THE HETEROGENEOUS BIOCHEMICAL MODIFICATION OF POROUS COLLAGEN SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

BY

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DISSERTATION

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Abstract

The extracellular matrix is a complex and dynamic collection of collagens, non-collagenous matrix proteins, glycosaminoglycans and growth factors. The ECM plays a significant role in guiding the behavior of cells in their tissue specific niche. The recapitulation of both the mechanical and biochemical cues of the ECM is an important objective towards in vitro engineering of functional tissue replacements. Herein I describe my efforts towards the biochemical modification of 3D, porous collagen scaffolds as ECM mimics. Benzophenone mediated photochemistry was used to decorate scaffolds with patterns of vascular endothelial growth factor, which were shown to induce endothelial cells towards a vasculogenic lineage. The use of a two photon excitation for BP patterning was proven affective for surface patterning but ineffective for patterning past a couple hundreds of microns. Heterogenous scaffold patterning in all three dimensions was achieved by collagen slurry layering prior to scaffold freeze drying. A single scaffold was created with two distinct compartment a high modulus “bone” compartment and a low modulus “cartilage” compartment. This technique was used to model the osteochondral interface.
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Chapter 1. Introduction and Background

Introduction

Historically, the extracellular matrix was thought to be a passive support structure, existing to fill the gaps between cells. It wasn’t until the 1981 discovery of integrins that the perceived insignificant role of the ECM gave way to a wave of scientific interest. Integrins are a large family of heterodimeric proteins that connect the intracellular cytoskeleton and the ECM. This interaction has been implicated in the downstream signaling of focal adhesion kinase (FAK) and phosphoinositide 3-kinase (PI3K) which mediates cell self-renewal and proliferation. In the past several decades, researchers have fleshed out the indispensable role of the ECM towards fundamental cell behavior[1].

The physical extracellular matrix structure is a three-dimensional assembly of collagens, glycosaminoglycans, proteoglycans, and non-collagenous proteins such as fibronectin, vitronectin, and tenascins. The physical structure works in conjunction with both soluble and sequestered growth factors to communicate tissue-specific directions to adhered cells. Each tissue type has a specialized set of mechanical and biochemical stimuli that code for a specific differentiation, quiescence, or self-renewal response. Perturbation of any singular stimuli can result in cell apoptosis, uncontrolled gene mutations, and tissue/organ failure [2-4].

The ECM’s most obvious and first recognized role is as a physical structure for cell anchorage and three-dimensional organization. Variations in ECM scaffold rigidity, topography, porosity,
and insolubility have been shown to dictate cellular division, polarity and migration [5-8]. Biochemically, cell attachment to the ECM is facilitated by transmembrane receptor interactions with matrix proteins. In addition to interactions with the cell membrane, non-collagen matrix proteins, collagens, and glycosaminoglycans are able to bind FGFs, HGFs, VEGFs, BMPs, and TGF-β [9, 10]. The sequestration of growth factors regulates their availability and delivery and establishes chemotactic biochemical gradients. The evolutionarily optimized combination of mechanical and biological behavioral cues is essential for proper cell, tissue, and organ functioning. The recapitulation of these cues is the primary goal of tissue engineers. The in vitro fabrication of functioning biomaterials is dependent on modeling the complex and dynamic extracellular matrix.

The most prevalent component of the ECM physical structure, collagen, is a popular choice for tissue engineering. In humans, collagen comprises one third of the body's total protein content and accounts for three fourths the dry weight of skin[11]. The structure of collagen is comprised of three parallel polypeptide strands in helical conformation coiled about each other to form a right-handed triple helix[12]. These triple helices then assemble to form macroscopic fibers and tissues.

There are a variety of collagen biomaterial formulations: sponges, fibers, hydrogels and microspheres[13]. For this research we investigate the use of three dimensional, porous collagen scaffolds as a simple ECM mimic. With robust mechanical properties and tunable degradation rates, porous collagen scaffolds have demonstrated their worth as a viable
platform for tissue engineering applications [14-23]. Collagen scaffolds were constructed via a freeze drying technique in which a network of ice crystals form and eventually sublimate leaving a dry, solid scaffold with an interconnected pore structure [19]. The mechanical and microstructural properties of these scaffolds have been thoroughly characterized [16, 19, 24, 25]. In this thesis, I present my efforts leveraging facile collagen scaffold fabrication with spatially selective biochemical modification techniques to model the physical and biochemical cues of the extracellular matrix for specific tissue engineering applications.

In chapter 2 I include my review of benzophenone photopatterning methods to introduce spatially selected biochemical patterns on cell culture substrates. When illuminated with 365 nm UV light, benzophenone undergoes an n to π* transition to form a transient radical intermediate. The intermediate species then undergoes a C-H insertion reaction with any proximal biomolecule. By restricting light exposure, through a photomask for example, immobilization of target biomolecules is patterned in a spatially selective manner. If no conjugation takes place, benzophenone can relax back to ground state and is able to be re-excited for further patterning applications. Previously, the utility of benzophenone patterned substrates to conduct biological investigations was demonstrated Herman et al [26, 27]. BP functionalized planar glass slides were patterned with selectins, key players in the initial flow-dependent tethering and rolling of leukocytes on vascular surfaces. [28-31] Leukocytes were subsequently flowed across the selectin modified surfaces and cell rolling was observed. The proof of principle study demonstrated the efficacy of BP patterning for the presentation biologically active ligands at controllably varied site densities and shape. Martin and Caliari
translated BP patterning to 3D porous collagen scaffolds[32]. Their seminar work demonstrated the efficacy of BP patterning for heterogeneous biomolecular modification of collagen scaffolds.

Chapter 3 will focus on my efforts using BP photopatterning to conjugate VEGF onto collagen scaffolds to induce endothelial cells towards a vasculogenic lineages. We showed that patterns of VEGF were able to stimulate human umbilical vein endothelial cells towards a vessel morphology compared to cell cultured on scaffolds without patterned VEGF. It was also determined that UV irradiation during patterning does not significantly affect scaffold stiffness nor crystallinity, as determined by the elastic modulus and X-ray diffraction, respectively.

Chapter 4 will focus on effort to make stratified collagen scaffolds as an osteochondral interface mimic. Collagen slurries of different composition were layered prior to freeze drying, producing a single scaffold with two distinct components, a “bone” compartment and a “cartilage compartment.” The different layers were shown to instruct mesenchymal stem cells towards two different lineages as was shown via reverse transcription polymerase chain reaction, cell morphology changes, and ECM deposition staining. Three dimensional scaffold modification will continue to be explored in chapter 5 where I will discuss on our attempts to introduce spatially selective photopatterning to all three dimensions in a collagen-GAG scaffold using a two photon excitation of BP. We demonstrated the successful two photon patterning of model protein Concanavalin A onto planar glass coverslips, planar collagen membranes and 3D collagen scaffolds.
**Figure 1.1. ECM-cell interactions** The cell lives in a specific niche for which it receives direction regarding fate and function. The extracellular matrix affects cells via anchorage, receptor binding, biomechanical forces, enzymatic remodeling, and growth factors. Adapted from *Extracellular matrix: A dynamic microenvironment for stem cell niche* [2].
References


Chapter 2. Benzophenone-based photochemical micropatterning of biomolecules to create model substrates and instructive biomaterials

Notes and Acknowledgements:


The article can accessed online at https://www.elsevier.com/books/micropatterning-in-cell-biology-part-c/thandampeacutery/978-0-12-800281-0

2.1. Introduction

Every living cell resides in an enormously complex and dynamic environment responsible for biological and biophysical cues that govern fate and function. This cellular niche is comprised of matrix proteins, proteoglycans, and other biomolecules working in conjunction with mechanical stimuli and paracrine signals to dictate a myriad of cell behavior such as adhesion, inflammatory response and stem cell differentiation.[1] The ability to mimic this complex cellular niche is of broad interest within the biomaterials research community.
We have developed a simple approach that takes advantage of the photochemical properties of benzophenone (BP). Upon illumination with 365 nm light, BP undergoes an $n \rightarrow \pi^*$ transition to form a transient diradical that can covalently attach to any proximal solution phase biomolecules to the surface via insertion into a C–H bond. When BP is immobilized to the surface of a substrate, the new C–C bond represents a covalent tether between the biomolecule and the substrate. If BP does not react with a nearby molecule within the excited state lifetime (up to ~120 μs) it relaxes back to the ground state from which it can be re-excited with subsequent optical excitation. Capitalizing on the fact that attachment is driven by UV excitation, geometric patterns and gradients of biomolecules can be generated by modulating the location and duration of exposure incident onto a BP-modified substrate. This approach has been previously utilized by our group to generate single and multi-component biomolecular patterns and gradients that were used for biological studies of cell-material interactions. [2-5]

The general BP immobilization and UV patterning procedures are easily adaptable for the patterning of almost any biomolecular target and convenient conjugation chemistry make it broadly amenable to a range of substrate materials. One particularly interesting adaptation is the extension from patterning on two dimensional substrates to three dimensional materials, which is uniquely enabled by this approach because physical contact is not required for patterning, as is the case for microcontact-based biomolecular attachment strategies. Photopatterning on corrugated glass substrates and 3-dimensional porous scaffolds further demonstrate the generality of the approach and open up potential avenues to studying the interplay of biomolecular cues, surface texture, and mechanical properties.
2.2. Experimental

2.2.1. Benzophenone patterning on glass slides

This process is based upon previously described methods. [2-5] It has been successfully applied to the spatial patterning of a diverse set of proteins, glycoproteins, and carbohydrates, including concanavalin A, P- and E-selectin, mannan, fibronectin, and ICAM-1. A schematic illustration of the surface chemistry and photopatterning scheme, along with images showing representative biomolecular patterns is shown in Figure 1.

In addition to patterning on planar microscope slides, corrugated glass substrates, created by photolithography and etching, may be substituted. [4] This section presents two approaches to surface chemical modification that omits or includes poly(ethyleneglycol) (PEG) moieties, as PEG is known to reduce non-specific binding of cells and proteins to surfaces.[6, 7]

Materials:

- Glass microscope slides (Fisher Scientific, Philadelphia, PA)
- Piranha solution (4:1 (v:v) concentrated H_2SO_4: 30% H_2O_2)
- Absolute ethanol (Decon Laboratories, King of Prussia, PA)
- 3-(triethoxysilyl)butyl aldehyde (Gelest, Morrisville, PA)
- 4-benzoyl benzylamine hydrochloride (Matrix Scientific, Columbia, SC)
- Sodium Cyanoborohydride (Sigma-Aldrich)
- Dimethylformamide (Fisher Scientific)
- Methanol (Fisher Scientific)
- Aldehyde-blocking buffer (0.1 M Tris, 200 mM ethanolamine, pH 7.0)
- Biomolecules to be patterned
- Phosphate buffered saline (Sigma Aldrich)
- Bovine serum albumen (Sigma Aldrich)
- Tween 20 (Sigma Aldrich)
- Glycine (Acros Organics)
- H₂N-PEG-CM (1000 MW, Laysan Bio, Inc., Arab, AL)
- N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC)
- N-hydroxysuccinimide (NHS)
- NHS-quenching buffer (0.1 M Tris, 100 mM ethanolamine, pH 8.5)

**Equipment:**

- Vacuum Desiccator
- Goniometer (Ramê-Hart, Netcong, NJ)
- Hot plate
- Parallel flow chamber (GlycoTech; Gaithersburg, MD)
- Silicone gasket of 127 μm thickness, 6 cm length, and 1 cm width
- Syringe pump (Harvard Apparatus, Hollison, MA)
- UV light source (Preferred: Argon ion laser, Coherent Innova 90–4)
- Refractive beam shaping optics (π-Shaper, Molecular Technologies, Berlin, Germany)
- Chromium-coated quartz photo masks
Opaque shutter

Computer controlled translational stage (Thorlabs Opto dc driver)

Optical power meter

Method:

Surface Functionalization: BP-Modified Glass Slides

1. Glass microscope slides are first cleaned with a Piranha solution (4:1 (v:v) concentrated H\text{2}SO\text{4}: 30% H\text{2}O\text{2}).[8] Heat solution and slides for 20 minutes.

