CONTROL OF COVALENT AND NON-COVALENT PRESENTATION OF
BIOMOLECULES WITHIN COLLAGEN GAG SCAFFOLDS

BY

JACQUELYN C. PENCE

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Chemical Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Adviser:

Assistant Professor Brendan Harley
ABSTRACT

Mass transport continues to be a major bottleneck in the development of biomaterials. The diffusive limitation of biomaterials restricts cell viability to regions where nutrients are readily available. To overcome this transport limitation, angiogenic biomaterials that utilize biomolecular cues such as Vascular Endothelial Growth Factor to drive vascularization are being explored. To develop these biomaterials, techniques to present these factors must first be investigated. Experiments were conducted with collagen glycosaminoglycan (CG) scaffolds to determine optimal conditions to control the levels of covalent and non-specific attachment of biotinylated Concanavalin A (ConA) via two distinct covalent patterning techniques: 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC) crosslinking and benzophenone (BP) biophotolithography. The results indicated that longer contact time of ConA with the CG scaffold independent of covalent attachment methods led to significantly higher amounts of fouling (non-specific attachment). The fouling was best minimized using a 1 hour wash followed by an overnight wash in a solution of 5% sucrose in PBS compared to no wash, PBS, 5% BSA in PBS, and 1% Tween in PBS. The total ConA immobilized by EDC chemistry was found to be strongly correlated to the amount of protein loading and was higher for an EDC:N-hydroxysuccinimide:Carboxyl ratio of 5:12.5:1 compared to 2.5:6.25:1 and 10:25:1. For BP chemistry, the UV exposure time was found to be significantly correlated to the total amount of ConA immobilized. Components of the scaffolds were also tested to determine their impact on covalent patterning and fouling. It was found that the Collagen Matrix yielded BP patterns with better resolution and less background noise caused by non-specific attachment. The use of Chondroitin Sulfate was found to have significantly less fouling than Hyaluronic Acid within
scaffolds, which was unexpected. Further, the Hyaluronic Acid yielded significantly higher total ConA immobilization.
ACKNOWLEDGMENTS

I would first like to thank my advisor Brendan Harley for his guidance and technical support over the course of my project. His enthusiasm and encouragement for this research helped me to pursue this project. I also owe a great debt of gratitude to all my fellow colleagues past and present within the Harley lab group, Steven Caliari, Sunny Choi, Bhushan Mahadik, Dan Weisgerber, Emily Gonnerman, Sara Pedron, Laura Mozdzen, Rebecca Hortensius, and Doug Kelkhoff who helped orient me on my commencement and offered continued moral support, insight, and assistance. I want to specifically thank Emily Gonnerman who worked side-by-side with me to understand the ConA fouling and develop the technique to quantify it.

Thanks to Teresa Martin and Steven Caliari who adapted the BP chemistry to CG scaffolds, without whom this project would not have been pursued. I appreciate the contributions of Dr. Bailey and Rory Turgeon whose collaboration and chemistry expertise helped shaped the direction of this project. Thanks to Kathryn Clancy and Romana Nowak whose knowledge on the endometrium will be indispensable in my future research.

I would like to acknowledge the support of the National Science Foundation under Grant No. DMR-1105300 which funded this research.

Finally, I want to recognize my family for their encouragement to return to school and the emotional support they have offered along the way. Thanks to my mom and dad, Chris and Elaine Greene, who were always available to listen and offer advice and encouragement. I appreciate all you have done for me over the years. A special thanks to my husband, Brandt Pence, whose love and support has been instrumental to my success.
## TABLE OF CONTENTS

CHAPERP 1: INTRODUCTION AND BACKGROUND ............................................................ 1

1.1 Vascularization ........................................................................................................... 1

1.2 Protein Immobilization Techniques ........................................................................... 9

1.3 Figures ......................................................................................................................... 13

CHAPTER 2: EXPERIMENTAL DESIGN .......................................................................... 15

2.1 CG Scaffold Fabrication ............................................................................................. 15

2.2 Protein Immobilization ............................................................................................. 15

2.4 Statistical Methods ................................................................................................... 19

2.5 Figures ......................................................................................................................... 22

CHAPTER 3: CONCANAVALIN A PATTERNING .......................................................... 25

3.1 Fouling ......................................................................................................................... 25

3.2 EDC ............................................................................................................................. 27

3.3 BP ................................................................................................................................ 30

3.4 Figures ......................................................................................................................... 35

CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS .................................................. 49
CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Vascularization

Mass transport continues to be a major bottleneck in the development of biomaterials. The diffusive limitation of biomaterials restricts cell viability to regions where nutrients are readily available. Dissimilar to biomaterial cultures, capillaries supply cells up to 200 microns away in vivo with necessary nutrients and eliminate waste (Lovett, Lee et al. 2009; Novosel, Kleinhans et al. 2011). Therefore, one approach to overcome the transport limitation currently under exploration utilizes the incorporation of vascular networks within biomaterials (Bramfeldt, Sabra et al. 2010; Novosel, Kleinhans et al. 2011).

Three major physiological vascularization processes have been identified: vasculogenesis, angiogenesis, and arteriogenesis (Fagiani and Christofori 2012). In vasculogenesis (Figure 1.1), progenitor cells are recruited to develop new vasculature, while the other two processes develop from pre-existing vessels. Angiogenesis is the process in which existing vessels sprout or split (intussusception), creating new vessel branches, whereas arteriogenesis occurs when increased fluid or pressure results in an increase in vessel diameter and recruitment of new cells.

A wide-range of methods that mimic angiogenic processes has been studied in the development of vascularized biomaterials. In angiogenesis, biological cues, such as growth factors and hypoxia, signal endothelial cells lining existing vessels to sprout (Carmeliet and Jain 2011). As gaps form in the endothelial cell junctions proteins are released from the vessels that form an extracellular matrix platform for the sprouting endothelial cells to migrate onto. This migration in turn results in the proliferation of new endothelial cells to line the new vessel and the
recruitment of pericytes to reinforce the new vessel. Angiogenic methods utilizing one or more cell types in addition to biomolecules to create vasculature within biomaterials are discussed here-in.

1.1.1 Growth Factor Driven Angiogenesis

A variety of angiogenic biomolecules including Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), and Transforming Growth Factor alpha (TGF-α), have been identified and used individually or jointly as angiogenic inducers within biomaterials (Kaully, Kaufman-Francis et al. 2009; Bramfeldt, Sabra et al. 2010).

In the body, VEGF often dictates the angiogenic process (Ferrara, Gerber et al. 2003).” VEGF signaling leads to endothelial cell proliferation, protects against endothelial cell apoptosis, and induces vascular permeability. Many techniques have been used in the incorporation of VEGF and other biomolecules into biomaterials (Figure 1.2) (Place, Evans et al. 2009). In regenerative medicine, some research has focused on the benefits of soluble VEGF therapy in tissue repair. New Zealand White Rabbits with unilateral hind limb ischemia that received intra-arterial bolus injections of VEGF had significantly better recovery, including higher capillary density and blood pressure, than their control counterparts (Takeshita, Zheng et al. 1994). Similar findings were made in Wistar Rats that received VEGF in a bolus intramuscular injection (Kofidis, Nolte et al. 2002). Other injuries in which bolus dosages of VEGF were found to improve revascularization include myocardial ischemia in pigs and brain edema in rats (Lopez, Laham et al. 1998; Harrigan, Ennis et al. 2003). In tissue engineering, however, this method may be
insufficient to vascularize the biomaterial in the window prior to bulk degradation of the tissue construct (Papavasiliou, Cheng et al. 2010). Strategies utilizing single doses of growth factor fail to mimic the complex spatio-temporal expression of these factors. This has been shown to lead to negative side effects, and single-dosing strategies are less efficacious (Papavasiliou, Cheng et al. 2010; Singh, Wu et al. 2012).

Current research in biomaterials focuses on growth factor delivery through immobilization or kinetic release methods. In kinetic release methods, growth factors are incorporated into a polymer or other material and are released over time through material degradation. In collagen coated polycaprolactone scaffolds containing VEGF, unwashed scaffolds resulted in a higher bolus release of VEGF compared to washed scaffolds (Singh, Wu et al. 2012). While the bolus release had little impact on the vascularization, it was suggested this could lead to systemic issues. A different study found that a bolus delivery of VEGF and/or PDGF did not result in stable increase in blood vessel density within a poly(lactide-co-glycolide) scaffold implanted in Lewis rats compared to the negative control over a four week period while controlled release of VEGF and PDGF did (Richardson, Peters et al. 2001).

Soluble presentation of factors may not have the same effect as growth factors immobilized within the ECM. Immobilized VEGF, for example, was found to interact with VEGFR-2 differently than soluble VEGF (Chen, Luque et al. 2010). Further, the activity of immobilized VEGF was maintained over a longer period of time compared to VEGF in solution. Therefore, immobilization techniques may provide a better method of growth factor presentation. Immobilization techniques are discussed in section 1.3.
While much research has focused on the use of VEGF alone in the development of angiogenic biomaterials, VEGF signaling by itself does not result in mature blood vessel formation (Richardson, Peters et al. 2001; Nillesen, Geutjes et al. 2007). For this reason, the use of VEGF in concert with other growth factors, such as FGF and PDGF, has also been studied. FGF is thought to be an upstream growth factor that regulates the expression of growth factors such as VEGF and PDGF in multiple cell lines (Murakami and Simons 2008). While the angiogenic actions of FGF have been found to be dependent on VEGF expression, the vessel formation induced by FGF was shown to be different than that of VEGF alone. The difference may be contributed to the other actions of FGF such as the regulation of PDGF. The complete angiogenic actions of FGF are not yet known (Nillesen, Geutjes et al. 2007). Both FGF and PDGF are important to the recruitment of mural cells that stabilize the neovascularization.

