in this study, we confirmed that tertiary encapsulation of FGF-2 within alginate microspheres and collagen scaffolds reduced the initial burst effect and controlled FGF-2 release. As such, with the control over the design and shape of this novel collagen-based system and its ability to support capillary formation, our collagen capsule may thus play a pivotal role in FGF-2 delivery for ischemia treatment applications.

6 APPENDIX

6.1 Genipin-Crosslinked Collagen Thermogel

In order to further increase the resilience of this collagen delivery system, collagen thermogels were also crosslinked. Since thermogels would house live cells, the natural, less cytotoxic genipin was used as a crosslinker. Collagenase digestion was performed *in vitro* to assess the effects of genipin crosslinking on hydrogel stability in the presence of collagenase enzymes. With respect to non-crosslinked collagen thermogels, percent residual mass of genipin-crosslinked collagen thermogels were significantly higher at all time points between 20 to 80 minutes, as seen from the collagenase degradation profile (Figure 6.1, A). Overall, in comparison to control non-crosslinked thermogels, the genipin-crosslinked thermogel averaged a 2-fold resistance against collagenase digestion (Figure 6.1, B; $p < 0.01$ a two-tailed Student *t*-test). Of most importance was the assessment of BOEC behaviour and viability within collagen thermogels, both with and without genipin crosslinking. In a 3-D culture model collagen thermogels BOEC encapsulation resulted in the formation of vessel-like structures (Figure 6.2, A & B) with over 85 % cell viability (Figure 6.2, C; $p < 0.01$). Immunostaining and confocal laser scanning microscopic analysis established that these structures were positive for CD31/Pecam1 endothelial cell marker (Figure 6.3, A) and uptake of DiI-Ac-LDL (Figure 6.3, B), suggesting that collagen thermogels and genipin crosslinking do not interfere with endothelial cell behaviour.