2. Rinse substrates three times in water and once in ethanol by carefully dunking the slides in each rinse component. After rinsing, the slides are dried under a stream of nitrogen.

3. Bake slides in an oven at 120 °C for 1 hour then cool to room temperature.

4. The cooled slides are positioned upright along the wall of a vacuum desiccator. The cap of a microcentrifuge tube containing 100 μL of 3-(triethoxysilyl)butyl aldehyde is placed in the center and vacuum applied. The silane is allowed to deposit onto the glass slide for 2.5 hours under vacuum.

5. Cure slides at 120 °C for 1 hour.

6. Rinse in absolute ethanol for 30 min and dry under a stream of nitrogen.

7. Measure water contact angle measurements on a goniometer to confirm successful silanization. The contact angles should be approximately 45°.

8. Prepare a solution of 20 mM 4-benzoyl benzylamine hydrochloride and 200 mM NaCNBH\text{3} in of 4:1 DMF:MeOH. Pipet 200μL of this solution between a pair of glass
slides such that the silanized slides of both slides are in contact with the inner liquid layer. Cover with foil and leave for 4 hours.

9. Quench the reaction by separating the sandwiched slides and immersing into aldehyde-blocking buffer (0.1 M Tris, 200 mM ethanolamine, pH 7.0) for 1 hour at room temperature.

10. Rinse thoroughly with water, DMF, methanol, and ethanol. Dry under a stream of nitrogen.

11. Measure contact angles to verify BP functionalization. The contact angles should be approximately 53°.

12. Store BP-functionalized slides in opaque slide holders in a desiccator at room temperature for up to a month.

Surface Functionalization: BP-Modified Glass Slides Incorporating Poly(ethylene glycol)

1. Treat slides as above through step 5.

2. Following silane curing, incubate slides for 4 hours at room temperature in the presence of 10 mM H₂N-PEG-CM and 100 mM NaCNBH₃ in water using the slide sandwich technique described above.

3. Quench the reaction by immersion in aldehyde-blocking buffer for 1 hour at room temperature.

4. Rinse slides with water, methanol, and ethanol. Dry under a stream of nitrogen.
5. Incubate slides in the dark for 4 hours at room temperature in the presence of 20 mM 4-benzoyl benzylamine, 75 mM N-(3-Dimethylaminopropyl)-N'-'ethylcarbodiimide hydrochloride (EDC), 30 mM N-hydroxysuccinimide (NHS) in PBS (pH 7.4).

6. Immerse slides in NHS-quenching buffer (0.1 M Tris, 100 mM ethanolamine, pH 8.5) for 1 hour at room temperature in the dark.

7. Rinse slides water, methanol, and ethanol. Dry under a stream of nitrogen.

8. Store the resulting BP-modified substrates in a desiccator in the dark until further use.

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**Photoimmobilization of Proteins or Carbohydrates onto Benzophenone Modified Glass Slides**

1. For highest resolution patterning, the argon ion laser beam should be homogenized using refractive beam-shaping optics and expanded to give a uniform illumination plane. The laser power should be adjusted to give a final illumination intensity of ~14 mW/cm² at the substrate surface. Alternatively, a UV LED (Clearstone Technologies) can be utilized for applications where high spatial pattern resolution is not required. Adjust LED output to ~14 mW/cm².

2. The protein and carbohydrate patterning targets should be reconstituted or diluted according to manufacturer’s instructions, aliquoted, and frozen at -80°C until time of use. Protein solutions to be used for patterning experiments are prepared fresh for each experiment. For photopatterning applications protein solutions of concentration 5µg/ml work well for most targets.

3. Solution phase patterning targets are introduced to the functionalized glass slides in a parallel-plate flow chamber. The chamber is separated from the glass substrate by a
silicone gasket. A vacuum is applied to hold the assembly together while the bimolecular solution is pulled through the chamber via a syringe pump.

4. Once assembled, the flow chamber is positioned face-down so the UV light is incident on the non-functionalized back of the glass slide. For spatial patterns, illumination is through chromium-coated quartz photomasks placed between the UV source and the BP-modified slides. For spatial gradients, an opaque shutter is attached to a translational stage and the stage is moved in time so as to create a spatial gradient in exposure time across the substrate during UV exposure.

5. Following UV exposure, the slide is separated from the chamber while immersed in a buffer rinse solution to prevent drying induced protein denaturation. Though the composition of the rinse solutions will vary based upon the biomolecule being patterned, successful past examples include: 0.5% (v/v) Tween 20 in PBS buffer for substrates presenting concanavalin A and fibronectin; 0.5 mg/mL sodium dodecyl sulfate (SDS) in Dulbecco’s PBS for substrates presenting mannann; and 0.5% (v/v) Tween 20 and 1% (w/v) BSA in Dulbecco’s PBS with Ca\(^{2+}\) and Mg\(^{2+}\) for substrates presenting P-selectin.

6. If patterning more than one target, flow a low-pH Glycine solution (pH 2.2) through the device to remove nonspecifically adsorbed biomolecules following the first exposure. Without disassembling the flow cell, then introduce PBS to first remove residual rinse solution components and then introduce the second biomolecule solution to the substrate and pattern as described above. Repeat the process for additional
components. When finished with all patterning, disassemble the flow chamber in pre-
determine rinse solution.

7. Store photopatterned substrates in 1% BSA/PBS solutions at 4 °C for up to a week.

2.2.2. Benzophenone Patterning on Porous Collagen Glycosaminoglycan Scaffolds

This process is based upon that described in [2]. It has been successfully applied to the spatial
patterning of proteins, including concanavalin A, fibronectin, VEGF, PDGF, and BMP2. In
addition to patterning on porous collagen-GAG scaffolds, this method is also amenable to
patterning on planar collagen-GAG membranes. [9] A schematic illustration of the surface
chemistry and photopatterning scheme used on collagen biomaterials, along with images
showing representative biomolecular patterns is shown in Figure 2.

[Insert Figure 2 here (caption provided at the end of the document)]

**Materials:**

- Freeze-dried collagen scaffolds
- Benzophenone isothiocyante [10]
- Dimethylformamide (Fisher Scientific)
- Absolute Ethanol (Decan Laboratories, King of Prussia, PA)
- Millipore Water
- Glass microscope slides (Fisher Scientific, Philadelphia, PA)
- Rubber o-ring
- Glass cover slips (Fisher Scientific, Philadelphia, PA)
- Phosphate buffered saline (Sigma Aldrich)
• Bovine serum albumen (Sigma Aldrich)
• Pluronic F-27 (Sigma-Aldrich)
• N,N-Diisopropylethylamine (Sigma Aldrich)

Equipment:
• UV light source (Preferred: Argon ion laser, Coherent Innova 90–4)
• Refractive beam shaping optics (π-Shaper, Molecular Technologies, Berlin, Germany)
• Chromium-coated quartz photomasks
• Translational stage
• Optical power meter

Method:

_Benzophenone Functionalization of CG scaffolds_

1. Collagen-glycosaminoglycan (CG) scaffolds are prepared via a previously reported freeze-drying technique[11, 12]
2. Benzophenone is immobilized when free amines on collagen are reacted with benzophenone isothiocyante. Prepare a 20 mM solution of benzophenone-4-isothiocyanate containing 0.5 mM N,N-diisopropylethylamine in dimethyl formamide (DMF).[13]
3. Add CG scaffolds to BP solution and allow to react on a shaker at room temperature in the dark for 48 hours
4. After functionalization, rinse the scaffold for 1 hour at least 3 times in DMF and once in ethanol before final storage in water. The rinsing process is complete when the center of the scaffold is no longer yellow.

**Photoattachment of biomolecules onto BP-presenting CG scaffolds**

1. Adjust the UV source such that it the power is ~20 mW/cm² at the patterning surface.

2. Prepare a solution of the biomolecule to be patterned. The concentration of each target must be optimized according to a working range of 1µg/ml to 100 µg/ml.

3. The scaffolds should be pre-soaked in a buffer solution containing the protein of interest for 1 hour at 4°C.

4. Place a rubber o-ring onto a glass slide to create a well. Carefully position the protein soaked scaffold in the middle of the rubber o-ring and pipette a 20 µL aliquot of protein solution onto the scaffold. Place a coverslip over top of the o-ring and scaffold to seal the chamber.

5. Following irradiation, immerse the scaffolds in a solution containing 0.2% pluronic F-127 in PBS for 1 hour.

6. Alternatively, for a 2-component patterning, wash the scaffold for 1 hour in PBS following the first excitation before incubating for 1 hour with the secondary component to be patterned and expose as described above.

7. Post-patterning, scaffolds are blocked in a 1% (w:v) solution of bovine serum albumin in PBS.
2.3. Discussion

Benzophenone photopatterning is a simple, versatile surface patterning technique. By modulating UV exposure across benzophenone functionalized substrates, spatial selectivity and controllable protein site density can be achieved. Slight modifications to the protocol may be required based upon the particular biomolecule of interest to be patterned. Common modifications include a change in biomolecule concentration and/or UV exposure time. This methodology is molecularly general and, due to a non-reliance on physical contact for patterning, as is the case with stamp-based patterning approaches, amenable to non-planar substrates, including topographically-complex substrates. The approach has also been demonstrated on corrugated glass slides, in addition to highly porous and non-porous collagen biomaterials.

Despite the enabling capabilities of this biomolecular patterning approach, an obvious concern lies in the ultimate bioactivity of the resulting patterns given the exposure to UV light. However, a several published and unpublished studies have confirmed that many different biomolecules still retain their native biological affect after photochemical patterning. Two particular investigations are presented below as examples.

2.3.1. Leukocyte rolling and adhesion on photochemically-patterned substrates

Irregular inflammatory and immune responses can result in a variety of diseases including rheumatoid arthritis, asthma, psoriasis, Crohn's disease, thrombotic disorders, and autoimmune disease.[14] Benzophenone photoimmobilization of biomolecular gradients and
patterns were used to conduct a high throughput investigation of the first step of the inflammatory response, leukocyte recruitment. A family of molecules known as selectins have been identified as key players in the initial flow-dependent tethering and rolling of leukocytes on vascular surfaces.[15-18] HL-60 promyelocytes and Jurkat T lymphocytes were introduced to substrates presenting gradients of either P-selectin or PSGL-1 under condition of physiological shear stress. Cell behavior was recorded at each combination of site density and shear stress. It was shown that HL-60 cells tether and roll on one component P-selectin gradient substrates over a wide range of site densities and wall shear stresses. Jurkat cells also tethered to and rolled on immobilized P-selectin, as well as PSGL-1, over a range of site densities and shear stresses. This proof of principle study demonstrates the efficacy of BP patterning for the presentation biologically active ligands at controllably varied site densities on a single substrate, allowing for large amounts of data to be acquired with minimal cost of time, materials, and effort.

2.3.2 Enhanced cell attachment to photochemically-patterned collagen scaffolds

Benzophenone photopatterning was successfully demonstrated on three dimensional collagen-GAG scaffolds. No significant changes to the bulk mechanical properties of BP modified scaffolds in response to the organic modification or exposure to UV light were observed. The efficacy of CG scaffolds as culture substrates was assessed by monitoring the proliferation of MC3T3-E1 preosteoblasts seeded onto scaffolds both with and without BP. It was shown that the addition of BP does not have a negative impact on scaffold bioactivity. Fibronectin was photoimmobilized on CB-BP scaffolds to demonstrate the ability to elicit a specific biological
response with photoimmobilized fibronectin promoting greater MC3T3-E1 cell attachment compared to scaffolds containing physiosorbed fibronectin or scaffolds that were not exposed to fibronectin. These results demonstrate that BP photoimmobilization is a viable method to introduce instructive biomolecular cues within collagen-GAG biomaterials for future studies in the areas of regenerative medicine and tissue engineering.