Studies with FGF-2 and VEGF in acellular collagen/heparin scaffolds implanted into Wistar rats found that the expression of the growth factors in concert yielded better vascularization with less hypoxia than the use of either factor alone (Nillesen, Geutjes et al. 2007). As previously mentioned, experiments of co-delivery of VEGF and PDGF through release from poly(lactide-co-glycolide) scaffolds were performed in Lewis rats and in NOD mice (Richardson, Peters et al. 2001). These studies found that VEGF led to a high density of immature blood vessels while PDGF led to no significant increase in blood vessels density over the control, but resulted in a higher percentage of mature vessels. When combined, VEGF and PDGF release led to a higher density of mature blood vessels. Rophael et al. took the research a step further in a factorial study with FGF-2, VEGF, and PDGF in matrigel/heparin gel implants in mice on angiogenesis and adipogenesis (Rophael, Craft et al. 2007). The research found that the three growth factors
combined yielded the highest percentage of vasculature by volume and the area of the new blood vessels was greater for combination treatment with VEGF and FGF-2 or with VEGF, FGF-2, and PDGF.

1.1.2 Multicellular Angiogenesis

Although growth factors provide imperative cues necessary for vasculature formation, other culture elements such as cell type, cell-cell interactions, and cell-matrix interactions, are essential in developing a mature vascular network as well (Davis and Senger 2005; Kaully, Kaufman-Francis et al. 2009). While vascular ECs are an obvious cell choice for developing vascularized biomaterials, the optimum type of EC to be used remains to be determined (Baiguera and Ribatti 2012). Other work has focused on the use of mesenchymal stem cells instead of ECs to form vasculature (Kaully, Kaufman-Francis et al. 2009; Duffy, McFadden et al. 2011).

The use of ECs alone results in immature vessels while vessels formed by co-cultures of ECs and an additional cell type such as smooth muscle cells or pericytes are more stable. In a multicellular model a secondary cell choice may depend on the type of tissue of interest and the cross-talk that cell has with the endothelial cells (ECs) (Kirkpatrick, Fuchs et al. 2011). Specific co-culture research on vascularization is summarized in Kirkpatrick et al., Baiguera and Ribatti, and Kaully et al. (Kaully, Kaufman-Francis et al. 2009; Kirkpatrick, Fuchs et al. 2011; Baiguera and Ribatti 2012).
1.1.3 ECM Role in Angiogenesis

The selection of biomaterial is important in directing angiogenesis due to cell-matrix interactions. In vivo, components of native ECM aid in a wide range of vascularization processes including preventing apoptosis, enhancing proliferation, mobilizing ECs, directing tubule formation, and sequestering angiogenic factors (Davis and Senger 2005; Hynes 2009; Bramfeldt, Sabra et al. 2010). Type I Collagen and vintronectin integrins within the ECM permit EC adhesion that activates the mitogen-activated protein kinase (MAPK) signal transduction pathway, encouraging EC proliferation while reducing apoptosis (Davis and Senger 2005). Further, Type I collagen induces the morphogenesis of EC sprouts whereas Laminin causes EC differentiation and tubule stabilization. Components of the ECM can also act like receptors to bind factors. Fibronectin not only is important for EC adhesion, but also bind VEGF, improving VEGF activity (Hynes 2009; Bramfeldt, Sabra et al. 2010). MMP degradation of the ECM causes chemotaxis of ECs. Without degradation, ECs can undergo haptotaxis through changes in the solid ECM (Davis and Senger 2005). The structure of the ECM also impacts angiogenesis. ECs can locate each other and organize by sensing cell generated tension within the ECM (Davis and Senger 2005). The mechanical properties of the ECM can also effect cell morphology which impacts tubule formation (Bramfeldt, Sabra et al. 2010).

An angiogenic biomaterial should mimic ECM by being both biocompatible and biodegradable as well as supporting cell adhesion (Bramfeldt, Sabra et al. 2010; Tian and George 2011). Further, the stiffness and pore structure should be optimized for vascularization. Specific biomaterials for prevascularized and angiogenic applications are discussed by Bramfeldt et al. and Tian and George (Bramfeldt, Sabra et al. 2010; Tian and George 2011).
For this research a collagen-glycosaminoglycan (CG) scaffold fabricated as described in Section 2.1 was chosen. The use of CG scaffolds as angiogenic biomaterials has already been established in vitro (Duffy, McFadden et al. 2011). CG scaffolds have been used in vivo for various tissue regenerative applications including skin, conjunctiva, peripheral nerve, and orthopedic tissue in vivo (Yannas, Lee et al. 1989; Harley, Spilker et al. 2004; Harley and Gibson 2008; Harley, Lynn et al. 2010). The lyophilization process forms an interconnecting network of pores within the CG structure with low relative density. CG scaffolds have superior fiber stiffness and degrade less quickly than scaffolds composed of collagen alone (Yannas, Burke et al. 1975). Both pore size and stiffness can affect the angiogenic potential of a biomaterial; the mean pore size of CG scaffolds is easily modifiable through alteration in the freezing kinetics (Harley and Gibson 2008). The mechanical behavior of the scaffold, which is modeled as elastomeric foam, can be modified through cross-linking (Harley, Leung et al. 2007). For example, the elastic modulus of a hydrated CG scaffold with pore size of 96 µm is approximately 200 Pa, but when cross-linked by EDC chemistry at a molar ratio of 5 EDC: 2 N-Hydroxysuccinimide (NHS): 1 Carboxyl was found to increase 7 fold (Harley, Leung et al. 2007).

1.1.4 Potential Challenges of Developing an Angiogenic Biomaterial

Co-cultures of multiple cell types can present many obstacles that can hinder the desired result. Many angiogenic co-culture experiments in vitro did not give rise to vessel formation (Kaully, Kaufman-Francis et al. 2009). Nutritional needs of each cell type to maintain phenotype and cell processes often differ; finding an optimal media for the use with multiple cell types can be difficult (Kirkpatrick, Fuchs et al. 2011). Not only could the order and time of seeding of cells impact the physiological outcome, the ratio of cell types has also found to be important. For
instance, the vascular network thickness and junction size in co-cultures of endothelial and mesenchymal stem cells were found to increase with increasing mesenchymal stem cells to endothelial cells present (Duffy, Ahsan et al. 2009).

The exogenous use of VEGF and other growth factors for vascularization of biomaterials raises some concerns. Complex regulatory relationships are responsible for controlling endogenous expression levels in vivo (Kirkpatrick, Fuchs et al. 2011). Endogenous overexpression of VEGF by tumor cells in vivo can lead to tumorigenesis and results in vessels that are tortuous, disorganized, and leaky (Chung and Ferrara 2011). VEGF further guides tumor formation by inducing the release of anti-apoptotic factors by ECM degradation resulting in an increase in local permeability and an influx of biomolecular signals (Folkman 2000). Therefore, an uneven expression of growth factor, such as a bolus dosage of VEGF, could therefore lead to uncontrolled and poorly organized vascularization. One proposed method to counteract this imbalance spatially expresses both angiogenic and antiangiogenic factors in different biomaterial compartments to better control growth (Yuen, Du et al. 2010) Platelets are thought to direct angiogenesis in this manner with differential expression of angiogenic and antiangiogenic factors by different platelet granules in vivo (Italiano, Richardson et al. 2008).

Further concern is warranted for the effects of VEGF beyond angiogenesis and tumorigenesis. In wound healing, for example, the presence of VEGF corresponds with a greater degree of scar formation in fetuses (Wilgus, Ferreira et al. 2008). Further, the amount of scar tissue formation could be reduced using an antibody against VEGF. The addition of other growth factors to wounds also negatively impacted the scar tissue formation.
The drawbacks of using exogenous angiogenic factors suggest a need to find other paradigms for introducing biomolecular agonists in a more controlled and non-pathological manner. The endometrium, the lining of the uterus where trophoblast implantation occurs, is a model system for developing an angiogenic biomaterial. The ovarian steroids estradiol and progesterone regulate local expression of factors such as VEGF to in turn regulate the various angiogenic processes that occur within the endometrium throughout the entirety of the menstrual cycle (Girling and Rogers 2009). Withdrawal of these factors correlate with the onset of menstruation and vascular breakdown occurs. Oestradiol and progesterone regulate VEGF expression in a temporal fashion (Girling and Rogers 2009; Rogers, Donoghue et al. 2009; Koos 2011). Initial low concentrations of oestradiol occur incongruence with VEGF expression from luminal epithelial cells and vessel permeability. An influx of plasma rich in oestradiol and other factors conducive to epithelial cell proliferation follows. As oestradiol decreases and progesterone increases, epithelial cell growth ceases and drives stromal cell proliferation and vessel maturation. Therefore, endometrial angiogenic cues could be expressed spatiotemporally within a biomaterial to drive angiogenesis.

1.2 Protein Immobilization Techniques

In order to develop a biomaterial that employs biomolecular cues to direct angiogenesis, methods to control their presentation must first be investigated. How protein is presented can impact its biological functions. For instance, it has previously been shown that VEGF receptor activity is different for covalently immobilized VEGF than its soluble form (Chen, Luque et al. 2010).
1.2.1 Ubiquitous Immobilization

Methods have been devised to uniformly immobilize biomolecules within a three dimensional biomaterial. One reaction commonly employed for protein immobilization within a collagen scaffold utilizes the coupling reagent EDC with the activating reagent NHS. In this method, a carboxylic group of the first component (collagen) forms an activated ester with NHS which can then form a zero-length cross-link with amines of the second component (protein) (Grabarek and Gergely 1990; Shen, Shoichet et al. 2008). Alternatively, the protein can react with NHS to form an activated ester that can react to amines within the collagen (Wissink, Beernink et al. 2001). EDC chemistry has previously been employed to immobilize biomolecules such as VEGF, heparin, and chondroitin sulfate within collagen materials (Pieper, Oosterhof et al. 1999; Wissink, Beernink et al. 2001; Chiu, Weisel et al. 2011). An alternative method to immobilize proteins onto collagen uses plasmids to engineer the desired biomolecule with a collagen binding domain. This allows for the protein to be immobilized direction to the collagen without a major impact on the protein activity (Lin, Li et al. 2012).