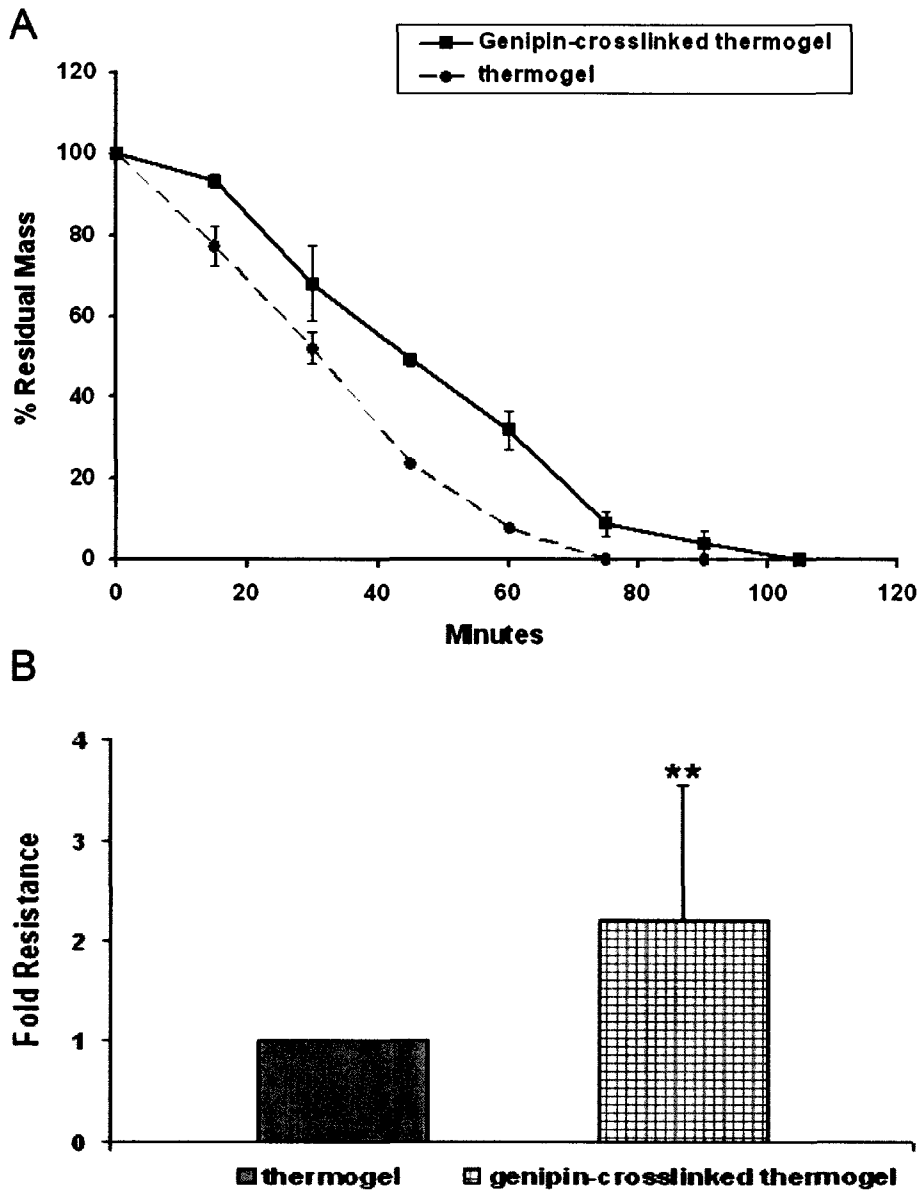


FIGURE 6.1. Collagenase degradation profile and averaged fold resistance of collagen thermogels. (A) Percent residual mass of genipin-crosslinked thermogels were significantly higher at all time points between 20 to 80 minutes and (B) averaged a 2-fold resistance between these time points with respect to non-crosslinked thermogels (N = 3). Double asterisks (**) represent significant difference ($p < 0.01$) by a two-tailed Student *t*-test. Data analyzed from results obtained from Alex Hyatt, M.Sc.