2.4. General Conclusions

Benzophenone mediated photochemical patterning offers a direct approach to generate complex, multi-component patterns or gradients on a range of substrate materials. Since this immobilization approach only requires the presence of a C–H bond, it is biomolecurally general and has broad applicability to a range of classes of biomolecules. Patterned planar and corrugated have been demonstrated as potential model substrates for subsequent studies of cellular behavior as have porous and non-porous collagen biomaterials. The BP moiety can be conveniently tethered to many substrate materials using straightforward conjugation chemistries and UV exposure to radiation in the ~350 nm wavelength range does not inactivate or otherwise damage biomolecules immobilized on the substrate surface. While some target biomolecules have been found to be more easily patterned using this approach than others, the general capability to create multicomponent biomolecular patterns and gradient with control over biomolecular deposition density should prove to be quite enabling for a myriad of studies investigating the biology-materials interface.
Figure 2.1. Photoimmobilization of biomolecules onto glass substrates (a) Schematic illustration showing the surface chemistry and subsequent approach to photopatterning. (b, left) Photoimmobilized “Illinois logo” pattern of biotinylated concanavalin A visualized with fluorescently labeled streptavidin. (b, right) Three-component pattern of mannan (stripes running from top right to bottom left), P-selectin (stripes running from top left to bottom right), and fibronectin (vertical stripes). Scale bars: 100 μm. (c) A single substrate displaying two overlapping gradients of P-selectin (high left, low right; red) and mannan (low left, high right; green). F.I. stands for fluorescence intensity. Scale bars: 500 μm. Reprinted with permission from (Martin, Herman, et al., 2011) Copyright 2011 American Chemical Society and (Toh et al., 2009) Copyright 2009 American Chemical Society.
**Figure 2.2.** Photoimmobilization of biomolecules onto highly porous collagen-glycosaminoglycan scaffolds (a) Schematic illustration showing the chemical derivatization and subsequent approach to photopatterning. (b) Photoimmobilized 100 μm stripes of biotinylated concanavalin A visualized fluorescently after post-pattern labeling with Qdot 525-streptavidin. (c) Photoimmobilized 100 μm stripes of N-cadherin (horizontal, red) and fibronectin (vertical, green) as fluorescently visualized using specific antibodies. Reprinted with permission from (Martin, Caliari, et al., 2011) Copyright 2011 Elsevier.
References

Chapter 3. Photopatterning of VEGF within collagen-GAG scaffolds can induce a spatially confined response in human umbilical vein endothelial cells

Notes and Acknowledgements:


3.1. Introduction

Composed of a heterogeneous and dynamic network of fibrillar proteins, proteoglycans, and other biomolecules, the extracellular matrix (ECM) provides structural, mechanical, and biomolecular signals that impact cell behavior. Analogs of the ECM fabricated from either synthetic or natural polymers have been developed for a wide range of in vitro and in vivo applications. It is commonly accepted that the structural, mechanical, and biomolecular properties of these biomaterials significantly impact cell response, thus providing impetus for optimization. The most abundant ECM protein, collagen, has been a popular choice for a range of biomaterials (e.g., 2D substrates, fibers hydrogels, sponges) [1]. Porous collagen-glycosaminoglycan (CG) scaffolds, prepared via freeze drying, have been developed for in vitro and in vivo regenerative medicine applications [2-5]. Fabrication methods have been described
that allow for tuning of the size and shape of the pore structure, as well as the degradation rate and mechanical properties [3, 6-10]. During optimization of these, and many other biomaterial systems, biomolecular supplementation (e.g., growth factors, cytokines) offers the potential to further influence cell response. While media supplementation is feasible in many in vitro cultures, diffusive loss and cost both limit their potential for clinical application. Recently, Hortensius et al. described incorporation of glycosaminoglycans (GAGs) with differential degrees of sulfation within CG scaffolds to mediate transient (non-covalent) sequestration of growth factors in order to extend the effect of exogenously added soluble molecules [11]. However, in the development of spatially or temporally complex biomaterial structures, multiple competing soluble factors may lead to unintended cell phenotypes [12]. Immobilization of factors to the biomaterial matrix offers the potential for increased signal life and improved specificity [13].

The inability to vascularize a biomaterial before transplantation into a living host is, arguably, the largest bottleneck to engineering large replacement tissues and organs. The transport of waste, nutrients, and oxygen are diffusion limited such that cells cannot survive more than 200 μm from the nearest capillary [14]. In response, many approaches have been explored to address this challenge, notably media supplementation, a wide range of growth factor delivery vehicles, channeled or multi-scale scaffolds, the use of perfusion bioreactors or microfluidics, and cell co-cultures [14-17]. Vascular endothelial growth factor (VEGF), a 40kDa dimeric glycoprotein, has been implicated as a particularly important ligand for angiogenesis [18]. VEGF binding to vascular endothelial growth factor receptor 2 (VEGFR2), activates signaling pathways
for actin remodeling, proliferation and cell survival. Soluble VEGF within culture media has long been shown to promote endothelial cell bioactivity [19, 20]. Recently, Segura et al. demonstrated that covalently immobilized VEGF was capable of activating VEGF receptor 2 (VEGFR2) [21, 22]. While soluble versus covalently bound VEGF activated distinct signaling cascades, covalently-bound VEGF lead to more sustained VEGFR2 phosphorylation. Complementing these results, covalently immobilized VEGF within biomaterial matrices has been shown to enhance endothelial cell activity [23-25] as well as promote endothelial cell mediated formation of immature vessel structures [26-28]. However, given the need to create complex vascular patterns, we are interested in exploring methods to create spatially-defined instructive signals within CG biomaterials in order to enhance angiogenic potential.

Recently, our groups [29-31] have described benzophenone (BP) mediated photolithography approaches to generate spatial patterns of biomolecules on a range of substrates. When excited with 365nm light, BP undergoes a C-H insertion reaction with nearby, solution-phase biomolecules, creating a covalent link between the substrate and biomolecules of interest. BP photochemistry has been used to immobilize a diverse set of proteins, glycoproteins and carbohydrates onto planar and corrugated glass slides. These patterned substrates were shown to retain biomolecular function and were applied to the study of leukocyte rolling during inflammation [32]. Translating this method to three-dimensional CG scaffolds, we demonstrated BP photolithography could be used to create spatially-defined patterns of model proteins within the CG scaffold, and that BP-immobilized fibronectin remained functional [30]. However, to date BP-photolithography has not been demonstrated to selectively immobilize a
functional growth factor within a CG scaffold. Here we describe the use of BP patterning to create spatially-defined patterns of VEGF within the CG scaffold and document response by human umbilical vein endothelial cells (HUVECs). Notably, we report the specificity and depth of penetration achievable when patterning within the CG matrix, as well as the impact of patterning conditions on the composition and mechanical properties of the CG scaffold. Finally, we demonstrate that photopatterned VEGF retains its bioactivity and is able to locally induce the formation of immature tubular networks.

3.2. Experimental

3.2.1. Fabrication of CG scaffolds

Porous CG scaffolds were fabricated according to a previously established method [2, 3]. Briefly, a 0.5% w/v% CG suspension was prepared by homogenizing type I microfibrillar collagen (Collagen Matrix, Oakland, NJ), chondroitin sulfate from shark cartilage (Sigma Aldrich, St. Louis, MO), and 0.5M acetic acid. The degassed slurry was poured into aluminum pans and placed into the freeze dryer (VirTis Genesis, Gardiner, NY). The slurry was cooled to a final temperature of -40 °C at a rate of 1 °C/minute [10]. The resulting ice crystal network was removed via sublimation under vacuum (200mTorr) leaving behind highly porous collagen scaffolds. The scaffolds were then subjected to dehydrothermal (DHT) crosslinking at 10 torr and 105 °C for 24 hours. Biopsy punches (Robbins Instruments, Inc.) were used to cut 6mm diameter disks from the 1.5mm thick CG scaffold sheet.
3.2.2. Conjugation of BP to CG scaffolds

A 20mM solution of benzophenone-4-isothiocyanate and 0.5M N,N diisoproylethlamine was prepared in DMF as previously reported [30]. Scaffolds disks were added to the BP solution at a density of 30 scaffolds per 10 ml of BP solution and reacted at room temperature in the dark on top of an orbital shaker. After 48 hours, the scaffolds were washed repeatedly with fresh DMF. Scaffolds were then soaked in ethanol before final hydration and storage in sterile 1x PBS at 4°C until use.

3.2.3. Photoattachment of VEGF to CG scaffolds

Lyophilized VEGF (R&D Systems, Minneapolis, MN) was reconstituted in sterile water to 100 μg/ml and stored at -80 °C until use. Scaffolds were subsequently soaked in 5 μg/ml VEGF in PBS for at least 1 hour prior to patterning. An argon-ion laser (Coherent Innova 90-4, Laser Innovations, Santa Paula, CA) was used for the UV exposures. The laser source was homogenized and expanded by beam shaping optics (π-Shaper, Molecular Technologies, Berlin, Germany) to give a uniform illumination profile. Homogeneous VEGF immobilization was achieved by illuminating scaffolds for 20 minutes at 20 mW/cm². To create spatial patterns laser illumination was attenuated by a photomask placed above the scaffold. After exposure, scaffolds were rinsed for 1 hour with 0.2% Pluronic F-27 (Sigma-Aldrich, St. Louis, MO) in PBS. CG scaffolds have distinct ‘pan’ and ‘air’ sides, corresponding to orientation in the pan during freeze drying, with ‘air’ sides showing slightly increased scaffold density [6]. As such, patterns were created on both surfaces and examined separately for pattern specificity and depth of patterning into the scaffold.
3.2.4. Fluorescent visualization of biomolecular patterns within the CG scaffold

A cocktail containing polyclonal rabbit anti human anti-VEGF IgG (1 mg/ml, Abcam, Cambridge, MA) and secondary Alexa Fluor 647 goat anti rabbit (2 mg/ml, Life Technologies, Waltham, MA) was premixed. Patterned scaffolds were blocked with 1% BSA in PBS, then incubated overnight in the staining solution at 4 °C. Scaffolds were rinsed in PBS prior to imaging on a LSM 710 confocal microscope (Carl Zeiss Microimaging, GmbH, Germany). Patterns were assessed using two methods. Pattern specificity was determined from images taken of the exposed scaffold surface. Separately, scaffold specimens were prepared to assess depth of patterning into the porous structure. Here scaffolds were embedded with Tissue Tek O.C.T Compound (Sakura Fintek) with the top/bottom surfaces of the scaffold disk oriented perpendicular to the base of the mold. 300 µm thick cross sections through the scaffold were generated via a cryostat (Leica, CM3050S cryostat) and mounted on a glass coverslip to facilitate imaging the depth of pattern penetration. In both cases, Image J Software (NIH, Bethesda, MD) was used to measure mean fluorescence intensity across the images to visualize pattern specificity and the maximum depth of penetration [33].

3.2.5. Determination of total immobilized VEGF within the scaffold

Total immobilized VEGF was determined by fluorimetry. Briefly, BP-functionalized scaffolds were soaked in VEGF solution and were subsequently exposed (+UV) or not exposed (-UV) to UV light (20 min, 20 mW/cm²). Scaffolds were stained for immunofluorescent analysis as before, then transferred to a 96 well plate well containing 300µL of 2.4 mg/ml papain from
carica papaya (Sigma Aldrich) in 5 mM EDTA (Sigma Aldrich). All subsequent steps were done under light-controlled conditions, with scaffolds incubated at 37°C until they were completely digested. The fluorescence released from each sample after digestion, which corresponds to the amount of immobilized VEGF, along with a standard curve of the fluorescent secondary was measured with a fluorimeter (TECAN XP-HP800, Maennedorf, Switzerland) with excitation at 620 nm and emission monitored at 670 nm.