1.2.2 Spatial Patterning

While bulk immobilization of biomolecules allows for cell experiments within biomaterials with well-defined biomolecular concentrations, it does not have the eloquence of physiological expressions of biomolecules. Biomolecular gradients, for example, have been found to be important in cell migration during angiogenesis (Bischoff 1995; Friedl and Brocker 2000). Therefore spatially patterned biomaterials can be beneficial in the study of cell behavior where an environment with a varying degree of biomolecular concentration is important. Cell migration along gradients has been studied for many cell types on 2-dimensional substrates. For example,
using a microfluidic device, a gradient of Laminin was immobilized via EDC chemistry onto a
self-assembled monolayer of ECM components adhered via a gold film to a glass slide to study
the effect of local concentration versus gradients on rat intestinal IEC-6 cell migration
(Gunawan, Silvestre et al. 2006). Gradients of biotinylated Concanavalin A (ConA biotin), P-
selectin, and mannan immobilized on benzophenone (BP) modified glass slides via
photolithography were employed to study the effects of biomolecule gradients on HL-60
promyelocytes attachment and rolling velocity (Toh, Fraterman et al. 2009). When exposed to
365 nm light, BP forms a transient diradical that can then bind a nearby biomolecule through a
C-H bond insertion. Multiple factors can be immobilized via repetitive excitement of the BP
molecule which returns to ground state if no reaction takes place. Cell experiments showed that
with increasing immobilization of P-selectin on the glass slides, HL-60 promyelocytes had
increasing attachment and decreasing rolling velocity. Carbodiimide and copper-catalyzed azide-
alkyne "click" chemistries have also been used successfully, as in a recent studying examining
the impact of peptide patterns and mesenchymal stem cell attachment (Hudalla and Murphy
2010). Importantly, many of these methods used to pattern molecules in 2-dimensions are now
being adapted for use in 3-dimensional biomaterials. The use of photo-coupling was
demonstrated in click-based PEGtide hydrogels utilizing thiol-ene to photo-pattern Alloc moiety
and fluorescently labeled ahxRGDSC (a cysteine containing peptide) gradients (Polizzotti,
Fairbanks et al. 2008). The BP method was modified to use BP-4-isothiocyanate, which binds to
free lysine side chains of collagen, allowing for the creation of multiple classes of biomolecules
in a model collagen-GAG (CG) scaffold system (Martin, Caliari et al. 2011).
Another technique that has been developed for patterning biomolecules utilizes inkjet printing (Phillippi, Miller et al. 2008; Miller, Phillippi et al. 2009). This method was used to immobilize bone morphogenetic protein-2 and insulin like growth factor-2 onto fibrin coated surfaces to direct osteogenic or myogenic differentiation of mouse stem cells.

In summary, lacking the vascular structures present in native tissue, most biomaterials are currently limited by their diffusive properties. The development of an angiogenic biomaterial may require the use of multiple cell types as well as growth factors such as VEGF. The use of exogenous VEGF alone could result in a highly disorganized dense bed of immature vessels within the biomaterial. A biomaterial that utilizes endometrium-inspired biomolecular cues may offer better control of VEGF and other angiogenic factors expressions. However, the spatiotemporal presentation of these biomolecules could play an important role in the formation of a mature vascular network. This thesis will explore the use of two strategies to tether factors to CG scaffolds with particular interest in controlling fouling and specific attachment as a precursor to total control over biomolecule presentation within this model scaffold system.
1.3 Figures

Figure 1.1: Angiogenesis (a&c) and Vasculogenesis processes (b). Angiogenesis can occur from sprouting of endothelial cells to form new vessels or from splitting of existing vessels. Vasculogenesis occurs from the recruitment of endothelial progenitor cells (EPCs) to create new vessels. (Carmeliet and Jain 2011)
Figure 1.2: Methods of growth factors or other biomolecule presentation within engineered biomaterials
CHAPTER 2: EXPERIMENTAL DESIGN

This chapter describes the general techniques used to study the covalent and non-specific attachment of ConA within CG scaffolds.

2.1 CG Scaffold Fabrication

CG scaffolds were fabricated from a degassed slurry of purified fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ), chondroitin sulfate isolated from shark cartilage (Sigma Aldrich, St. Louis, MO), acetic acid (Acros Organic, NJ), and deionized water. The slurry was degassed at greater than 10 Torr and 4º C, which removed entrapped air while not boiling the slurry, prior to being pipetted into an aluminum mold. Over a one hour period, the slurry was cooled from 20º C to -40º C where it was held for one hour to ensure freezing of slurry. The temperature was then raised to 0º C over a 40 minute period under vacuum at 200 mT. The water in the slurry was then sublimated off. The scaffolds were then sterilized and dehydrothermally cross-linked at a pressure less than 10 torr and 105ºC for 24 h. (Harley, Spilker et al. 2004). Prior to experiments, the scaffold sheets were cut down for experimental samples using 6 mm or 8 mm biopsy punches (Miltex, York, PA) and hydrated by soaking in ethanol followed by PBS.

2.2 Protein Immobilization

2.2.1 Fouling

Fouling, or non-specific attachment, was evaluated using in Concanavalin A-AlexaFluor 647 conjugate (Invitrogen, Carlsbad, CA). For comparison purposes, scaffolds were initially treated similarly to patterned groups. Control scaffolds underwent the same EDC reaction as described below, with the omission of ConA. After an overnight wash in PBS at pH 8 following the EDC
reaction, the fouling scaffolds then underwent the same ConA treatment but without the EDC chemistry followed by the same wash steps as their protein immobilized counterparts.

2.2.2 EDC Chemistry

Concanavalin A AlexaFluor 647 conjugate (Invitrogen, Carlsbad, CA) was ubiquitously immobilized within the CG scaffolds using a 2-step reaction with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) (both from Sigma Aldrich, St. Louis, MO) carried out in PBS at either a 10:25:1, 5:12.5:1, or 2.5:6.25:1 EDC:NHS:-COOH ratio (Figure 2.1).

Moles of –COOH were first determined using the weight of the scaffolds (0.0012 mol –COOH per g collagen) prior to hydration, and the amounts of EDC and NHS required were determined. The EDC and NHS were dissolved in a volume of PBS that would allow the scaffolds to be full submerged. The hydrated scaffolds were then placed in the solution at 37°C on a shaker for 20-30 min depending on the experiment. The scaffolds were then removed from the solution. The excess moisture was wicked off the scaffolds using a Kimwipe and each scaffold placed either on a plate lid or in the well of a 96-well plate. ConA was diluted in PBS to the desired concentration. A total of 10 µL of protein solution was pipetted onto each scaffold. The protein was allowed to attach for 1 hour at room temperature. The excess protein solution was removed using a Kimwipe and each scaffold was placed into 2 mL of PBS solution on a shaker at room temperature for 1 hour. After 1 hour, the PBS solution was changed and left overnight. Control scaffolds, used to determine fouling, were treated with PBS rather than protein solution. After 24 hours, control scaffolds were then treated with 10 µL of protein solution and allowed to sit at room temperature for 1 hour prior to undergoing further washing.
2.2.3 BP Chemistry

Benzophenone Isothiocyanate (Invitrogen, Carlsbad, CA) was covalently immobilized into the scaffolds in dimethylformamide (DMF) and N-Diisopropylethylamine (DIEA) solution in the dark at room temperature for 48 hours (Figure 2.2) (Martin, Caliari et al. 2011). Unbound BP was removed with repeated DMF, ethanol, and PBS washes. Biotinylated ConA (Vector Labs, Southfield, MI) was dissolved in PBS to the desired concentration. Prior to patterning, 10 µL of protein solution was pipetted onto the scaffolds that were dabbed dry and placed on glass slides. A coverslip was placed on top of the scaffold with or without a photomask depending on the type of patterning. The scaffolds were then exposed to 350-365 nm UV light for the desired time duration. The scaffold was then turned over and exposed again for the same length of time. The scaffolds were placed in a pluronic acid solution, to deactivate BP, for 1 hour at room temperature prior to undergoing further washes.

2.3.1 Determination of ConA Immobilization by Fluorescent Microscopy

Scaffolds with ConA biotin immobilized via BP chemistry as described in the preceding section, were blocked with BSA, treated with Streptavidin-AlexaFluor conjugate (Invitrogen, Carlsbad, CA) for 1 hour on a shaker and washed with PBS for a minimum of 1 hour prior to imaging. Scaffolds were imaged in glass coverslip bottom dishes (In Vitro Scientific, Sunnyvale, CA) using the FITC cube on a fluorescent microscope (Leica DMI4000B with Qimaging camera). Exposure conditions were kept constant throughout individual experiments to allow for comparison between samples.
2.3.2 Quantification of ConA Immobilization Through EDC Chemistry

A standard curve of ConA AlexaFluor conjugate masses was generated by digesting control scaffolds spiked with known amounts of soluble ConA and measuring the resulting mean fluorescent intensity. Scaffolds immobilized with ConA by EDC chemistry were placed in papain digest solution (Sigma, St. Louis, MO) in a 60º C water bath for 1 hour until digestion was complete. The samples were then vortexed and plated in a clear 96 well plate. The wells were excited at 620 nm (20 nm bandwidth) and the emission was read at 670 nm (25 nm bandwidth) using a fluorometer (Tecan, Switzerland). The readings were then normalized to a blank scaffold digest and compared to the standard curve to obtain the amount of ConA present in the digest.