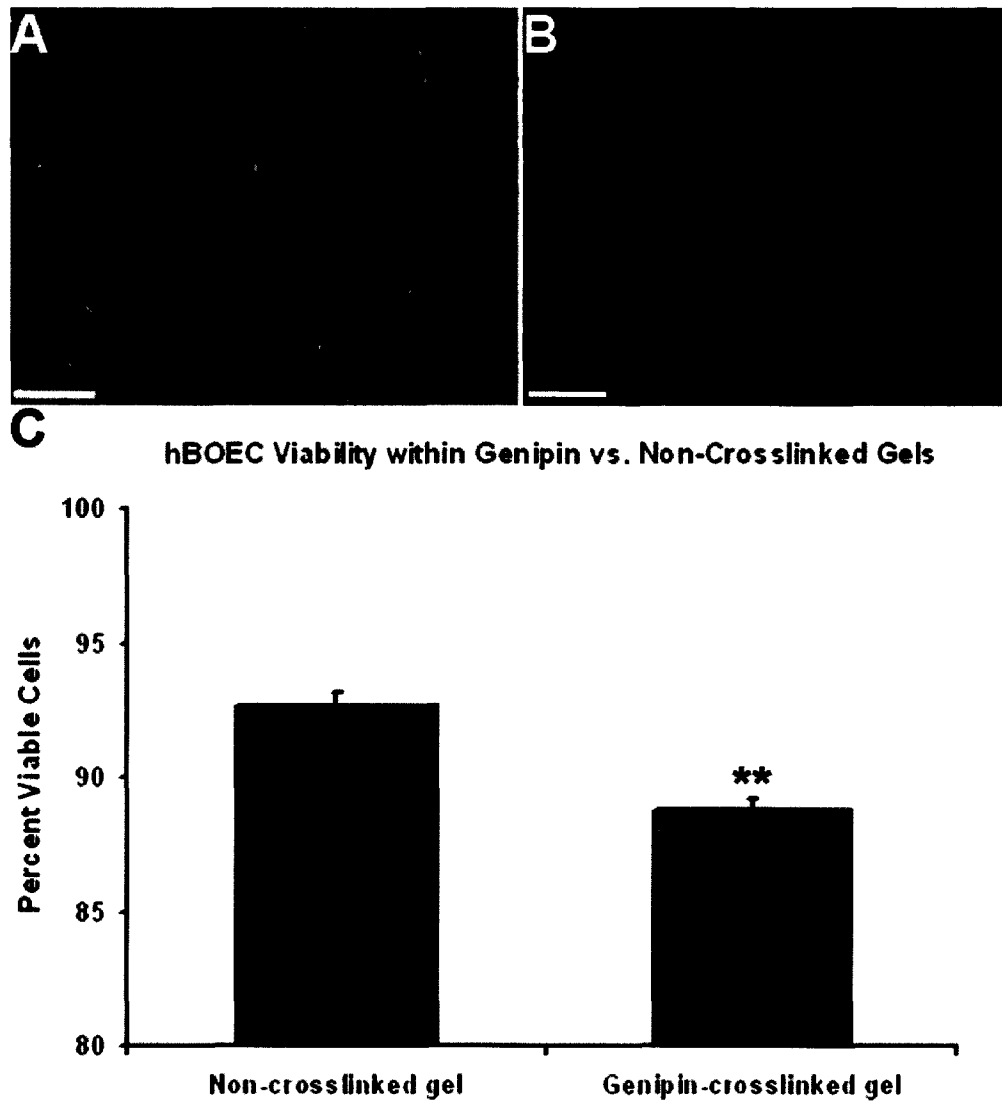


FIGURE 6.2. Human BOEC (hBOEC) morphology and viability within genipin-crosslinked collagen thermogels. (A) Vessel-like and spherical structures of cells are viewed under phase contrast 7-days post incubation. (B) Representative LIVE/DEAD assay image also shows vessel-like formation of live (green) cells and few spherical dead (red) cells accounting for (C) over 85% cell viability in collagen thermogels. Scale bars = 100 μ m. Values are represented as mean \pm SD. Double asterisks (**) represent significant difference ($p < 0.01$) by a two-tailed Student *t*-test. N= 4. Data analyzed from results obtained from Alex Hyatt, M.Sc.

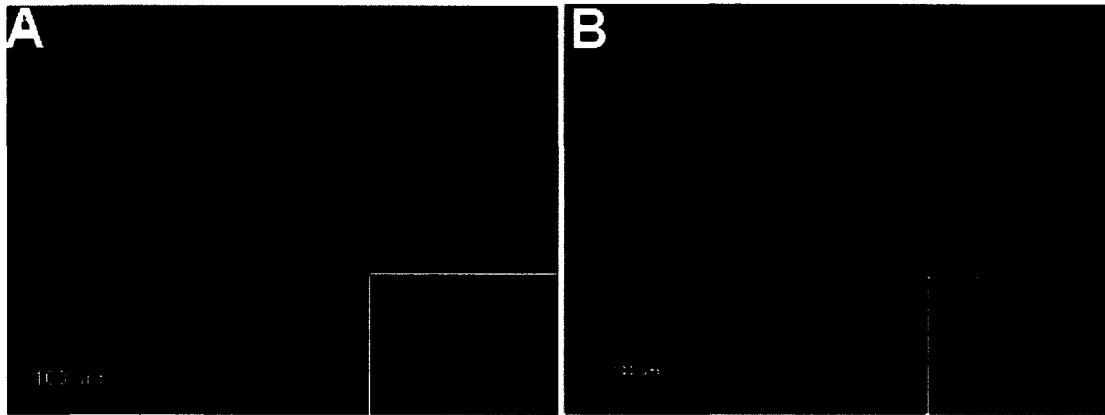


FIGURE 6.3. Human BOECs encapsulated within genipin-crosslinked collagen thermogels showing (A) positive staining for CD31 and (B) uptake of DiI-acetylated LDL. Inset A: Negative control- cells stained with only secondary antibody. Inset B: Negative control- neural cells (NDCs) stained for uptake of DiI-acetylated LDL. Scale bars = 100 μ m. Images courtesy of Alex Hyatt, M.Sc.