3.2.6. Scaffold mechanical properties

The tensile modulus of CG scaffolds at all stages of BP photopatterning was assessed via previously described methods [30, 34]. Briefly, CG scaffolds were cut into 8 x 18 mm strips. The following specimens, corresponding to all stages of scaffold preparation were tested: CG scaffold alone (CG); scaffold exposed to UV light (CG+UV); scaffold exposed to DMF solvent (DMF); scaffold exposed to DMF solvent and UV light (DMF+UV); scaffold with immobilized BP (CG-BP); scaffold with immobilized BP exposed to UV light (CG-BP+UV). For UV exposure, scaffolds were exposed to 20 mW/cm² for 20 minutes. Tension tests were subsequently performed using an MTS Insight Electromechanical load frame (MTS, Eden Prairie, MN) with a 250 N load cell and 2.5 cm rubber-coated clamps [7]. Tensile tests were performed on specimens (9 mm wide, 18 mm gauge length) at a rate of 1mm/min until failure. Tensile moduli were determined from the elastic region of the corresponding stress-strain curve as previously described [30, 34, 35].
3.2.7. X-ray diffraction (XRD) analysis of collagen crystallinity

The impact of UV exposure on the collagen structure within the CG scaffold was assessed via X-ray diffraction (XRD) analysis [30, 36, 37] using a D-5000 diffractometer (Siemens/Bruker AXS, Madison, WI) with a CuKα source from 5 degrees to 50 degrees at a rate of 0.5 °/min with an increment of 0.02°. Scaffolds at each relevant stage of preparation (CG, CG+UV, CG-BP, CG-BP+UV) were tested to assess any changes in collagen crystallinity [37]. The resulting spectra were analyzed using MDI Jade (Materials Data Inc., Livermore, CA).

3.2.8. Cell culture and assessment of cell bioactivity within the scaffold

Pooled human umbilical endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). HUVECs were grown in medium200 (Life Technologies, Waltham, MA) supplemented with low serum growth supplement (LSGS, Life Technologies, Waltham, MA), 1% L-glutamine, and 1% penicillin/streptomycin at 37°C and 5% CO₂. Cell media was changed every 2 days. Cells were passaged via 0.05% trypsin with EDTA (Fisher-Mediatech) at room temperature and used for experiments at passage 3 or 4. Prior to seeding, HUVECs were starved in unsupplemented media for 6 hours [38]. Scaffolds were seeded via a previously described static seeding method [3, 5, 39, 40]. Briefly, scaffolds were first transferred from 0.2% pluronic (storage) solution to unsupplemented media for 1 hour. Excess media was then removed by gentle dabbing with a sterile Kimwipe and scaffolds were placed in a low attachment 24 well plate (Corning Costar, Sigma Aldrich) to reduce cell loss. HUVECs were suspended in unsupplemented media at a concentration of 2x10⁶ cells/ml (20,000 cells per 10µL). A 20µL aliquot of HUVECs were subsequently pipetted onto each side of the scaffold.
(~80,000 HUVECs per scaffold). After 1 hour incubation at 37°C for attachment, 2 ml of supplemented media was added to each well and the scaffolds maintained in culture 18 hours.

3.2.9. Fluorescent analysis of cell morphology

After 18 hours of culture, HUVEC-seeded scaffolds were fixed in 10% formalin. Fixed HUVECs on the scaffolds were permeabilized in 0.1% Triton X100 in PBS. Scaffolds were then stained to visualize the actin cytoskeleton and nucleus. F-actin was stained with phalloidin-TRITC (Life Technologies, Waltham, MA) for 30 min with DAPI (Life Technologies, Waltham, MA) counterstaining of the nuclei. Stained scaffolds were stored in the dark at 4°C until imaging. Confocal imaging was performed on a Zeiss LSM 710 multiphoton confocal microscope (5x objective, 63x oil objective) equipped with a Spectraphysics Mai-Tai Ti-Sapphire laser. Cell spreading was assessed by total actin area per cell.

3.2.10. Statistical methods

Statistical analysis was performed via one-way analysis of variance (ANOVA). Mechanical characterization experiments used at least n = 8 scaffolds per group, biomolecular patterns used n = 3 scaffolds per group, cell penetration analyses used n = 3 scaffolds per group, and cell spreading analysis used n=4. Significance was set at p < 0.05. Error bars are reported as standard deviation unless otherwise noted.
3.3. Photoimmobilizing VEGF within the scaffold

VEGF was successfully immobilized onto CG scaffolds via BP photochemistry. Illumination through a photomask, yielded patterns, such as stripes (Figure 1) and squares (Figure 5). In Figure 1A, 100 μm wide stripes with 400 μm spacing are clearly discernable and demonstrate high on-to-off pattern specificity (line trace below). The stripes pattern was used to determine the achievable patterning depth within the CG scaffold. Here, the VEGF pattern was visualized in transverse cryostat sections through the patterned scaffold. BP-photopatterning was able to achieve patterning depths on the order of 500 μm into the scaffold on the ‘pan’ side (Figure 1B). A line scan also included shows a clear gradient above baseline of attachment as a function of depth from the surface. Patterning was uniformly found to not be as deep on the ‘air’ side due to a higher density scaffold region that results from fabrication. A repeating squares pattern was subsequently used in cell response studies. To demonstrate the specificity of BP-photopatterning, VEGF immobilization was compared between sets of CG-BP scaffolds soaked in 1 μg/ml VEGF that were either exposed (+UV) or not exposed (-UV) to UV irradiation (Figure 1C). The group where VEGF was specifically photoimmobilized contains significantly more VEGF than the group containing non-specifically physisorbed VEGF.

3.4. Mechanical and structural analysis

While we had previously shown that attachment of BP to the scaffold network did not impact the structural and mechanical integrity of the CG scaffold [30], that study did not examine the impact of UV exposure on the scaffolds. Here, UV exposure was not found to significantly impact scaffold mechanical properties at any stage of processing (CG scaffold alone; CG scaffold...
in DMF solvent; BP-immobilized CG scaffold; Figure 2a). Further, X-ray diffraction (XRD) analysis indicated that all scaffold variants displayed a broad peak at $2\theta = 20^\circ$ (Figure 2b) representing the characteristic interchain spacing of the collagen triple helix [41]. Additional peaks that do emerge in the XRD spectra after chemical and/or UV treatment likely arise due to minor crosslinking However, mechanical testing indicates that these crosslinks do not impact bulk mechanics.

3.5. Ubiquitously patterned VEGF stimulated HUVEC morphology and depth of penetration

Previous reports indicated that VEGF exposure induces significant changes in HUVEC morphology [42]. Therefore, we next chose to examine the impact of photoimmobilized VEGF on the bioactivity of HUVEC cells within the CG scaffold, first using ubiquitous photoimmobilization. HUVECs were starved in unsupplemented media for 6 hours before being seeded onto the CG scaffolds in order to discern the impact of matrix-photoimmobilized VEGF on cell activity. Immobilized VEGF was observed to impact both HUVEC morphology (Figure 3) as well as penetration into the matrix (Figure 4) at 18 hours post-seeding. HUVECs seeded into scaffolds with BP-immobilized VEGF displayed an elongated morphology with multicellular features such as branching, alignment, and extensive network formation that are characteristic of activated HUVECs (Figure 3A). Conversely, HUVECs without VEGF supplementation displayed rounded or cuboidal morphologies (Figure 3B). Actin area per cell, which is indicative of VEGF induced cell spreading, is significantly higher for HUVECs treated with VEGF compared to those that were not (Figure 3C). Further analysis of Figure 3B highlighted that only ~66% of HUVECs
on non-VEGF-treated scaffolds had any cell-cell contact, which is in contrast to the pervasive connectivity observed on VEGF-treated scaffolds.

Enhancing the speed or depth of penetration of cells within a tissue engineering scaffold, particularly those able to contribute to angiogenesis, is of particular interest for biomaterial design. Unlike hydrogel systems, scaffold-based biomaterials rely on active cell penetration through the pores to populate the matrix. Comparing the effects of VEGF photoimmobilization on the depth of HUVEC penetration, VEGF promoted enhanced cell infiltration into the scaffold (Figure 4). After 18 hours, HUVECs traveled on average 454± 109 μm into the scaffolds containing BP-photoimmobilized VEGF, as measured on the pan side, compared to 242 ± 35 μm without VEGF (p = 0.05).

3.6. Photopatterned VEGF impacts early HUVEC network formation

Conglomeration of HUVECs was observed in CG scaffolds with and without photoimmobilized VEGF. However examining the response of HUVECs on scaffolds with VEGF photopatterned into geometric designs (500 μm squares) revealed networks of elongated HUVECs concentrated on the VEGF-photopatterned region (Figure 5A). Comparatively, HUVECs on scaffolds without photopatterned VEGF displayed significantly reduced organization (Figure 5B).
3.7. Discussion

In the field of tissue engineering a significantly barrier to continued progress is diffusion limited transfer of oxygen, nutrients, and waste within a 3D biomaterial. In vivo, cells cannot survive more than 200 μm from the nearest capillary, making rapid formation of vascular structures a particularly important objective in tissue engineering [14]. In vitro techniques such as the use of perfusion bioreactors can partially overcome diffusion limitations [10], but without the formation of a robust vascular network, internal cell necrosis is common upon transplantation into an in vivo host. Furthermore, while perfusion bioreactors can improve nutrient transport through a porous biomaterial, resultant shear stresses on individual cells within the network can be significantly impacted by the scaffold structure [43, 44], introducing an unintended parameter needing to be optimized. To combat this fundamental challenge, there has been much interest in engineering biologically compatible materials with pre-formed, spatially controlled vasculature. However tools to guide the formation of such vascular networks within a three-dimensional biomaterial construct remain limited. In this paper we describe the integration of a collagen scaffold platform with biomolecular photo-patterning techniques that may represent an early step in creating neo-vascular structures.

Collagen-glycosaminoglycan scaffolds offer several key advantages for tissue engineering such as facile fabrication, biocompatibility, and tunable mechanical and microstructural properties [3, 6, 7, 36]. Notably, the collagen backbone provides the potential for further biochemical modification. Indeed, previous investigations have explored the use of chemical crosslinking methods to ubiquitously immobilize growth factors within the scaffold network [23, 27].
Further, the proteoglycan content of these scaffolds offers the potential to use non-covalent interactions to transiently sequester growth factors to the scaffold surface for enhanced bioactivity [11]. However, none of these approaches offers a robust method for locally tuning the growth factor dose presented to cells within the matrix. Here a photoreactive benzophenone moiety was attached to the CG scaffold struts thus affording the ability to undergo UV light induced conjugations with proximal biomolecules. We explored the use of BP photolithography to create defined patterns of a functional biomolecule, VEGF, within CG scaffolds. Immobilized VEGF had been previously shown to activate endothelial cells towards vasculogenic phenotypes [23, 25, 26]. However, these immobilization strategies were not capable of producing defined-spatial patterns of VEGF.

We first confirmed the ability to create well-defined patterns of VEGF within the scaffold (stripes, Figure 1). We observed VEGF had a low non-specific binding affinity for the CG scaffolds in the absence of BP-mediated photoimmobilization, as observed by the high on:off pattern ratio. Further, we observed BP-mediated covalent attachment led to significantly (p < 0.05) higher VEGF immobilization than physiosorption alone (Figure 1C). However, UV-based immobilization strategies depend on sufficient depth of penetration of UV radiation in order to catalyze factor crosslinking. Previously Martin et al. reported an estimated patterning depth of 300 μm into the scaffold [30]. However, it was not clear whether this estimate was based upon visualization or patterning limits. In order to determine the true patterning depth, thick CG scaffolds (1.5 mm thick) were first photopatterned, then transverse cross sections analyzed via fluorescence microscopy (Figure 1B). This approach revealed a reproducible patterning depth of
500 μm, which is deeper than previously reported. However, patterning depth varied with scaffold orientation. When the CG suspension is freeze-dried to create the scaffold structure the side of the suspension exposed to the freeze drier (‘air side’) cools via convective cooling forming a thin, denser scaffold layer [6]. Comparatively, the CG suspension immediately adjacent to the mold (‘pan side’) cools via conductive cooling and lacks a densified region. Qualitatively, scaffold patterning depth appears to be reduced on the ‘air side’ due to the dense collagen layer, establishing that patterning depths are greater for the ‘pan side’ (data not shown).