2.3.3 Quantification of ConA Immobilization Through BP Chemistry

A combination of ConA biotin and ConA AlexaFluor conjugate was used to determine the mass of ConA fouling and immobilized by BP chemistry within the scaffolds. A standard curve was prepared using ConA AlexaFluor conjugate as described in the previous section. Under the assumption that the fouling of ConA biotin and ConA AlexaFluor conjugate were equivalent, a fouling experiments as described in Section 2.4.1 was performed on scaffolds immobilized with BP but with no UV exposure with known loading concentrations of either ConA biotin or ConA AlexaFluor conjugate. Following the overnight wash, the ConA biotin scaffolds were treated for 1 hour on a shaker with Streptavidin AlexaFluor conjugate (Invitrogen, Carlsbad, CA) and washed for a minimum of 1 hour on a shaker prior to digestion as previously described. The digest was excited at a wavelength of 485 (20 nm bandwidth) and the emission was measured at 535 (25 nm bandwidth) via fluorometer (Tecan, Switzerland). The scaffolds fouled with ConA
AlexaFluor conjugate were also digested and analyzed using a fluorometer (Tecan, Switzerland) as described in Section 2.3.2. The amount of ConA AlexaFluor conjugate fouling was determined using the standard curve and this information was then used to correlate the biotinylated ConA fouling readings to known amounts of ConA.

Scaffolds with ConA biotin immobilized by BP chemistry as described in Section 2.2.3 were treated for 1 hour on a shaker with Streptavidin-AlexaFluor conjugate (Invitrogen, Carlsbad, CA) and washed with PBS for a minimum of 1 hour on a shaker prior to being digested and analyzed as previously described. The samples were then compared to the ConA biotin fouling versus mean fluorescent intensity curve as described above to determine the amount of ConA biotin immobilized within the scaffold.

2.3.4 Determination of Depth of Patterning

Scaffolds were imaged as described in Section 2.4.4 and then oriented with the stripes vertically up and down and immersed in Optimal Cutting Temperature solution (OCT) and frozen at -80°C. The scaffolds were then cut perpendicularly to the circular surface and washed in PBS. The scaffolds were placed with cut side down in a microscopy dish and imaged using a fluorescent microscope (Leica DMI4000B with Qimaging camera). Fluorescent images were inverted and autocorrected for brightness and contrast in ImageJ.

2.4 Statistical Methods

Unless otherwise stated, significance was reported for p<0.05.
2.4.1 Fouling Statistical Methods

The effect of 1 (n=24) or 2 (n=30) 24 hour washes was determined via independent samples t-test. The effects of different wash solutions were determined by 1-way ANOVA and Dunnett’s test with 5% sucrose as the control. The temporal effects of sucrose were examined further using ANOVA and Bonferroni’s post hoc test.

2.4.2 EDC Statistical Methods

The optimal EDC: NHS: Carboxyl ratio was evaluated through linear regression in SPSS with total immobilization as the dependent variable and loading, dummy coded variables for EDC ratio 2.5:6.25:1 and 10:25:1, and products of the dummy variables and protein loading masses as independent variables. The effect of EDC ratio on fouling was analyzed similarly with fouling as the dependent variable. 7-8 samples at each protein loading mass/pH combination were used. The effects of pH on 1 and 2 step EDC immobilization were analyzed through 1-way ANOVA and contrasts outlined in Table 2.1 (n=6).

Linear regression with total immobilization or fouling as the dependent variable and mass of ConA loading, dummy variable coding for Hyaluronic Acid, and dummy variable times mass of ConA loading as independent variables was used to test the significance of GAG on total immobilization and fouling (n=3 scaffolds per GAG/pH step/ConA loading mass).

2.4.3 BP Statistical Methods

The evaluation of different patterning steps on ConA immobilization was done with a minimum of 3 samples were group by 1-way ANOVA and Bonferroni post hoc testing. The correlation of UV exposure time on ConA immobilization was tested at $\alpha = 0.05$, power = 0.8, and n=40 (n=5
per time point). The effects of pH on total ConA immobilization was analyzed using a 1-way ANOVA and Bonferroni post hoc testing between pHs (n=9 per pH).
2.5 Figures

Figure 2.1: Two step EDC reaction scheme
Figure 2.2: BP Conjugation to CG scaffold and photopatterning schematic utilizing a photomask (Martin, Caliari et al. 2011)
Table 2.1 Contrast Coefficients for pH effects on 1 or 2 step EDC ConA immobilization

<table>
<thead>
<tr>
<th>Contrast</th>
<th>pH 5.5, 1 step</th>
<th>pH 7.4, 1 step</th>
<th>pH 5.5, 2 step</th>
<th>pH 5.5 then pH 7.4</th>
<th>pH 7.4, 2 step</th>
<th>Fouling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.5</td>
<td>.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>-1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>0</td>
</tr>
</tbody>
</table>
3.1 Fouling

Covalent immobilization of protein onto CG scaffolds is molecule and fouling dependent. When protein is introduced to the scaffolds, some of the protein will non-specifically bind to the ECM components present such as GAGs (Place, Evans et al. 2009). In the case of ConA, non-specifically binding occurs with collagen (Soderstrom 1987). This fouling may result in a different cellular response compared to immobilized protein and can impair resolution of the spatio-patterning signal as illustrated in Figure 3.1. For this reason, fouling should be minimized as best as possible. Many patterning and processing parameters have the potential to affect fouling including materials used (such as collagen and GAG), contact time of the protein with the scaffolds, and the wash steps used, among others. These parameters will be discussed in detail in the subsequent sections.

Due to its previous use in benzophenone patterning research (Toh, Fraterman et al. 2009; Martin, Caliari et al. 2011), biotinylated Concanavalin A (ConA biotin) was chosen as a test protein. ConA binds to glucose residues within collagen and therefore is a good target protein for studying fouling (Soderstrom 1987).

3.1.1 Effect of Scaffold Materials

The molecular chemistries of the CG scaffolds can have a large impact on the binding affinity of ConA. The collagen used in the fabrication of the scaffolds for this research undergoes processing in its isolation and purification that can alter the fibrillar structure. The effects on fouling which is addressed in more detail in Section 3.3.2 were also found to vary depending on
the collagen used. Native ECM contains different types of GAGs depending on the tissue type the ECM is isolated from (Place, Evans et al. 2009). The impact of GAGs on fouling and covalent immobilization is further discussed in Section 3.2.3

3.1.2 Wash Dependency

Wash steps were optimized for duration, number, and wash solution to reduce the amount of fouling accrued during the immobilization process (Figure 3.2).

No difference between 1 or 2 day washes was found (p=0.694), but a significant effect was detected between wash groups (p<0.001). 5% sucrose wash was significantly better at reducing fouling than all other washes (p<0.05 for all tests). This result is unsurprising since, as previously discussed, ConA binds to sugars. Other proteins may have different optimal washes depending on their binding affinities and will have to be investigated.

3.1.3 Effects of Sucrose on Protein Binding and Exposure Time Dependency of Protein Fouling

Upon determining that 5% sucrose was the optimal wash solution, the wash was optimized further by determining when sucrose addition would minimize fouling by adding sucrose either prior to protein addition or after protein addition (Table 3.1). The data suggest a significant difference between sucrose after protein (16%) and both sucrose before protein (27%, p=0.001) and PBS treatments (27%, p<.001). The results agree with the hypothesis that the sucrose has a higher binding affinity than the CG scaffold to ConA and is extracting the protein.

In this study, exposure times of protein to the scaffolds were also explored. The results found that the longer protein exposure time (20 min) resulted in greater fouling (Table 3.1), confirming
that in the CG scaffold system that fouling is time sensitive and the exposure time should be
limited when possible.

3.2 EDC
Ubiquitous ConA immobilization in CG scaffolds was achieved via EDC chemistry. A variety of
conditions were explored to determine optimum immobilization parameters and effects on
fouling.

3.2.1 EDC Ratio Optimization
The molar ratio of EDC: NHS: Carboxyl Groups was varied while keeping the molar ratio of
EDC: NHS constant to determine the impact of EDC concentration on overall Con A protein
immobilization and fouling at varying protein concentration (Figure 3.3).

The intercept was found to be higher for the 5:12.5:1 ratio compared to the intercepts of
2.5:6.25:1 (p=0.014) and 10:25:1 (p=0.015) ratios. No differences were detected between slopes
(p>0.6 for all tests), implying that the change in protein immobilization per change in protein
loading is constant for all three ratios at the protein concentrations evaluated.

This suggests that the maximal ConA immobilization occurs at the 5:12.5:1 ratio. An increased
reaction rate likely explains the higher resultant immobilization of 5:12.5:1 ratio to 2.5:6.25:1.
However, it is less clear why the 10:25:1 ratio was lower than the 5:12.5:1. One explanation is
that the reaction rate at 10:25:1 is so high such that almost all free –COOH bonds present in the
collagen are consumed during the duration of the first step and, therefore, aren’t available to bind
ConA.
The effects of EDC concentration on fouling were also examined (Figure 3.4). Fouling was found to be a function of protein loading concentration (p<0.001). No significant differences in slope were detected between EDC ratios (p>0.05 for all tests). However, there was a significant difference in intercept between the 5:12.5:1 and 10:25:1 groups (p=0.015). Overall, these data suggest that the rate of fouling, the change in ConA attached per the change in ConA loaded, is not different between EDC ratios.

3.2.2 Impacts of pH and Simultaneous 1 or 2 step EDC Reaction on ConA Immobilization

Two other variables that may impact the amount of ConA immobilized include pH and EDC/NHS crosslinking versus protein addition kinetics. Notably, EDC crosslinking may be utilized in 2 distinct reaction schemes: one step versus two step attachment. In the one step method, the protein is added at the same time as the EDC and NHS. In the two step method, the scaffold is first soaked in the EDC and NHS to activate the NHS. The protein is then added in the second step to complete these reactions. Both the activation and crosslinking of the protein are pH sensitive (Grabarek and Gergely 1990; Sehgal and Vijay 1994; PierceBiotechnology 2011). The NHS activation is best performed with the pH range of 4.5 to 7.2 (PierceBiotechnology 2011). However, the denaturization of the collagen must be considered, therefore PBS at a pH of 5.5 was chosen. The amine reaction is optimal at a higher pH than the NHS activation, at a pH of 7.2-7.5 (PierceBiotechnology 2011). Therefore we designed model one step and two step reaction sequences. One step crosslinking was performed at pH 5.5 or pH 7.4. Two step crosslinking was carried out at pH 5.5 for both steps, pH 7.4 for both steps, or at a hypothesized optimal condition of pH 5.5 for the first step and pH 7.4 for the second step.
Percent immobilization of 50 ng of ConA within scaffolds at an EDC ratio of 5:12.5:1 were determined at different pH conditions for one and two step chemistries (Figure 3.5). One step EDC chemistries resulted in significantly higher immobilization rates than fouling (p=0.003), but no pH effect was found (p=0.559). Two step EDC chemistry at pH 7.4 resulted in significantly higher immobilization than its 1 step counterpart (p<0.001), while two step EDC chemistry at pH 5.5 had no significant effect (p=0.179). Further, more protein was found to be immobilized at pH 7.4 than at pH 5.5 (p<0.001), but no difference was detected at the sample size between pH 5.5 first step followed by pH 7.4 second step and pH 7.4 for both steps (p=0.455). These results suggest that two step crosslinking with the second step performed at pH 7.4 is most optimal for eliciting biomolecule immobilization within CG scaffolds.