6.2 Primary, Secondary and Tertiary FGF-2 Encapsulation

The use of a multiple encapsulation strategy demonstrated reduced and controlled release of FGF-2 over various time points of incubation. ELISA values of media samples, demonstrated that primary encapsulation of FGF-2 within alginate microparticles resulted in an average daily burst release of 700 pg/mL FGF-2 each day over the first 10 days of incubation. Incorporation of microspheres within a genipin-crosslinked collagen thermogels reduced FGF-2 release to an average daily release of 500 pg/mL and further encapsulation within collagen-coated EDC-crosslinked capsules reduced FGF-2 release to an average daily release of 300 pg/mL every 10 days for over 29 days incubation (Figure 6.4). This suggests that increased encapsulation of FGF-2 within various scaffolds further decreases the initial FGF-2 release resulting in a more sustained, continuous FGF-2 release over long-term incubations.

6.3 Western Blot

To further confirm bioactivity of FGF-2 on pre-seeded endothelial cells, western blot analyses were conducted on FVIII-expressing blood outgrowth endothelial cells. FGF-2 in control media samples demonstrate an increase in the levels of phosphorylated ERK-1 and ERK-2, with respect to total ERK-1/2 proteins, suggesting that extracellular FGF-2 works through the Raf1-MEK-MAPK (ERK1/2) pathway ultimately leading to increased phosphorylation of ERK-1 and ERK-2 and eventual cellular survival and proliferation.

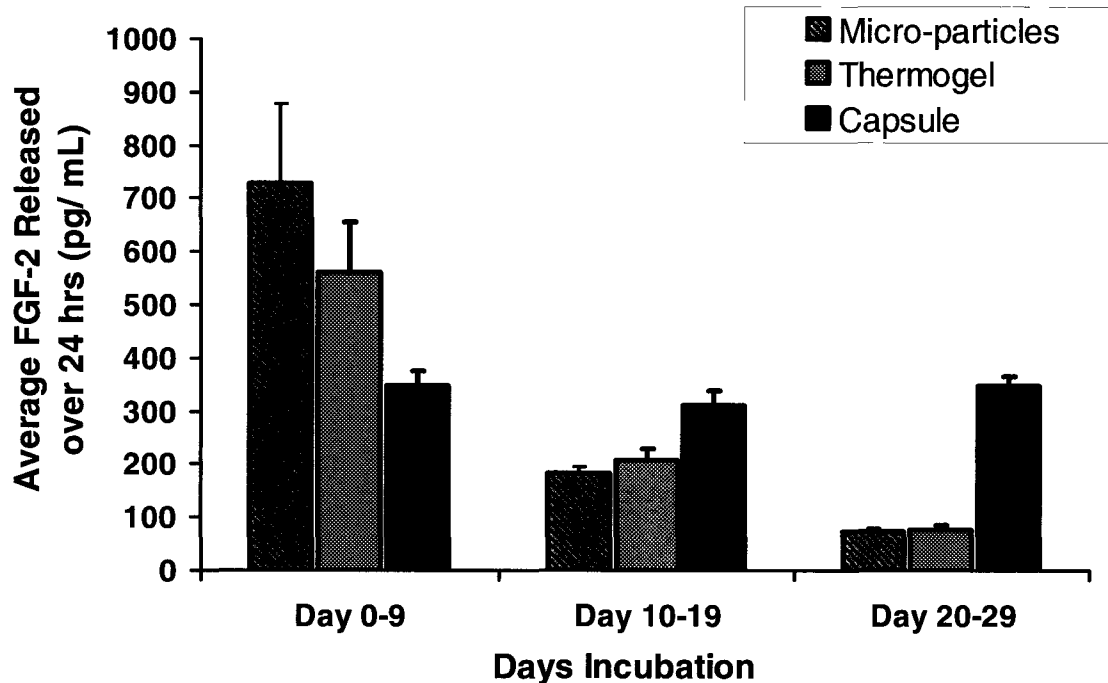


FIGURE 6.4. Primary, secondary and tertiary encapsulation of FGF-2. Human recombinant FGF-2 loaded within alginate microparticles (primary encapsulation) released on average 700 pg/mL of FGF-2, each day over the first 10 days of incubation. Incorporation of microspheres within a genipin-crosslinked collagen thermogel (secondary encapsulation system) reduced FGF-2 release to 500 pg/ mL each day over the first 10 days of incubation. Tertiary encapsulation of the microparticle-containing thermogels within collagen-coated EDC-crosslinked capsules further reduced FGF-2 release to 300 pg/ mL every 10 days up to 29 days of incubation. Values are represented as mean \pm SD.