Further, we examined the mechanical and compositional properties of the CG scaffold in response to BP-photopatterning (Figure 2). Given the known impact of the mechanical properties of both a biomaterial substrate [45, 46] or 3D scaffold [5, 47] on cell behavior, we wanted to confirm that BP-photoimmobilization could modify growth factor attachment orthogonally from mechanics. Further, maintaining the structural integrity of the collagen fibrils is a critical aspect of the bioactivity of CG scaffolds [2, 48, 49]. Notably, previous investigations suggested that there was no significant change in scaffold mechanical properties in response to the chemical process of attaching benzophenone to the scaffold [30]. However, while this study compared properties of the scaffold during all stages of functionalizing the BP to the scaffold surface, it did not consider the impact of UV exposure. Here, tensile testing showed that there was no significant change in the elastic moduli of benzophenone-functionalized scaffolds when exposed to UV light (Figure 2) even at considerably longer exposure times than used to create biomolecular patterns (60 vs. 20 minutes). Similarly, no significant differences were observed in
scaffold composition as a function of UV exposure (Figure 2B). All variants display a broad peak at $2\theta = 20^\circ$ representing the characteristic interchain spacing of the collagen triple helix [41]. No sign of collagen denaturation (loss of the broad collagen peak) was observed for any of the samples. The increase in peak height for the CG+UV, CG-BP, and CG-BP+UV variants is indicative of increased collagen crystallinity relative to the unmodified, likely due a crosslinking effect from DMF and UV exposure, consistent with previous findings [30]. New peaks that emerge are also likely associated with minor crosslinking events. However, these effects were not sufficient to significantly impact scaffold tensile modulus (Figure 2A). Together, these results suggest the ability to create defined lateral patterns of factors across the CG scaffold without impacting structural or mechanical properties.

We examined whether selective patterning of VEGF to the scaffold surface could drive functional changes in HUVECs. Previously, Segura et al. demonstrated immobilized VEGF on two-dimensional was sufficient to induce VEGF receptor activation on human umbilical vein endothelial cells [21, 22, 50], while Radisic et al. showed covalent immobilization of VEGF within a model collagen matrix improved penetration, proliferation, and bioactivity of and H5V and D4T endothelial cells [23-25]. The potential for BP-photoimmobilization to decouple VEGF-attachment and scaffold crosslinking spurred our investigation. HUVECs were starved for 6 hours before seeding into the scaffold then were kept in minimal media conditions to explore the potential for photopatterned VEGF to improve cell bioactivity over an 18 hour time period. HUVECs seeded onto scaffolds that did not contain VEGF remained in a mostly quiescent state (Figure 3A), with little observed elongation and minimal cell-cell contact indicative of activated
HUVECs [42]. Conversely, BP-mediated photoimmobilization (ubiquitous) of VEGF to the CG scaffold induced a significant change in cell morphology (Figure 3B). HUVECs were more elongated and increased cell-cell contact was observed. Features such as branching, sprouting, network formation and polygon formation are clearly visible. Further, cross sectional images of the cell laden scaffolds demonstrated that HUVECs migrate deeper into scaffolds in response to photoimmobilized VEGF (Figure 4). Interestingly, the porous structure of the CG scaffolds may also contribute to cell alignment into larger organized structures [17]. While manipulation of the microstructural features of the scaffold (pore size, shape) has previously been demonstrated to optimized cell attachment and tissue-specific morphologies [3, 7, 51, 52], such manipulations have not been explored in the context of HUVEC bioactivity, but are expected to contribute significantly to future optimization of CG biomaterials in order to enhance HUVEC bioactivity.

Lastly, we were curious whether spatial-organization of immobilized VEGF could impact HUVEC bioactivity. While gradients of growth factors have been described as being able to impact endothelial cell activity [25], BP-photolithography has the potential to create both gradients and discrete patterns [29, 30]. Here, CG scaffolds were patterning with VEGF in a squares motif. Low magnification fluorescent images show that in both the cases of no VEGF and patterned VEGF, HUVECs often form islands on the surface of the scaffold (Figure 5). However, higher magnification assessment demonstrated significant morphological differences as a function of immobilized VEGF. Notably, in the absence of VEGF (Figure 5b), HUVECs showed quiescent morphology with little organization, while the presence of photoimmobilized VEGF (Figure 5A)
encouraged cell elongation and the beginning stages of vessel-like network formation. These results suggest a paradigm for using on and off pattern cues to guide long term bioactivity of HUVECs within a CG biomaterial. A limitation of this technique is the laser penetration through the scaffold, limiting the depth of photopatterning to approximately 500\(\mu\)m. However, if thicker biomaterials are required, multiple 500 \(\mu\)m thick scaffolds could be individually patterned and then assembled into a composite, multi-layered construct. Given our capacity to generate biomaterials containing spatially-graded structural and biochemical properties [53, 54] and to investigate the combined effects of multiple biomolecular signals on cell bioactivity [12, 39], future studies integrating multiple biochemical signals (e.g., growth, phenotype) will be critical for developing biomaterial tools sufficient to enhance angiogenesis.

3.8. Conclusions and Future Work

In this paper, we show that benzophenone mediated photopatterning is a promising method to create biochemically modified CG scaffolds with spatial control over factor presentation. The exposure to UV in the presence of BP is not detrimental to the scaffolds mechanical properties or the structural organization of collagen fibrils. Importantly for future applications of this methodology, we showed that CG scaffolds can be functionalized with growth factors in a spatially controllable manner and elicit a functional cell response. As proof of principle, HUVECs were directed towards a more activated phenotype with photopatterned VEGF. Given the potential for BP approaches to create multiple and even overlapping biomolecular patterns, there is the potential to use this technique to pattern multiple growth factors across a single scaffold to create complex instructive signals within a three-dimensional biomaterial.
**Figure 3.1.** BP photopatterning of VEGF onto CG scaffolds. (A) The surface of a CG scaffold patterned with stripes of VEGF (100µm stripes; 400 µm spacing). Line scan showing on:off pattern intensities shown below. (B) Cross section of CG scaffold showing depth of penetration of VEGF stripes. Line scan showing on pattern intensities (black), on pattern intensities linear fit (blue), and average background signal (red) shown below. (C) Amount of VEGF bound to the benzophenone decorated CG scaffold either with (+UV; specific attachment) or without (-UV; non-specific fouling) UV exposure. *: p < 0.05.
Figure 3.2. BP photoimmobilization does not impact scaffold mechanical properties. (A) Elastic modulus of CG scaffolds at all stages of processing: CG scaffold alone (CG), UV exposed CG scaffolds (CG+UV), DMF treated CG scaffolds (DMF), UV exposed DMF-treated CG scaffolds (DMF+UV), benzophenone-coasted CG scaffolds (CG-BP), and UV exposed BP-modified scaffolds (CG-BP+UV). No significant change in modulus was observed. (B) XRD spectra from CG, CG+UV, CG-BP, and CG-BP+UV scaffolds show a broad peak at 20° characteristic of the interchain spacing of the collagen triple helix remains throughout processing.
**Figure 3.3.** Photoimmobilized VEGF leads to an activated HUVEC cell morphology. (A) Representative images of TRITC-phalloidin (actin, green) and DAPI (nucleus, blue) stained HUVECs seeded onto CG scaffolds containing benzophenone photoimmobilized VEGF exhibit an activated (spread) morphology. (B) Representative images of HUVECs seeded into scaffolds without photoimmobilized VEGF reveal cells remain in quiescent state with little elongation, branching, or cell-cell contact. Images acquired 18 hours post-seeding. (C) Actin spreading per cell for plain scaffolds and scaffolds containing photoimmobilized VEGF. *: p < 0.05.
Figure 3.4. Photoimmobilized VEGF leads to greater HUVEC scaffold penetration depth. Representative cross section of the pan side of CG scaffolds reveal differences in HUVEC penetration for (A) benzophenone immobilized VEGF versus (B) unmodified CG scaffold. Dashed lines indicate the edges of the scaffold. (C) HUVEC penetration was significantly greater with VEGF photoimmobilization. *: $p < 0.05$. Images acquired 18 hours post-seeding.
Figure 3.5. Photopatterned VEGF leads to spatially driven phenotypical differences in HUVEC cells. (A) Representative images of HUVECs seeded onto CG patterned with VEGF (blue) in a square motif imaged with both a 10x objective (left) and a 63x oil objective (right). Cells in contact with square islands of VEGF exhibit branching, elongation, and increased cell-cell contact. (B) Representative images of HUVECs seeded onto an unmodified CG scaffold imaged with both a 10x objective (left) and a 63x objective (right). HUVECs remained clumped and do not exhibit activated morphology. Images acquired 18 hours post-seeding.


Chapter 4. Spatially-directed osteochondral differentiation of mesenchymal stem cells using bilayered collagen scaffolds

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4.1. Introduction

Osteochondral defects can develop a variety of ways: from trauma related injuries to the natural degradation over time\cite{1}. As the baby boomer generation ages and the overall average life expectancy rises, so does the number of cases of age related osteochondral degradation, termed osteoarthritis. As of 2008, there were over 20 million Americans suffering from osteoarthritis, a number that is expected to double by 2020\cite{2}. The most common clinical repair strategies include bone marrow stimulation and allograft transplantation. Bone marrow stimulation, while demonstrating marked success, only works at as palliative treatment rather than stimulating full regeneration\cite{3}. Allograft transplantation introduces the risks of immune rejection and disease transmission and requires a suitable donor to be located. Thus, there is a pressing need to recapitulate the complex mechanical, biomolecular, and cellular characteristics of the osteochondral interface into a suitable biomaterial substitute. This complexity is further
compounded by the limited self-renewal capacity of native cartilage tissue. There has been much work on engineering mimics of either cartilage or bone independently. Some commonly used materials include PLGA, PCLA, hydroxyapatite, hyaluronic acid, and collagen [4-9]. However, single phase scaffolds do not effectively model dynamic or multi-tissue targets. Many researchers have begun to investigate the use of biphasic scaffolds systems with a cartilage mimic component and a bone mimic component as a suitable osteochondral substitute[10]. Chen et al prepared a biphasic scaffold composed of a low modulus collagen region and a higher modulus PLGA region[11]. The researchers used this construct to regenerate an osteochondral defect in the knee of a 1 year old beagle dog. Dormer et al approached the biphasic scaffold challenge from another angle. Rather than concentrating on independent stiffness regimes, the researchers created protein-loaded microsphere based scaffolds to create a gradient of lineage specific growth factors[12]. It is likely that a combination of mechanical stimuli and biomolecular factors is the promising direction for osteochondral research. To this end, Castro and Obrien developed a novel material composed of two biocompatible polymers, a poly(ethylene glycol) (PEG) hydrogel cartilage-like layer and a poly(caprolactone) (PCL)-based bone-like layer, containing tissue-specific growth factor loaded microspheres[13]. Here we detail our development of stratified freeze-dried collagen scaffolds with independent compartments for the dual differentiation of mesenchymal stem cells towards bone or cartilage lineages via both stiffness and biochemical cues.

Collagen is the most abundant extracellular matrix (ECM) protein and a popular platform for tissue regeneration [14]. Collagen scaffolds prepared via freeze drying have tunable mechanical,
structural, and biochemical properties that can be optimized to facilitate a variety of biomedical applications [15-19]. Previous research has investigated the use of chemical crosslinking, photochemical modification, and proteoglycan content to enhance cell activity [20-23].