3.2.3 Effects of GAGs on EDC Immobilization and Fouling of ConA

As previously discussed in Section 3.1, the materials used in the fabrication of the CG scaffolds can impact fouling. The materials can also impact the immobilization process. GAGs have previously been shown to mediate non-covalent interactions between ECM and biomolecules in vivo most likely via the degree of GAG sulfation (Gama, Tully et al. 2006; Hudalla and Murphy 2011). There are four major classes of GAGs, heparin/heparin, chondroitin/dermatan, keratan, and hyaluronic acid which vary in degree of sulfation but are all linear polysaccharides with repeating disaccharide units (Raman, Sasisekharan et al. 2005). GAG presentation on model surfaces has been shown to impact biomolecule sequestration from the media and subsequent cell activity (Hudalla, Koepsel et al. 2011). The effect of GAGs on fouling and total immobilization of ConA during the EDC process was examined using CG scaffolds prepared
with either chondroitin sulfate or hyaluronic acid with 10, 25, or 50 ng ConA loading and different pHs (Figure 3.6).

Combining the pH results (Figure 3.7), linear regression of ConA loading versus fouling for the two GAGs detected differences in both slopes (p=0.024) and intercepts (p<0.001). This suggests that the total fouling was not only higher for hyaluronic acid, but had a different rate of fouling dependent on ConA loading. Linear regression of ConA loading versus total immobilization (both fouling and covalent immobilization) detected a difference in intercepts (p<0.001) but not in slopes (p=0.355). Therefore, the total immobilization of ConA was found to be significantly higher for hyaluronic acid functionalized scaffolds, but there was no difference in the amount of ConA immobilized per amount loaded between the groups.

3.3 BP

Many parallels can be drawn between the parameters of EDC and BP chemistry. In Section 3.2.1 the immobilization rate of ConA by EDC and NHS was optimized by adjusting the ratio of EDC and NHS to carboxyl groups present within the collagen scaffolds. In BP chemistry, the analog would be power of the UV laser beam used for the photo-immobilization of the ConA. Along with altering the EDC ratio, the total immobilization as well as fouling in EDC chemistry can be increased or decreased by altering the lengths of EDC reaction steps. Similarly, in BP chemistry, the total concentration of ConA cannot only be altered by UV intensity, but also by changing the exposure time. Still further, the chemistries of the scaffolds themselves and the concentrations of ConA can alter the EDC and BP reactions. Some of these parameters are discussed in more detail in the subsequent sections. For BP photochemistry, the patterning aspect adds in some extra considerations including depth of patterning as well as pattern contrast and localization.
3.3.1 Verification of BP Immobilization by UV Exposure

To determine which steps in the BP process resulted in ConA immobilization, the mean fluorescent intensities were measured with fluorescent microscopy for scaffolds with different processing conditions. These data are depicted (Figure 3.8).

ConA biotin was added to BP-functionalized scaffolds and then washed without any UV exposure to determine whether the presence of ConA biotin to BP’d scaffolds alone could lead to immobilization. The post hoc analysis found that these scaffolds with MFI of 28.5 and SE of 1.7 had a significantly higher fluorescence than that of BP’d scaffolds alone with an MFI of 9.0 and SE of 2.1 (p<.001) (Figure 3.8). This analysis did not, however, determine whether the presence of ConA was due to BP immobilization or fouling. Most likely this increase in fluorescence can be attributed to fouling; additional experiments performed on non-BP’d scaffolds also saw a degree of immobilization and was assumed to be from fouling.

The impact of UV exposure on ConA biotin immobilization was next investigated. Some scaffolds were exposed with UV prior to ConA biotin addition to determine whether the UV could prime the scaffold to immobilize ConA biotin without the ConA biotin present. No significant difference was detected between UV exposure prior to ConA biotin addition and ConA biotin addition to BP’d scaffolds without UV exposure (p=1.00). Although no increase in ConA biotin concentration was found with UV exposure prior to protein addition, further studies should be performed using UV exposure with photo masks prior to ConA addition to determine whether there is a change in where the ConA is immobilized and the clarity of the pattern.

Finally, UV exposure in the presence of ConA biotin was evaluated and was found to have a significantly higher MFI (mean 59.4 with SE of 4.3) than all other groups examined (p<.001 for
all comparisons. Therefore, BP-photolithography patterning is successful at sequestering ConA biotin into scaffolds.

### 3.3.2 Effects of UV Exposure Time on ConA Immobilization

Lengthening UV exposure hypothetically increases the overall immobilization by allowing more time for the photoreaction to take place. This results in a higher probability of reaction between benzophenone and the biomolecule. The time effects of fouling discussed in Section 3.1.3 were minimized by maintaining the same protein contact time for the scaffolds while adjusting the UV exposure time. Total immobilization of ConA was observed to increase with exposure time (Figure 3.9).

The correlation (R = 0.88) between attachment and total exposure times of 15 to 240 seconds (7.5 to 120 seconds per side) was significant (p<0.001), suggesting that ConA immobilization by BP chemistry can be controlled through alteration of UV exposure at exposure times less than 4 minutes. Importantly, this work extends observations regarding graded factor immobilization on 2-dimensional substrates as a function of UV exposure time (Toh, Fraterman et al. 2009) to fully 3-dimensional biomaterials where light scattering and depth of penetration are concerns.

### 3.3.3 pH on BP Immobilization

Since the general chemistry behind BP and EDC protein immobilization methods differ, the effects of pH on ConA were reexamined in the context of BP during patterning and wash steps (Figure 3.10). As with EDC patterning, pH 7.4 was determined to be the optimum pH for BP patterning of ConA displaying significantly higher levels of immobilization compared to pH 5 (p=.004) or pH 9 (p=.01).
3.3.4 Collagen Dependency

As CG scaffolds are fabricated from different collagen sources, we examined the effect of collagen source on biomolecule attachment. Collagen isolated from bovine Achilles tendon (Sigma Aldrich, St. Louis, MO) and purified fibrillar collagen (Collagen Matrix, Franklin Lakes, NJ) were evaluated for fouling and patterning with a ConA protein concentration of 5 µg/mL and exposure times of 5 min and 3 min for Sigma collagen and Collagen Matrix collagen respectively. **Figure 3.11** depicts representative patterns of the two collagens under the same patterning conditions.

The results suggest that the fluorescent intensity of the collagen from bovine Achilles tendon is greater than that of the fibrillar collagen and therefore is more prone to fouling. Further, the patterned square on the fibrillar collagen appear sharper with a greater signal/noise ratio. The results suggest that the processing and origin of the collagen will impact patterning of collagen GAG scaffolds. As a result, the fibrillar collagen source was chosen to conduct further experiments due to its superior patterning abilities.

3.3.5 Depth of Patterning

While 2-dimensional patterning allows for spatial patterning and control of biomolecular density on a biomaterial surface, 3D patterning allows for an additional parameter: depth of patterning. Patterning depth can have physiological consequences to cultures within a biomaterial. For example, if angiogenic biomolecules were patterned with BP chemistry into a scaffold, the patterning depth could regulate or limit which cells receive the cues based on their own depth in the scaffold. For some applications it may be desirable to have depth controlled patterns while in others it might be desirable for the pattern to be present throughout the entire depth of the
material. Preliminary results using 0.5 % CG scaffold (-40°C freezes temperature with an approximate pore size of 100 um) suggest that conventional BP photopatterning can be used to generate patterns ~1 mm into the scaffold (Figure 3.12, 3.13).
3.4 Figures

Figure 3.1: CG scaffold with BP patterning of ConA
Figure 3.2 Non-Covalent Con A immobilization in CG scaffolds after washes. 50 ng Con A immobilized in hydrated 0.5% collagen matrix CG scaffolds with freezing temperature of -40º C. Scaffolds were washed for one hour before undergoing 1 or 2 24 hour washes.
Table 3.1: **Protein exposure times** - Effects of 50 ng ConA exposure times on fouling of 0.5% collagen matrix CG scaffolds with freezing temperature of -40\(^\circ\) C.