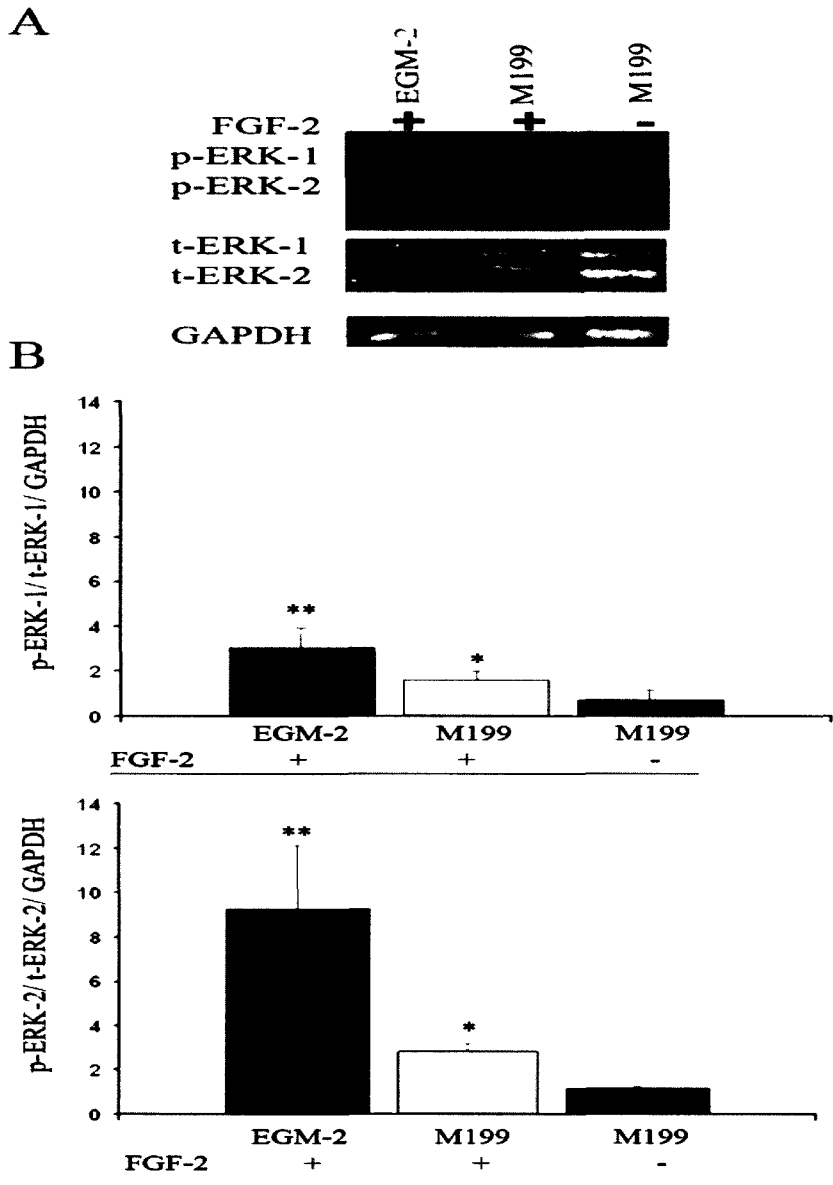


Figure 6.5. Western blot analysis of FGF-2 on ERK-1/2 phosphorylation of FVIII-producing BOECs in culture. (A) Densitometry quantification of phosphorylated ERK-1 and ERK-2 (p-ERK-1 and p-ERK-2) western blot bands demonstrates that in the presence of FGF-2-containing media (EGM-2 and M199) (B) band intensities of p-ERK-1 and p-ERK-2 are significantly higher than growth factor- and serum-free M199. Single and double asterisks (**) represent significant difference ($p < 0.01$ and $p < 0.05$, respectively; $N=3$) by an ANOVA test.

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