Homogeneous freeze dried collagen scaffolds have previously been shown to promote MSCs towards bone, tendon, and cartilage lineages. Caliari \textit{et al} investigated the roles of scaffold structure in combination with soluble biomolecule media supplementation [24]. It was determined that mineralized collagen scaffolds in conjugation with osteogenic media induce MSCs towards osteogenic differentiation. Conversely, chondrogenic media containing TGF-β in conjunction with lower density scaffolds pushes MSCs towards a chondrogenic lineage. However, when chondrogenic and osteogenic medias are mixed it was found that the lower density, non-mineralized scaffolds promoted an osteogenic lineage while the higher density non-mineralized scaffolds induced a chondrogenic response[25]. Clearly, the scaffold structure (density, stiffness, mineralization, etc...) and biochemical culture cues play a key role in stem cell behavior. There has yet to be a platform that combines the facile and tunable fabrication of collagen scaffolds with biomolecular cues to promote spatially controlled multi-linage stem cell differentiation in a single biomaterial construct.

Other researchers have investigated the viability of bilayered collagen scaffolds for osteochondral reconstruction. Levingstone \textit{et al} developed a layered scaffold via a method they termed “iterate-freeze drying[26].” Their osteochondral scaffold was shown to have a seamless layer
structure and allowed cell attachment. There was not, however, a demonstration of instructing cell behavior independently in each compartment.

Our work leverages collagen scaffold technologies, spatially defined biomolecular cues, and mesenchymal stem cells to show a single scaffold containing two compartments that independently guide stem cell behavior.

4.2. Experimental

4.2.1. Fabrication of collagen scaffolds

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Two different collagen slurries, a low density (LD) and a high density (HD) were prepared via a previously established method[27]. High density, mineralized collagen slurry was produced from collagen (1.9 w/v%) with calcium salts (Ca(OH)$_2$, Ca(NO$_3$)$_2$ · 4H$_2$O). Low density slurry was composed of 0.5% collagen type I microfibrillar collagen (Collagen Matrix, Oakland, NJ) with hyaluronic acid. Low density slurry was also supplemented with TGF-β3 to create a 3rd slurry type (LD+). All slurry was degassed prior to freeze drying. One component scaffolds were made from freeze drying slurry either in single scaffold molds or as a sheet before punching out individual scaffolds with biopsy bunches. Two-component layered scaffolds were freeze dried in single scaffold molds. The tips of 1000 ul pipets were cut to increase the area of the liquid delivery at
the tip of the pipette tip. The high density slurry was pipetted into the bottom of each well. The well plate was agitated to settle the slurry into an even layer. The LD slurry was pipetted directly on top of the HD slurry. The scaffolds were immediately freeze dried to a final temperature of -40 degrees Celsius 1°C/min.

Scaffolds were hydrated briefly (less than 20 minutes) in ethanol prior to another brief hydration in sterile 1xPBS. The scaffolds were crosslinked via 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) crosslinking with the ratio of 5:2:1 (COOH:EDC:NHS), in sterile 1xPBS, pH=7.4) for 30 minutes. The scaffolds were then stored in sterile 1XPBS until time of use. Prior to use in cell experiments the scaffolds were transferred to the appropriate cell media and warmed to 37°C.

4.2.2. Mesenchymal stem cells grown on collagen scaffolds

Single donor human mesenchymal stem cells (Lonza, Switzerland) were passaged according to manufacturer suggestions. Cells were fed twice a week and used at passage 4. Warm, cell media laden scaffolds were placed in pairs in a 24 well plate. MSCs were seeded on both sides of collagen scaffolds at a density of 7.5 x 10^5 cells/ side using the static seeding method[28]. The cells were allowed to attach for 30 minutes before 1 ml of media was added to each well. MSCs were then grown for 21 days at 37 degrees Celsius. The cell laden scaffolds received fresh media twice a week. MSCs were cultured on the scaffolds in either Growth Basal Media (GBM, Lonza,
Switzerland) or a 1:1 mixture of Osteogenic induction media (Lonza, Switzerland) and Chondrogenic media supplemented with TGF-β3 (Lonza, Switzerland).

### 4.2.3. Alkaline Phosphatase Assay

An ALP assay kit (Invitrogen, Waltham, MA) was used to assess the ALP activity in cell media due to MSC ALP secretion. Briefly, cell media was collected from each well and stored at -80 degrees Celsius. The ALP assay buffer as well as cell media samples were brought to room temperature prior to the assay. The kit was used as directed. Briefly, p-nitrophenyl phosphate (pNPP), a phosphate substrate, turns yellow (λ=405nm) when dephosphorylated by ALP. Standard concentrations of ALP were prepared in order to quantify unknown ALP activity in cell media samples. Cell media containing the assay Stop Solution was used as a background control. Optical density was measured with a plate reader (BioTek Instruments, Inc., Winooski, VT).

### 4.2.4. RNA isolation, reverse transcriptase, and real-time polymerase chain reaction

Isolation of RNA from MSC-seeded scaffold was performed at days 10 and 21 using the RNeasy Mini Kit (Qiagen, Valencia, CA). Scaffolds were added to RLT lysis buffer supplemented with 1% β mercaptoethanol and digested for at least 5 minutes with periodic vortexing. RNA was extracted from the lysate according to manufacturer instructions. RNA was then converted to cDNA using Quantitect Reverse Transcriptase kit (Qiagen) and a Thermal Cycler (Bio-rad T-100, Hercules, CA). Real-time polymerase chain reaction (PCR) reactions were conducted in triplicate using the
Quantitect SYBR Green PCR kit (Qiagen) in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Primer sequences were taken from the literature[29].

4.2.5. Visualization of MSCs on collagen scaffolds

After 21 days of culture, MSC-seeded scaffolds were fixed in 10% formalin. Scaffolds were then stained to visualize the actin cytoskeleton and nucleus. F-actin was stained with phalloidin-TRITC (Life Technologies, Waltham, MA) overnight with DAPI (Life Technologies, Waltham, MA) counterstaining of the nuclei. Stained scaffolds were stored in the dark at 4°C until imaging. Confocal imaging was performed on a Zeiss LSM 710 multiphoton confocal microscope (20x objective) equipped with a Spectraphysics Mai-Tai Ti-Sapphire laser.

4.2.6. Scaffold sectioning

Scaffolds were embedded with Tissue Tek O.C.T Compound (Sakura Fintek) with the top/bottom surfaces of the scaffold disk oriented perpendicular to the base of the mold. 150 µm thick cross sections of the scaffold were generated via a cryostat (Leica, CM3050S cryostat), mounted on a glass coverslip, and allowed to dry prior staining analysis.

4.2.7. Alcian blue and alizarin red staining

To assess the amount of GAG deposition, scaffold sections were stained with 3% Alcian blue in water (pH 2.5). The sections were stained for 10 minutes and monitored under the microscope.
After staining the sections were carefully rinsed with water. A separate set of sections were stained for calcium deposition with 2% Alizarin Red in water (pH 2.6) for 30 seconds and gently rinsed with water. Stained sections were imaged with a light microscope (Zeiss 40C Invertiskop, Carl Zeiss Inc.).

4.2.8. Scanning electron microscopy imaging

Larger LD/HD scaffolds (diameter = 11.4mm) were fabricated according to the above protocol. Cross-sectional slices were obtained via slicing with a razorblade. The sample was fixed to an aluminum sample stud with carbon tape. Scanning electron micrographs were obtained using a Hitachi S-4700 field emission gun scanning electron microscope (SEM) after sputter coating the sample with roughly 7.5 nm of a Pd/Au alloy. The SEM was run in normal resolution, low magnification mode with a 5 kV accelerating voltage, 10 µA extracting current, and 12 mm working distance using combined backscattered and secondary electron emission signals.

4.2.9. Compression testing

Large HD, LD, and LD/HD scaffolds (diameter = 11.4mm) were fabricated according to the above protocol. Uniaxial compression tests were performed on dry scaffold samples at a rate of 1 mm/min using a mechanical testing system (Insight, MTS Systems). Elastic modulus was determined via linear regression of the linear portion of the stress-strain curve.
4.3. Multilayered scaffolds fabrication

We have successfully developed scaffolds presenting layers of biomolecular variability. As proof of principle, stratified scaffolds containing layers supplemented with either streptavidin Alexafluor 647 (Invitrogen, Waltham, MA) or streptavidin Alexafluor 568 (Invitrogen, Waltham, MA). Two different layering techniques were investigated. First, a layer of slurry substituted with streptavidin 647 was pipetted into the bottom of the cylindrical mold. The bottom layer was allowed to freeze in a -80°C freezer until hard. Then, the next layer of slurry, containing the other fluorophore was pipetted on top of the initial frozen layer. A second layered technique was executed concurrently which involved pipetting a second type of slurry directly on top of an unfrozen slurry layer. Both types of layered slurry constructs were freeze dried to make a porous, layered collagen scaffold. The scaffold for which the first layer was frozen suffered from delamination between the layers. There was much less delamination observed for the scaffolds prepared without an intermediate layer freezing step. A line scan of each fluorophore demonstrates a continuous structure with chemically different layers joined together by a mixed, opposing gradient interface, reminiscent of in vivo tissue. Thus, it was determined that the latter method would be used for subsequent biological studies.

4.4. SEM imaging

SEM images were taken of a cross-section of a dry low density/ high density (LD/HD) layered scaffold. Images were collected of the HD region, the LD region, and the interface between the two. A differential pore structure is clearly visible between the LD and HD regions.
4.5. Mechanical testing: compression testing

Compression testing was conducted for dry low density, high density, and low density/ high density layered scaffolds. The elastic moduli of each was extracted from their associated stress-strain curves. HD scaffolds had the elastic modulus of 382.29 kPa and LD scaffolds had the elastic modulus of 15.15 kPa. When HD and LD are layered, the composition elastic modulus was determined to be 45.591 kPa. Error bars are reported as standard error of the mean.

4.6. ALP activity

ALP activity for mesenchymal stem cells grown on LD, LD+, and HD scaffolds grown in either growth basal medium or 1:1 chondrogenic : osteogenic media was assessed after 10 and 21 days of growth. At day 10 secreted ALP activity was significantly higher for MSCs grown on either LD or LD+ grown in GBM compared to all other scaffold and media conditions. However, by day 21 the HD scaffolds had around the same ALP activity as the low density scaffolds both with and without the addition of TGF-β3. All scaffolds grown in 1:1 chondrogenic: osteogenic media at both 10 and 21 days exhibited depressed ALP activity in comparison to other conditions.

4.7. Polymerase chain reaction

RNA was extracted from MSCs grown on LD, LD+ and HD scaffolds and was converted to cDNA before eventual qPCR. Primers were chosen from the literature and have been show to indicate
changes in MSC phenotype during differentiation. All changes in gene expression are given relative to MSCs grown on LD scaffolds in GBM. At day 10 the relative ALP gene expression of for HD (OC) scaffolds was higher than both LD (OC) and L+ (OC). By day 21, HD (OC) has higher relative gene expression than all the LD samples. The HD (GBM) samples had higher average ALP expression levels, but varied greatly and could not therefore be deemed statistically significant. At day 10 The LD+ (OC) had a statistically significant greater relative gene expression than LD+ (GBM) for the RUNX2 gene. By day 21 there were no significant differences between any conditions for RUNX2. LD (OC) was had significantly higher ACAN gene expression than LD+ (GBM) at day 10, but by day 21 HD (OC) had higher relative ACAN expression than all the conditions except HD (GBM). Once again, HD(GBM) had higher average relative gene expressions but showed such variability that it could not be determined statistically significant. At day 10, LD+ (OC) had a significantly higher collagen I gene expression than every condition other than HD (OC). PCR data presented, but not specifically mentioned was determined to be significantly insignificant.

4.8. Fluorescent imaging of MSC cell morphology

MSCs were grown on collagen scaffolds for 21 days before fixation and subsequent staining. Cell morphology was assessed via F-actin staining with DAPI nuclear backstaining. Cells grown on either low density scaffolds and high density mineralized scaffolds in either chondrogenic : osteogenic media or basal growth media all displayed low cell-cell contact and low cell spreading. On the other hand the cells cultured on LD scaffolds containing TGF-β3 in GBM media showed
much larger cell-cell contact and spreading. Furthermore, when LD scaffolds containing TGF-β3 that were cultured in osteogenic: chondrogenic media they were even more spread and had even greater cell-cell contact.