<table>
<thead>
<tr>
<th>Time</th>
<th>No Sucrose</th>
<th>Sucrose After</th>
<th>Sucrose Before</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>22%</td>
<td>13%</td>
<td>28%</td>
</tr>
<tr>
<td>20 min</td>
<td>31%</td>
<td>19%</td>
<td>25%</td>
</tr>
</tbody>
</table>
Figure 3.3 Total Con A Immobilization by EDC
Figure 3.4: Con A Fouling of EDC'd Scaffolds
Figure 3.5: Effects of pH and one or two step EDC chemistry
Figure 3.6: Percent EDC immobilization and fouling of 50 ng Con A in CG scaffolds with Chondroitin Sulfate or Hyaluronic Acid
Figure 3.7 A. Hyaluronic acid total immobilization results (left) and fouling (right) B. Chondroitin Sulfate total immobilization results (left) and fouling (right).
Figure 3.8 MFI for different BP steps for patterning of 5 µg/mL Con A biotin (bars that share a letter are not statistically different)
Figure 3.9 Effects of UV exposure times on attachment
Figure 3.10 Effects of ConA loading and pH on total immobilized ConA via BP patterning
Figure 3.11: CG scaffold with BP patterning of ConA: A.) Left: Fluorescent image at 10x magnification of patterning on collagen isolated from bovine Achilles tendon. Right: Profile plot of patterning. B.) Left: Fluorescent image at 10x magnification of patterning on fibrillar collagen. Right Profile plot of patterning
Figure 3.12 Depth of patterning in CG scaffold: image taken with fluorescent microscope longitudinal cross-section (left) surface image of scaffold taken prior to cutting (right)
Figure 3.13 Scaffold cross-sections for determining patterning depth: images were inverted in Image J and contrast was increased using auto section to better determine the depth of patterning.
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

In this work, we examined the use of multiple biomolecule immobilization strategies to create patterns of biomolecules within a 3-dimensional CG scaffold system for the eventual purpose of generating vascular networks to improve scaffold bioactivity. EDC and BP chemistries were individually characterized and optimized for improved control over covalent and nonspecific binding of a model protein, Con A, within CG scaffolds. Notably:

- The amount of ConA immobilized with EDC chemistry was found to be highly correlated with the amount of ConA loaded onto the scaffold. Therefore, one method to increase the amount of ConA immobilized would be to increase the amount of ConA loaded.

- While varying the EDC:NHS:COOH ratio did not result in a significant difference in the change of total ConA immobilization per change in ConA loaded into the scaffold, the optimal ratio was found to be 5:12.5:1 due to a higher amount of ConA immobilized. The data supported previous reports that performing the EDC reaction in two steps where in EDC and NHS were introduced in a phosphate buffer solution to the CG scaffold to form an active ester prior to the introduction of the second component, ConA, are more efficient at immobilizing ConA (Chiu, Weisel et al. 2011).

- Studies examining pH dependence found no significant differences between EDC immobilization at pH 5.5 and pH 7.4. This agrees with previous coupling versus pH curve in which an optimal peak for coupling was found at pH 6.5 (Sehgal and Vijay 1994). Significant differences between pH 5.5 and 7.4 were found with the two step
process. Using pH 7.4 for both steps or pH 5.5 for the first step and a pH 7.4 were both found to have a higher total immobilization compared to using pH 5.5 for both steps.

- An interrogation on the BP process verified that ConA immobilization occurred due to the simultaneous presence of ConA and UV light. Further, the efficiency of ConA immobilization within CG scaffolds via BP chemistry was determined to be dependent on the length of UV exposure as was previously shown with gradients in 2-dimensions on glass substrates (Toh, Fraterman et al. 2009). Like with EDC, a phosphate buffer solution at pH 7.4 was found to yield the highest total ConA immobilization with BP photochemistry.

- Molecules like ConA which bind to collagen and GAGs are prone to non-specific binding. Many factors were therefore investigated for their impact on the severity of ConA fouling. Exposure time was found to positively correlate with ConA fouling. A sucrose solution was found to minimize the fouling compared to other washes studied. However, more work will need to be done to optimize the wash parameters for other biomolecules of interest.

- Beyond processing, fouling was found to greatly depend on the origin and components of the CG scaffolds to which they were introduced. GAGs with higher degrees of sulfation could lead to an increase in nonspecific binding of biomolecules. However, this research found that the ConA bound more covalently and nonspecifically to scaffolds containing hyaluronic acid which has no sulfation compared to scaffolds containing chondroitin sulfate which is partially sulfated. More experiments will need to be performed to verify these findings.
• The processing of the materials was also found to be important. When two different Type I collagens from Achilles bovine tendon were used for BP patterning, it was found that the one purchase from Sigma had a higher background noise due to fouling compared to the pattern generated on Collagen Matrix.

In the future, further investigation into BP patterning with additional biomolecules with different size and affinities to the collagen GAG scaffold including VEGF will be performed to better characterize and optimize the control of patterning within CG scaffolds. The bioactivity of these patterns will be assessed with in vitro cultures of Human Umbilical Vein Endothelial Cells (HUVECs) and will extend to explore the spatial effects of the pattern on 3-dimensional vascular network organization. Upon validation of VEGF bioactivity, intricate spatial patterns with multiple physiological endometrial cues will be explored using co-cultures of human Endometrial Epithelial Cells (EECs) and HUVECs to model endometrial angiogenesis.
APPENDIX A- ABBREVIATIONS

Biotin- Biotinylated
BP- Benzophenone
ConA- Concanavalin A
CG- Collagen-GAG
DIEA- N,N-Diisopropylethylamine
DMF- Dimethylformamide
E2- 17-β Estradiol
EC- Endothelial Cell
ECM – Extracellular Matrix
EDC- 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride
EEC- Endometrial Epithelial Cell
FGF- Fibroblast Growth Factor
GAG- Glycosaminoglycan
HUVEC- Human Umbilical Vein Endothelial Cell
MFI- Mean Fluorescent Intensity
NHS- N-Hydroxysuccinimide
OCT- Optimal Cutting Temperature Solution
PDGF- Platelet Derived Growth Factor
SE- Standard Error
TGF-α- Transforming Growth Factor α
VEGF- Vascular Endothelial Growth Factor
APPENDIX B: EXPERIMENTAL PROTOCOLS

B.1 CG Suspension Preparation Protocol

Reference: Yannas, Lee et al. 1989; O'Brien, Harley et al. 2004; Caliari and Harley 2011; Gonnerman, McGregor et al. in preparation

Reagents

- Collagen from bovine Achilles tendon (Sigma-Aldrich C9879); store at 4°C
- Chondroitin sulfate sodium salt from shark cartilage (Sigma-Aldrich C4384); store at 4°C
- Glacial acetic acid (Sigma-Aldrich 71251)
- Ethylene glycol (VWR BDH1125-4LP)
- Deionized water

Equipment and Supplies

- Recirculating chiller (Fisher Isotemp Model 900)
- Rotor-stator (IKA 0593400)
- Disperser (IKA 3565001)
- Jacketed beaker (Ace Glass 5340-115)
- Freeze-dryer (VirTis Genesis)
- Beakers
- Parafilm
- Spatula

Procedure

*This procedure describes how to make 300 mL of 0.5% CG suspension. Scale collagen and GAG content appropriately to create different volumes of suspension.

1. Fill recirculating chiller with a 50/50 mix of ethylene glycol and deionized water, making sure that the cooling coils are completely immersed in the liquid. Set the recirculating chiller to 4°C.
2. Attach recirculating chiller to jacketed beaker so that the coolant enters at the jacketed beaker’s base and exits at the beaker’s top. Allow for the temperature to equilibrate to 4°C, about 30 minutes. Maintaining this temperature is important, as it will prevent the collagen from denaturing during the blending process.

3. Prepare a 0.05 M solution of acetic acid by adding 0.87 mL of glacial acetic acid to 300 mL of deionized water.

4. Weigh 1.5 g of collagen and add to the jacketed beaker.

5. Pour 250 mL of the 0.05 M acetic acid into the jacketed beaker.

6. Assemble the rotor-stator and attach it to the disperser. Lower the rotor-stator into the suspension. The rotor-stator should be vertical and centered in the beaker.

7. Blend the suspension at 15,000 rpm for 90 min at 4°C. The height of the rotor-stator may need to be adjusted during the blending process: If the rotor-stator is positioned too high, the holes on its side will be visible; if it is too low, the suspension will bubble excessively. Periodically check to see if the rotor-stator is clogged with collagen; remove clogs with a spatula as needed.

8. Add 50 mL of 0.05 M acetic acid to a 50 mL centrifuge tube. Weigh out 0.133 g of chondroitin sulfate (GAG) and add to the centrifuge tube. Vortex until the GAG is fully dissolved. Let the GAG solution rest in the refrigerator (4°C) for at least 10 minutes.

9. Add the GAG solution drop-wise to the collagen suspension while it is being mixed at 15,000 rpm at 4°C. Periodically manually stir in any GAG that remains on the surface of the suspension using a spatula. It may be necessary to stop and unclog the rotor-stator with a spatula during this process.

10. Once all of the GAG solution has been added, blend at 15,000 rpm for 90 min at 4°C. Periodically check to ensure the rotor-stator is lowered to the correct depth, as the suspension will gradually become less viscous and creep up the sides of the jacketed beaker. Periodically check to see if the rotor-stator is clogged; remove clogs with a spatula as needed.

11. Store the suspension for at least 18-22 h at 4°C.

12. Degas the suspension to remove any air bubbles prior to use. It is recommended to degas approximately 20 mL at a time, until the solution starts to boil. To minimize suspension loss during the degassing process, cover the beaker with slit Parafilm.

13. Store the suspension at 4°C. Periodically check the CG suspension; if not homogenous, re-blend at 15,000 rpm for at least 30 min at 4°C.
B.2 DHT Crosslinking Protocol

Reference: (Yannas, Lee et al. 1989; Harley, Leung et al. 2007)

Supplies and equipment

- Sterile air filter (Millipore SLGP033RS)
- Vacuum oven (Welch Vacuum, Fisher 13-262-52)
- Welch DirecTorr Gold synthetic pump oil (Fisher 01-184-105)

Procedure

*Note: Periodically check vacuum pump oil levels. Change oil at least once every 6-12 months. Change sterile air filter on ‘Purge’ line regularly.

1) Turn on vacuum oven and set the temperature to 105°C.

2) Once vacuum oven has reached temperature set point, place scaffolds in opened aluminum pouches carefully inside the oven. Close the oven door.

3) Close the ‘Purge’ valve, located on the lower right face of the vacuum oven. Completely open the ‘Vacuum’ valve.

4) Turn on the vacuum pump and make sure vacuum is pulled to a sufficiently low level (< 1 in Hg). Allow scaffolds to crosslink for 24 hours.

5) After crosslinking is complete, turn off the vacuum pump, close the 'Vacuum' valve, open the 'Purge' valve, then carefully remove scaffolds from the oven. Quickly seal the aluminum pouches, taking care to ensure that the aluminum pouches are sufficiently “puffed” so that the scaffolds will not be crushed during storage. Store sealed pouches with scaffolds (now sterile) in desiccator until time of use.
B.3 Scaffold Cutting and Hydration

Reference: (Olde Damink, Dijkstra et al. 1996; Harley, Leung et al. 2007; Caliari and Harley 2011; Gonnerman, McGregor, et al., in preparation)

Reagents

- Sterile phosphate-buffered saline (PBS)
- 200 proof (100%) ethanol

Equipment and Supplies

- 50 mL centrifuge tubes (Fisher 14-432-22)
- Dual range balance (Mettler Toledo XS105)
- 6 mm biopsy punches (Fisher NC9551417)
- Pasteur pipettes

Procedure

* Note: all steps should be performed in the laminar flow hood unless otherwise noted.

1. Prior to cutting scaffolds, ensure that all materials (including gloves) are completely dry.

2. Cut scaffolds from scaffold sheets using a biopsy punch. Ensure that the biopsy punch is oriented perpendicular to the scaffold sheet. Holding the top of the biopsy punch, gently spin the biopsy punch downward to cut through the sheet, applying pressure at the end only if necessary. If the scaffold remains lodged in the biopsy punch, gently poke it out using a Pasteur pipette. Place cut scaffolds in labeled pre-weighed 50 mL conical tubes.

3. If scaffolds are to be EDC’d, weigh scaffolds prior to hydration.

4. Hydrate cut pieces in 100% ethanol overnight. (Do not hydrate scaffolds for BP)

5. Rinse scaffolds PBS for 1 hour, then let soak in PBS for 24 hours prior to crosslinking.
B.4 Scaffold Cutting and EDC Crosslinking Protocol

Reference: (Olde Damink, Dijkstra et al. 1996; Harley, Leung et al. 2007; Caliari and Harley 2011; Gonnerman, McGregor, et al., in preparation)

Reagents

- 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Sigma-Aldrich E7750); store at -20ºC
- N-hydroxysulfosuccinimide (NHS, Sigma-Aldrich H7377); store in desiccators
- Sterile phosphate-buffered saline (PBS)

Equipment and Supplies

- 6-well plates (Fisher 08-772-1B)
- 50 mL centrifuge tubes (Fisher 14-432-22)
- Syringe and syringe filter (Fisher 148232A)
- MTS 2/4 digital microtiter shaker (IKA 3208001)
- Dual range balance (Mettler Toledo XS105)
- Razor blades
- 6 mm biopsy punches (Fisher NC9551417)
- Pasteur pipettes

Procedure

1. Note: all steps should be performed in the laminar flow hood unless otherwise noted.

2. Determine the EDC and NHS concentrations to be used in the crosslinking solution. The calculations shown below are done with a 5:2:1 EDC:NHS:COOH molar ratio, where COOH is carboxylic acid groups in CG material based on a conversion factor of 1.2 mmol COOH per gram of collagen (Olde Damink, Dijkstra et al. 1996). The mass of EDC and NHS required can be calculated as follows:

   \[ M_{EDAC} = M_{scaffold} \left( \frac{0.0012 \text{ mol}_{COOH}}{g_{collagen}} \right) \left( \frac{5 \text{ mol}_{EDAC}}{1 \text{ mol}_{COOH}} \right) \left( \frac{191.7 \text{ g}_{EDAC}}{1 \text{ mol}_{EDAC}} \right) \]
5. Mix the EDC and NHS in PBS. Approximately 1 mL of solution will be needed per scaffold piece (6-8 mm diameter, 3-5 mm thick).

6. In the laminar flow hood, sterile filter the solution and add to 6-well plates. One can crosslink up to 6 scaffolds per well. If volume is insufficient to cover scaffolds, add additional PBS, keeping the volume constant for all wells.

7. Add scaffolds to crosslinking solution and place well plate on digital microtiter shaker in 37°C incubator. Allow scaffolds to crosslink under moderate shaking for 1 hour 30 minutes. Crosslinking time should be increased for less permeable constructs and higher solids content scaffolds.

8. Remove EDC/NHS solution and rinse scaffolds in sterile PBS under moderate shaking for 1 hour.

9. Remove PBS wash solution and replace with fresh PBS. Rinse under moderate shaking overnight.

10. Store in fresh sterile PBS at 4°C until use.

4. \[ M_{NHS} = M_{\text{Scaffold}} \left( \frac{0.0012 \text{ mol COOH}}{g_{\text{collagen}}} \right) \left( \frac{2 \text{ mol}_{\text{NHS}}}{1 \text{ mol COOH}} \right) \left( \frac{116.0 \text{ g}_{\text{NHS}}}{1 \text{ mol}_{\text{NHS}}} \right) \]
B.5 Scaffold Cutting and EDC ConA Crosslinking Protocol

Reference: (Olde Damink, Dijkstra et al. 1996; Harley, Leung et al. 2007; Caliari and Harley 2011; Gonnerman, McGregor, et al., in preparation)

Reagents

- 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Sigma-Aldrich E7750); store at -20°C
- N-hydroxysulfosuccinimide (NHS, Sigma-Aldrich H7377); store in desiccators
- Sterile phosphate-buffered saline (PBS)
- 200 proof (100%) ethanol
- Biotinylated ConA (Invitrogen)

Equipment and Supplies

- 6-well plates (Fisher 08-772-1B)
- 24-well plates (Fisher 08-772-1)
- Clear 96 well plate (Fisher 12-565-66)
- 50 mL centrifuge tubes (Fisher 14-432-22)
- Syringe and syringe filter (Fisher 148232A)
- MTS 2/4 digital microtiter shaker (IKA 3208001)
- Dual range balance (Mettler Toledo XS105)
- 6 mm biopsy punches (Fisher NC9551417)
- Pasteur pipettes
- Tweezers

Procedure

1. Note: all steps should be performed in the laminar flow hood unless otherwise noted.
2. Prior to cutting scaffolds, ensure that all materials (including gloves) are completely dry.
3. Cut scaffolds from scaffold sheets using a biopsy punch. Ensure that the biopsy punch is oriented perpendicular to the scaffold sheet. Holding the top of the biopsy punch, gently spin the biopsy punch downward to cut through the sheet, applying pressure at the end only if necessary. If the scaffold remains lodged in the biopsy punch, gently poke it out using a Pasteur pipette. Place cut scaffolds in labeled pre-weighed 50 mL conical tubes.

4. Weigh scaffolds prior to hydration.

5. Hydrate cut pieces in 100% ethanol overnight.

6. Rinse pieces in PBS for 1 hour, then let soak in fresh PBS for 24 hours prior to crosslinking.

7. Prepare protein solutions by adding desired amounts of biotinylated Con A (i.e. 1 mg, 2.5 mg, or 5 mg) to 1 mL of sterile PBS and vortex.

8. Determine the EDC and NHS concentrations to be used in the crosslinking solution. The calculations shown below are done with a 5:2:1 EDC:NHS:COOH molar ratio, where COOH is carboxylic acid groups in CG material based on a conversion factor of 1.2 mmol COOH per gram of collagen (Olde Damink, Dijkstra et al. 1996). The mass of EDC and NHS required can be calculated as follows:

9. \[ M_{EDAC} = M_{scaffold} \left( \frac{0.0012 \text{ mol} \text{COOH}}{g \text{collagen}} \right) \left( \frac{5 \text{ mol} \text{EDAC}}{1 \text{ mol} \text{COOH}} \right) \left( \frac{191.7 \text{ g} \text{EDAC}}{1 \text{ mol} \text{EDAC}} \right) \]

10. \[ M_{NHS} = M_{scaffold} \left( \frac{0.0012 \text{ mol} \text{COOH}}{g \text{collagen}} \right) \left( \frac{2 \text{ mol} \text{NHS}}{1 \text{ mol} \text{COOH}} \right) \left( \frac{116.0 \text{ g} \text{NHS}}{1 \text{ mol} \text{NHS}} \right) \]

11. Mix the EDC and NHS in PBS. Approximately 1 mL of solution will be needed per scaffold piece (6-8 mm diameter, 3-5 mm thick).

12. In the laminar flow hood, sterile filter the solution and add to 6-well plates. One can crosslink up to 6 scaffolds per well. If volume is insufficient to cover scaffolds, add additional PBS, keeping the volume constant for all wells.

13. Add scaffolds to crosslinking solution and place well plate on digital microtiter shaker in 37°C incubator. Allow scaffolds to crosslink under moderate shaking for 20-30 minutes.

14. Remove scaffolds from EDC solution and dab dry on Kimwipe to remove excess solution.

15. Lay scaffolds flat within individual wells of a 96 well plate.

16. Pipette 5 μL of protein solution to top surface of scaffolds.

17. Let scaffolds sit in dark at room temperature for 30 min.
18. Flip scaffold over and pipette 5 μL of protein solution to the top surface of the scaffold

19. Let scaffolds sit in dark at room temperature for an additional 30 min.

20. Remove scaffolds from 96 well plate and place in wells of 24-well plate with 2 mL per well of desired wash solution (i.e. PBS, 5% BSA in PBS, 5% sucrose, or 1% tween)

21. Allow sample to rinse under moderate shaking at room temperature for 1 hour

22. Remove wash solution and replace with 2 mL fresh wash solution per scaffold. Rinse under moderate shaking at room temperature overnight.