4.9. Alizarin red and alcian blue staining

Bilayered scaffolds were sectioned into 150um thick slices using a cryostat. The fragile slices were collected by melting onto room temperature equilibrated glass slides. As evident in Figure X, many of the bilayered scaffolds became delaminated during the slicing process. The slices were allowed to dry to ensure adherence to the slides during staining. Upon drying, a drop of alizarin red stain was applied to the cross section. The drop was allowed to sit for several minutes while being monitored under the microscope, the section was rinsed carefully with water before being imaged with a visible light microscope. The scaffolds culture in growth basal medium exhibited much greater mineral staining than those grown in chondrogenic:osteogenic media. For all the conditions, except the LD+ scaffolds in O:C media, the mineralization is primarily localized in the high density compartments. The TGF+ scaffold has a much more evenly distributed calcium staining signal.

Concurrently, bilayered collagen scaffolds slices were staining with Alcian blue for GAG deposition. Interestingly, the LD scaffolds that do not contain TGF-β3 also present greater GAG staining in the high density mineralized region. On the other hand, while still exhibiting high Alcian blue staining in the HD layer, the LD+/HD scaffold had upregulated GAG deposition staining
in the low density layer. The scaffolds containing immobilized TGF-β3 and were cultured in O:C media do not have the same dark GAG staining in the higher density region.

4.10. Discussion

With an aging baby boomer generation, the number of osteochondral defects due to natural aging related degradation is on the rise. Current medical intervention often merely seeks to minimize associated painful symptoms in the short to medium term, but no technique has been shown to fully regenerate and repair an osteochondral injury[10]. This is largely due to the complexity of the dynamic multi-tissue interface and the inability for cartilage self-repair. A successful osteochondral implant must contain both a hard tissue region for bone connected to a soft tissue region for cartilage. To meet this need, tissue engineers are shifting focus from singular component scaffolds to sophisticated families of stratified materials. We leverage our tunable collagen scaffold fabrication technology to create a set of stratified structures with two independent connected compartments able to promote MSCs towards two different lineages.

Collagen scaffolds have several attributes that make them an ideal platform for tissue engineering applications: biocompatibility, facile fabrication, and modalities for further modification[15, 17, 27]. Previously, our group used the photolithographic moiety, benzophenone, to create cell responsive biomolecular patterns for vasculogenesis and bone-tendon applications [22, 23]. However, it was determined that the light generated patterns were unable to penetrate and modify more than 500µm into collagen scaffolds. This technique proved
ineffective for any applications that sought to create biomolecular heterogeneity at depths greater than 500um. Instead, heterogeneity was introduced prior to freeze drying. Three different collagen slurries were prepared: a low density slurry (LD), a high density mineralized slurry (HD), and a low density slurry supplemented with TGF-β3 (LD+). By carefully pipetting the slurry iteratively on top of one another, we created single stratified scaffolds containing both a high density bone mimic and low density cartilage mimic. Two types of bilayered scaffolds were investigated: LD/HD scaffolds and LD+/HD scaffolds. It was shown by Banks et al that mechanical stiffness of collagen was the single greatest driver of MSC differentiation towards osteogenesis. However, chondrogenesis is primarily initiated by the transforming growth factor-beta (TGF-β) superfamily, the most investigated biologically active substance investigated in the field on cartilage tissue engineering. TGF-β is a 25kDa polypeptide homodimer[30]. Treatment with the isoform TGF-β3, promotes MSC to cartilaginous ECM production[31]. Thus, the effects of adding TGF-β3 to the LD region of our layered collagen scaffold were investigated.

The fabrication of bilayered, porous collagen scaffolds was achieved two different ways. The first method involved the freezing the bottom layer prior to addition of the next layer of slurry. The second method involved pipetting each layer subsequently at room temperature. Each method was able to successfully create bilayered scaffolds, but when one layer was frozen before the other, this resulted in a high amount of delamination between the layers. This is due to a small amount of water freezing first at the outside edges of the slurry, creating a thin layer of ice. This ice inhibits diffusion between the two layers of slurry. Layering slurry at room temperature,
however, allows the two slurries to mix slightly at their interface. As is seen in Figure X the fluorescent signal of each layer component meets the other in an opposing gradient motif.

Our osteochondral stratified scaffold contains both a “bone” region and a “cartilage region.” The different layers were qualitatively discernable with the naked eye with the HD layer appearing white and the LD layer appearing off-white. The elastic moduli of LD, HD and LD/HD scaffolds were obtained. Non-surprisingly, HD scaffolds had the highest elastic modulus at an average of 382.29kPa while LD scaffolds had a much lower modulus of only 15.15kPa. The complete layered scaffold had an elastic modulus of 45.59 kPa, which was in between the moduli of its individual components but much closer to the LD scaffold elastic modulus.

To prove that each later operated independently to induce a specific, desired cell behavior, we created single component scaffolds to model the LD, LD+ and the HD layers. As the interface between the layers is gradated, it would be very difficult to separate each layer distinctly for analysis.

Cell morphology on each scaffold type was investigated via F-actin staining with DAPI nuclear counterstaining. MSCs grown on low density scaffolds containing immobilized TGF-β3 displayed the most different morphological differences compared to all the other scaffold conditions. Both the HD and the LD scaffolds induced a rounded cell morphology. Conversely, in the presence of
immobilized TGF-β3, the cells exhibited a polygonal cell morphology with greater cell-cell interactions. This effect is amplified when comparing the TGF-β3 scaffolds cultured in growth basal medium versus those cultured in mixed 1:1 osteogenic:chondrogenic media. The MSCs cultured in the presence of OC media displayed even high polygonal cell spreading and cell density. This makes sense as OC media contains soluble TGF-β3 and is amplifying the effects observed by immobilized TGF-β3 alone.

ALP analysis of cell secretions further highlights the independent cell behavior induced by different scaffold types and different culture media. At day 10 all scaffold types cultured in OC media had low ALP activity. Both LD and LD+ scaffolds cultured in GBM had significantly higher ALP activity than the high density scaffolds. However, by day 22 all the scaffolds cultured in GBM media had similar ALP activities that were significantly higher than all of the MSCs cultured in OC media.

Analysis of homogenous collagen scaffolds show their capabilities to dictate MSC behavior via structural and chemical changes to the scaffolds and the media. For these materials to be successful candidates for interface engineering, it must be shown that when put together the layers can independently effect cell lineage outcomes. MSCs were cultured on layered collagen scaffolds for 21 days in either GBM or OC before subsequent fixation and cryosectioning. The slices were stained with either Alizarin red for calcium deposition (osteogenesis marker) or Alcian blue for glycosaminoglycan deposition (chondrogenesis marker). The layered scaffolds lacking
TGF-β3 (LD/HD) exhibited the most intense alizarin red staining, located in the HD layer. The LD/HD scaffolds cultured in GBM media had stronger calcium staining than the LD/HD scaffolds cultured in OC media. Surprisingly, the Alcian blue staining on the LD/HD scaffolds is also primarily located in the highest density layer with much lighter blue staining in the lower density region. The alizarin red staining of LD+/HD also shows high calcium staining in the high density region. Alcian blue staining shows high GAG deposition in both the high density compartment but also in the low density compartment. This suggests that the presence of immobilized TGF-β3 induced MSCs towards the chondrogenesis lineage in a spatially defined manner.

We have illustrated the successful implementation of collagen slurry layering to create a series of stratified collagen scaffolds that instruct MSCs towards two different lineages in a spatially defined manner on a single substrate.

4.11. Conclusions

Due to the rising prevalence of osteochondral injury and degradation, there has been much research on designing a construct to facilitate complete repair and regeneration. However, the complex and dynamic nature of the interface combined with cartilage’s low self-repair capabilities makes this a particularly challenging problem. We have successfully designed a sophisticated set of bilayered scaffolds with a high density, mineralized “bone” mimic compartment and a low density “cartilage” mimic compartment that come together in an opposing gradient motif. This soft/hard stratified scaffold was seeded with mesenchymal stem
and after a period of 21 days it was shown to induce differential cell behavior in a spatially defined manner. These scaffolds are promising candidates for \textit{in vivo} osteochondral repair studies.
Figure 4.1. (top, left) Fluorescent image of scaffold layered at room temperature with corresponding line scans of fluorescent intensity. Scale bar = 1mm. (bottom, left) Fluorescent image of scaffold composed of pre-frozen layers. Scale bar = 500µm. (top, right) An SEM image of an DH/LD scaffold interfacial region. (bottom, right) Elastic modulus of HD, LD, and LD/HD scaffolds as determined by compression testing.
**Figure 4.2.** Cell morphology (nucleus, blue) (actin, green) of MSCs seeded onto LD, HD, and LD+ scaffolds at 10 days.
Figure 4.3. Alkaline phosphatase activity of MSCs seeded onto LD, HD, and LD+ scaffolds at 10 and 21 days. Error bars = SEM
Figure 4.4. Imaging of layered scaffold cross-section slices. Scaffolds oriented such that low density layer is on the top. Calcium deposition was stained with Alizarin red (red) and GAG deposition was stained with Alcian blue (blue). Layered scaffolds were either cultured in GBM media (top) or OC media (bottom) Scale bars = 650 µm.
Figure 4.5. Reverse transcriptase polymerase chain reaction of MSCs grown on collagen scaffolds for 10 days. Error bars reported as SEM.
Figure 4.6. Reverse transcriptase polymerase chain reaction of MSCs grown on collagen scaffolds for 21 days. Error bars reported as SEM.
References


Chapter 5. Two-photon photolithography of collagen-glycosaminoglycan scaffolds
functionalized with benzophenone.

5.1. Introduction

The extracellular matrix (ECM), a conglomerate of collagens, glycosaminoglycans, proteoglycans, non-collagenous matrix proteins, and growth factors, works in conjunction with cell-cell signaling to dictate each individual cell’s phenotypic development and tissue specific roles [1, 2]. The recapitulation of these specialized instructions is the fundamental task of tissue engineers towards the fabrication of fully functioning scaffolds for organ/tissue replacement or regeneration. There has been much fruitful effort towards the design of a suitable biomaterial. Ideally such a material would satisfy both the physical needs of the cell, such as texture and stiffness, as well as the biochemical needs of the cells. There has been a lot of work regarding the design and fabrication of scaffolding materials for tissue engineering applications. Popular fabrication techniques include: soft lithography, 3D printing, porogen leaching, electrospraying, and lyophilization[3]. Equally as important as the physical scaffold structure is the ability to decorate said structure with the appropriate tissue specific biochemical cues. In vivo, there are two modes in which biochemicals are made available to cells: in the surrounding solution or anchored to the ECM. Soluble cues are easy to model in vitro, as they can be added directly into cell culture media. Additionally, ubiquitous immobilization biomolecular cues can often achieved via facile conjugation chemistry, physisorption, or pre-fabrication incorporation to a slurry or hydrogel solution [4, 5]. The more challenging task, is the recapitulation the heterogeneous, patterns and gradients of biomolecular cues that decorate the ECM. To this end, several
techniques have been employed to introduce biomolecular features with micro-scale resolution
to biomaterials including sequential photolithography, soft lithography patterning, and
microfluidic patterning [6, 7]. As previously discussed, our group has capitalized on the photo-
reactive moiety, benzophenone, to biochemically modify the surface of planar glass slides,
corrugated glass slides, 2D collagen membranes and 3D collagen scaffolds[8-11]. BP is
immobilized to collagen scaffolds via a free lysine side chain. When exposed to UV light, BP will
undergo an insertion reaction with any proximal biomolecule. Forcing the light to pass through a
photomask prior to scaffold irradiation, we gain spatial control of the target biomolecule
attachment. The immobilization density is the greatest on the surface of the scaffold and forms
a gradient of decreasing density into the scaffold[12]. The limited depth patterning capabilities
can be attributed to light scattering in collagen which inversely proportional to the wavelength
of incident light [13, 14]. Thus, here I describe my attempts to excite the benzophenone with a
longer wavelength two photon infrared laser rather than the higher energy single photon
uv light laser

For a multiphoton interaction to occur, the light is pumped such that two or more photons meet
at the exact same spatial and temporal location[13]. When this occurs the photons combine to
create an event that has the energy of a photon half their original wavelength. Thus, it is possible
to selectively excite photo-responsive moieties exclusively where the photons meet, leaving all
the surrounding material un-excited by single photons of low energy. This technique affords
better spatial patterning in all three dimensions as well as minimizes photodamage or
photobleaching in surrounding areas. We seek to combine our BP patterning technologies with the excitation abilities of a two photon laser[15].