23. Rinse scaffolds in 2 mL of PBS per scaffold.

24. Store in fresh sterile PBS at 4°C until use or analysis.
B.6 Analysis of Digested Scaffolds of Protein Immobilization by EDC

Reagents

- Papain buffer (100 mL); store at 4°C
  - 100 mL PBS
  - 1 mL 0.5 M EDTA (pH = 8.0, Sigma-Aldrich EDS); store at 4°C
  - 79 mg cysteine-HCl (Sigma-Aldrich 00320)
- Papain from Carica papaya (Sigma-Aldrich 76218); store at -20°C

Supplies and equipment

- Black 96-well plates (Fisher 14-245-177)
- Vortex (Fisher 02-215-365)
- Water bath (60°C, Fisher 15-460-2SQ)
- Fluorescent spectrophotometer (Tecan F200)
- Microcentrifuge tubes (1.5 mL)
- Conical centrifuge tubes (15 and 50 mL)
- Kimwipes
- Sterile blunt-nosed tweezers

Procedure

1. Prepare papain solution by adding 2.4 mg of papain to 1 mL of papain buffer. Let solubilize in 60°C water bath for ~10 minutes. Vortex thoroughly to mix.

Standard and 100% samples

2. Pipette 200 μL digest solution into each standard tube.
3. Dab blank scaffolds dry on Kimwipe and place in 1.5 mL microcentrifuge tubes with papain solution
4. Add 10 μL of the protein solution used for immobilization at the desired concentration into each standard tube. For blank scaffolds, use 10 μL of PBS
5. Place tubes in 60°C waterbath for 1 hour or until scaffolds are digested.
6. Vortex each tube thoroughly.
7. Pipette 100 μL from each tube into a black 96-well plate.
8. Immediately read plate on Tecan F200 fluorometer. Load protocol. For AlexaFluor 488 streptavidin use excitation and emission. For AlexaFluor 647-Con A biotin use: excitation: 485 (20 nm bandwidth) and emission: 535 (25 nm bandwidth) excitation: 620 nm (20 nm bandwidth) and the emission: 670 nm (25 nm bandwidth).
9. Plot normalized intensity (y axis) vs. amount of protein (ConA) (x axis).

\[
\text{Normalized intensity} = \frac{\text{Average sample intensity}}{\text{Blank intensity}}
\]
10. Fit the curve with the best fit line.

Sample Analysis

1) Pipette 200 μL of papain solution into each labeled 1.5 mL microcentrifuge tube.
2) Dab blank scaffolds dry on Kimwipe and place in 1.5 mL microcentrifuge tubes with papain solution.
3) Place tubes in 60°C waterbath for 1 hour or until scaffolds are digested.
4) Vortex each tube thoroughly.
5) Pipette 100 μL from each tube into a black 96-well plate.
6) Immediately read plate on Tecan F200 fluorometer. Load protocol. For AlexaFluor 488 streptavidin use excitation and emission. For AlexaFluor 647-Con A biotin use: excitation: 485 (20 nm bandwidth) and emission: 535 (25 nm bandwidth) excitation: 620 nm (20 nm bandwidth) and the emission: 670 nm (25 nm bandwidth).
7) Determine amount of protein immobilized by subtracting the blank and using the curve determined by the standard.
B.7 Analysis of Digested Scaffolds of Protein Immobilization by BP

Reagents

- Papain buffer (100 mL); store at 4°C
  - 100 mL PBS
  - 1 mL 0.5 M EDTA (pH = 8.0, Sigma-Aldrich EDS); store at 4°C
  - 79 mg cysteine-HCl (Sigma-Aldrich 00320)
- Papain from Carica papaya (Sigma-Aldrich 76218); store at -20°C

Supplies and equipment

- Black 96-well plates (Fisher 14-245-177)
- Vortex (Fisher 02-215-365)
- Water bath (60°C, Fisher 15-460-2SQ)
- Fluorescent spectrophotometer (Tecan F200)
- Microcentrifuge tubes (1.5 mL)
- Conical centrifuge tubes (15 and 50 mL)
- Kimwipes
- Sterile blunt-nosed tweezers

Procedure

1. Prepare papain solution by adding 2.4 mg of papain to 1 mL of papain buffer. Let solubilize in 60°C water bath for ~10 minutes. Vortex thoroughly to mix.

AlexaFluor 647- Con A biotin Standard and 100% samples

2. Pipette 200 μL digest solution into each standard tube.
3. Dab blank scaffolds dry on Kimwipe and place in 1.5 mL microcentrifuge tubes with papain solution
4. Add 10 μL of the protein solution used for immobilization at the desired concentration into each standard tube. For blank scaffolds, use 10 μL of PBS
5. Place tubes in 60°C waterbath for 1 hour or until scaffolds are digested.
6. Vortex each tube thoroughly.
7. Pipette 100 μL from each tube into a black 96-well plate.
8. Immediately read plate on Tecan F200 fluorometer. Load protocol. For AlexaFluor 488 streptavidin use excitation and emission. For AlexaFluor 647-Con A biotin use:
   - excitation: 485 (20 nm bandwidth) and emission: 535 (25 nm bandwidth) excitation: 620 nm (20 nm bandwidth) and the emission: 670 nm (25 nm bandwidth).
9. Plot normalized intensity (y axis) vs. amount of protein (ConA) (x axis).

\[
\text{Normalized intensity} = \frac{\text{Average sample intensity} - \text{Blank intensity}}{\text{Blank intensity}}
\]
10. Fit the curve with the best fit line.

Sample Analysis

11. Pipette 200 μL of papain solution into each labeled 1.5 mL microcentrifuge tube.
12. Dab blank scaffolds dry on Kimwipe and place in 1.5 mL microcentrifuge tubes with papain solution.
13. Place tubes in 60°C waterbath for 1 hour or until scaffolds are digested.
14. Vortex each tube thoroughly.
15. Pipette 100 μL from each tube into a black 96-well plate.
16. Immediately read plate on Tecan F200 fluorometer. Load protocol. For AlexaFluor 488 streptavidin use excitation and emission. For AlexaFluor 647-Con A biotin use:
   excitation: 485 (20 nm bandwidth) and emission: 535 (25 nm bandwidth) excitation: 620 nm (20 nm bandwidth) and the emission: 670 nm (25 nm bandwidth).

AlexaFluor 488-Streptavidin Standard

17. Determine amount of protein immobilized for the AlexaFluor 647 samples by subtracting the blank and using the curve determined by the standard.
18. Make a plot of the fluorescence measured for the AlexaFluor 488 streptavidin/Con A biotin fouling samples versus the quantity of Con A determined to be in the corresponding AlexaFluor 647 samples.
19. Make a best-fit line through the curve.
20. Use this curve to determine the quantities of protein immobilized in the AlexaFluor 488 streptavidin/Con A biotin samples.
B.8 Benzophenone Chemistry

Reagents

- Dimethylformamide (DMF) (Sigma)
- N-Diisopropylethylamine (DIEA) (Sigma)
- Benzophenone (BP) (Invitrogen)
- Sterile phosphate-buffered saline (PBS)
- 200 proof (100%) ethanol
- ConA biotin
- 0.5% pluronic acid solution in PBS

Supplies and equipment

- Glass scintillation vials
- Aluminum foil
- Conical centrifuge tubes (15 mL)
- Kimwipes
- Sterile blunt-nosed tweezers
- MTS 2/4 digital microtiter shaker (IKA 3208001)

Procedure

1. Add 100 mg of BP to 10 mL of DMF and 500 μL DIEA in glass scintillation vial covered in foil. (NOTE: Do not use plastic)
2. Place scaffolds into BP solution and let sit in the dark at room temperature for 48 hours.
3. Remove BP solution and rinse with multiple DMF washes until scaffolds are no longer yellow and appear white.
4. Rinse scaffolds in ethanol for 1 hour. Replace ethanol and leave scaffolds overnight.
5. Rinse scaffolds in PBS for 1 hour. Replace PBS and leave scaffolds overnight.
6. Prepare the protein solution in PBS to a concentration of 1 mg/mL, 2.5 mg/mL, or 5 mg/mL
7. In dark, remove scaffold from PBS and remove excess solution by dabbing scaffold on Kimwipe.
8. Place scaffold inside a small O-ring (to prevent sample from being smashed) on glass coverslip on top of a glass slide
9. Add 10 μL of protein to the scaffold and place coverslip on top (forming a coverslip sandwich) If mask is to be used, place on top of top coverslip
10. Turn on the laser and expose the scaffold at 365 nm with intensity between 18-20 mW for the desired time.
11. Turn off laser and flip the coverslip sandwich over and replace to slide
12. Turn on laser and expose the scaffold for the previous time length
13. Make sure the protein loading time between samples are the same. (i.e. protein should be left in scaffolds for 10 min despite exposure times).
14. After protein loading time has expired, dry scaffold with Kimwipe and place in 2 mL of pluronic acid.
15. After a minimum of one hour, remove scaffold from pluronic acid solution and rinse under moderate shaking at room temperature in desired wash solution overnight.
16. Rinse scaffold under moderate shaking at room temperature in PBS for 1 hour.
17. Add 1 μL of AlexaFluor 488 labeled streptavidin in 2 mL 3% BSA in PBS to each well with scaffold in a 24 well plate.
18. Let samples label under moderate shaking for 1 hour at room temperature.
19. Remove solution and rinse scaffolds with 2 mL PBS for a minimum of 1 hour.
20. Analyze samples as described in B.7.
B.9 Imaging Depth of Patterning

Reagents

- OCT solution
- PBS

Supplies and equipment

- Cryo-mould
- Tweezers
- -80 C freezer
- Razor blades
- Leica DMI4000B with Qimaging camera
- Image J

Procedure

1. Fill mould half full with OCT solution.
2. Place scaffold in microscopy dish under microscope.
3. Rearrange scaffold so that stripes are in desired orientation (left and right)
4. Remove excess PBS from scaffold by dabbing on Kimwipe while maintaining orientation.
5. Place scaffold in cryo-mould with stripes oriented left and right and fill cover over with OCT solution.
6. Allow sample to sit until shape is regained.
7. Place sample in freezer until sample is frozen.
8. Remove sample from slide, maintaining the stripe orientation left and right.
9. Chop sample down middle perpendicular to stripes.
10. Soak scaffolds pieces in PBS to remove OCT solution.
11. Place scaffold cut side down onto microscopy dish and image with microscope.
12. Open image in Image J
13. Auto-Contrast and invert image colors.
14. Save image.
REFERENCES