Currently, several groups employ two photon photolithography to modify materials for biological applications. The West group developed a collagenase sensitive poly (ethylene-glycol-co-peptide) diacrylate hydrogel that was decorated with cell adhesive ligand (RGDS) via 2 photon lithography[16]. The Shoichet group employed two photon lithography to heterogeneously modify hydrogels[17, 18]. Photoexposed agarose thiols formed disulfide bonds with proximal free cysteines on patterning targets. In subsequent work, they extended their patterning capabilities to multiple target patterning different via orthogonal binding pairs: barnase-barstar and streptavidin-biotin. Briefly, maleimide is conjugated to streptavidin (or barnase) which can then form disulfide bonds with the agarose thiols. A subsequent soak in a protein conjugated with biotin (or barstar) immobilizes the protein. A second target can be added using the other binding pair. The researchers report patterning 100um x 100um x 40um boxes at depths of 400um in the hydrogel.

Two photon patterning had been successfully reported in hydrogel systems, but yet to be shown in a porous scaffolds. We sought to demonstrate two photon photolithography on collagen-glyosaminoglycan scaffolds. As proof of principle, we started with two photon photopatterning on BP coated glass coverslips and collagen membranes. Patterning parameters such as excitation wavelength, power and number of exposures was investigated using collagen membranes. Finally, two photon patterning was conducted on 3D collagen-GAG scaffolds.
5.2. Experimental

5.2.1. Benzophenone functionalization of glass coverslips

Preparation of BP-modified glass substrates has been previously reported [8, 10, 11]. Briefly, glass coverslips were cleaned with Piranha solution (4:1 (v:v) concentrated H$_2$SO$_4$: 30% H$_2$O$_2$). Substrates were rinsed 3 times with water, once with ethanol, and dried under a stream of nitrogen. Coverslips were baked in an oven at 120 °C for 1 h. After cooling back to room temperature, the glass substrates were carefully positioned upright along the wall of a vacuum desiccator, with 100 μL of 3-(triethoxysilyl)butyl aldehyde (Gelest, Morrisville, PA) contained in the cap of an eppendorf tube placed in the center of the chamber. Vacuum was applied to the sealed chamber and vacuum deposition of silane onto the glass slides was allowed to occur for 2.5 h. Slides were again cured at 120 °C for 1 h, soaked in ethanol for 30 min, rinsed with ethanol, and dried under a stream of nitrogen. Successful silanization was confirmed via measurement of water contact angles (Ramê-Hart Goniometer, Netcong, NJ).

Slides were then incubated in the dark for 4 h at room temperature in the presence of 20 mM 4-benzoyl benzylamine hydrochloride (Matrix Scientific, Columbia, SC) and 200 mM NaCNBH$_3$ in a solution of 4:1 DMF:MeOH. The reaction was stopped via immersion in aldehyde-blocking buffer (0.1 M Tris, 200 mM ethanolamine, pH 7.0) for 1 h at room temperature. Slides were rinsed thoroughly with water, DMF, methanol, and ethanol, and then dried under a stream of nitrogen. The resulting BP-modified substrates were stored in a desiccator in the dark until further use.
5.2.2. Preparation of collagen slurry

Porous CG scaffolds were fabricated according to a previously established method [19, 20]. A 0.5% w/v% CG suspension was prepared by homogenizing type I microfibrillar collagen (Collagen Matrix, Oakland, NJ), chondroitin sulfate from shark cartilage (Sigma Aldrich, St. Louis, MO), and 0.5M acetic acid.

5.2.3. Preparation of collagen membranes

Collagen-glycosaminoglycan membranes were fabricated by allowing CG-GAG slurry to evaporate at ambient conditions overnight in a plastic Petri dish.

5.2.4. Preparation of collagen scaffolds

Degassed slurry was poured into aluminum pans and placed into the freeze dryer (VirTis Genesis, Gardiner, NY). The slurry was cooled to a final temperature of -40 degrees Celsius at a rate of 1.0 degree per minute. Ice crystals formed in the scaffold underwent sublimation by vacuum, leaving behind an interconnected pore structure throughout the collagen material. Scaffold discs were harvested from the porous sheet with 6mm biopsy punches.

5.2.5. Benzophenone functionalization of collagen substrates

A 20mM solution of benzophenone-4-isothiocyanate and 0.5M N,N diisopropylethlamine was prepared in DMF. Scaffolds disks were added to the BP solution at a density of 30 scaffolds per 10ml BP solution. The attachment of BP to the collagen was allowed proceeded for 48hrs, at
room temperature, in the dark, on top of a Belly Dancer™ undulating orbital shaker (Sigma-Aldrich, St. Louis, MO). After 48 hours, the scaffolds were washed repeatedly in DMF until no longer bright yellow. Scaffolds were hydrated in ethanol prior to final storage in sterile 1xPBS at 4°C.

5.2.6. Two photon patterning of 2D and 3D collagen materials

Lyophilized Concanavalin A (ConA) conjugated with biotin was reconstituted in sterile water to the recommended concentration of 5mg/ml and stored at -80 degrees Celsius until use. Scaffolds or membranes were subsequently soaked in 1ug/ml ConA-biotin for at least 1 hour prior to patterning. Protein soaked membranes or scaffolds were placed on a coverslip and mounted on to the confocal microscope stage. Visual light was used bring the microscope into focus onto the surface of the collagen. The focus was moved onto the scaffold to the desired patterning area. The scaffolds were patterned with the Mai Tai Titanium Sapphire two photon pulsed laser tuned to 720 nm. The effects of zoom, number of scans, and power with regard to patterning efficiency were all investigated.

5.2.7. Fluorescent visualization of biomolecular patterns within the CG scaffold

Patterned scaffolds were blocked in 1% BSA/PBS solution, then incubated overnight in the staining solution of streptavidin-Alexafluor647 at 4°C. Scaffolds were rinsed in PBS prior to imaging on a LSM 710 confocal microscope (Carl Zeiss Microimaging, GmbH, Germany). Pattern specificity was determined from images taken of the exposed scaffold surface. Image J Software
(NIH, Bethesda, MD) was used to determine to create line profiles of pattern intensity across patterning conditions.

5.3. Discussion

Here we describe our use of two photon patterning techniques to decorate a benzophenone functionalized collagen scaffold with a biomolecular target. It was hypothesized that a 2 photon excitation of benzophenone with infrared light will result in greater scaffold penetration compared to that of single photon UV laser patterning. In extension, the use of two photon lithography could be a useful technique to create complex three dimensional bimolecular patterns completely inside collagen-GAG scaffolds. As with single photon patterning, unreacted benzophenone relaxes back to its ground state and is available for further irradiation and conjugation. The opportunity for multiple component patterning is conserved going from a single photon excitation to a multiple photon excitation.

All two photon patterning was conducted on a Zeiss 710 inverted confocal microscope with a titanium:sapphire laser using the 20x objective. Patterning was first demonstrated on model linear substrates, glass coverslips functionalized with benzophenone as previously described. All protein patterning, rinsing and fluorescent staining was conducted in a non-removable adhesive chamber with both a solution inlet and outlet port. The two photon laser focal point was scanned in a user defined manner to “draw” shapes of immobilized protein onto the BP functionalized surface of a coverslip. After patterning, the coverslips were rinsed, fluorescently tagged and
imaged with the confocal microscope. The fluorescent images of the slides confirmed successful patterning of ConA-biotin onto the surface of benzophenone functionalized coverslips.

Our goal was to heterogeneously modify collagen scaffolds and not glass, so we quickly transitioned to a model planar collagen-GAG model system, non-porous membranes. Planar collagen surfaces lend themselves to a much easier image analysis than porous 3D scaffolds, due to the lacking complex pore structure. Patterning on the glass coverslips was conducted at 740nm, an initial proof-of-concept guess. Theoretically, 2 photon excitations are approximately, but not necessarily, double the single photon excitation wavelength[14]. BP single photon excitation occurs at 365nm, so a variety of wavelengths were investigated for optimum patterning efficiency as assessed via fluorescent intensity analysis of stained patterned regions. Successful patterning was observed for 720, 740, 760 and 780nm excitation, but the optimum patterning wavelength was determined to be 720nm. Exposure at this wavelength produced patterned regions with the highest protein immobilization as determined by fluorescence intensity as measured with ImageJ intensity analysis. The effects of laser power, multiple scans across the same region, and pixel dwell time were also evaluated. Non-surprisingly, it was found that increased laser power, slower scanning speed, and multiple exposures over the same pixel result in a higher immobilization density.

After optimization on collagen membranes, 2 photon patterning protocols were translated to 3D collagen scaffolds. Benzophenone functionalized collagen scaffolds were successfully patterned with ConA-biotin using a two photon excitation according to the objective, wavelength and laser
power regimes previously established. The pattern resolution was dictated by size of the pores which can be tuned during freeze drying to range anywhere from tens to hundreds of microns. All patterning trends observed for collagen-GAG membrane patterning were conserved when moving to the porous 3D platform. The highest patterning density is achieved at high powers and long exposure times that afford the opportunity for multiple BP excitation and relaxation cycles. Initial success of two photon patterning on the surface of the scaffolds was encouraging, but the ultimate goal of this endeavor was to increase our 3D patterning capabilities inside the scaffold. Using the “z stack” function, a series of 3D boxes were “drawn” with the laser focus several hundred microns into the benzophenone functionalized CG scaffold. To our dismay, patterning efficiency declined at a few hundred microns into the scaffold. Two major issues with 2 photon patterning of benzophenone functionalized collagen scaffolds were discovered. Benzophenone has an inherently low two photon cross section compared to typical two photon initiators\[13, 14\]. For small two photon cross sections, a large photon flux is required. At high photon fluxes, significant photo-degradation of CG-GAG scaffolds was observed. Collagen autofluorescence is also excited around 365nm. During patterning, the confocal microscope collects the auto-fluorescent signal, providing an image of the collagen scaffold pore and strut structure in real time. When subjected to high intensity two photon irradiation, the scaffold struts were observed to stretch and thin, fray and finally break apart. Eventually, holes the size and shape of the patterning regions would form into the scaffold. Moreover, confounding this issue is inability to visualize deep patterns. Limited by scattering, fluorescent detection of patterned targets deeper than 200 μm into the scaffold is not feasible. Considering the significant damage caused by high intensity two photon irradiation, combined with the imaging difficulties, it was determined that
two photon patterning would be not a robust or viable method for the heterogenous decoration of collagen scaffolds in the z direction.
**Figure 5.1.** Two photon patterning of Concanavlin A on a BP functionalized planar collagen-GAG membrane. Patterned regions are separated into three columns of two replicates representing varied duration of beam dwell time on each pixel. Each row represents a different percentage of total laser power used during patterning.
Figure 5.2. Two photon patterning of concanavalin A on a collagen-GAG membranes with the patterned circle regions being exposed multiple times as labeled on the figure.
**Figure 5.3.** Two photon patterning of Concanavalin A on collagen-GAG membranes patterned using two objectives on a confocal microscope, 10x and 20x. Below is the line scans associated with the various patterning regions.
Figure 5.4. Examples of two photon patterning of Concanavalin A onto the surfaces of collagen-GAG scaffolds.
References