I, Alex E. Bell, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering.

It is entitled:
Microscale Additive Manufacturing of Collagen Cell Culture Scaffolds

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Microscale Additive Manufacturing of Collagen Cell Culture Scaffolds

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By

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Abstract

Communication between cells and their environment is a key influencing factor on cell fate in tissues. Through integrin receptors, cells can sense and respond to structural features of the extracellular matrix. Signaling-factor receptors can detect gradient changes in concentration of a multitude of soluble ligands. Both mechanical and biochemical signals are presented to cells in defined spatial patterns. One of the most important sets of signals for developing or regenerating tissue are those guiding the formation of a vascular supply. Vascular development is crucial to recapitulation of further tissue function. Replicating these 3D patterns in vitro is a considerable challenge for the long-term goal of engineering functional tissue replacements.

Multiphoton crosslinking (MPC) is an additive manufacturing process using light-activated chemistry to produce crosslinking by the absorption of multiple photons. MPC is quadratically dependent on light intensity, allowing isolation of the reaction to the focal plane. With diffraction-limited optics, this provides 1 μm resolution. MPC has the potential to give unlimited 3D control of the structural and biochemical cues imparted by functional proteins and peptides at a sub-cellular scale. Potential benefits of MPC include both the ability to spatially isolate presentation of a given factor in a concentration-controlled manner and sequestering the factor to provide a sustained dose over an extended period of cell culture and tissue regeneration.

The MPC technique is essentially applicable to any protein, as several amino acids can react with an excited radical species. MPC of protein substrates has not yet been utilized toward functional tissue engineering applications. This is due to multiple factors, including the need for the substrate protein to be soluble, form a stable 3D structure, and have the unexposed portion readily removed. Thus, MPC has been limited in use primarily to highly soluble proteins such as
albumin, which is not effective as a tissue scaffold material. Patterning of functional proteins onto scaffolds using MPC has also been limited by the need for the unexposed materials to be removed from the scaffold following the patterning process. This has meant that the technique, thus far, is largely employed on synthetic hydrogels with peptide fragments.

The first specific aim of this dissertation was to expand the capabilities of MPC by developing methods to use relevant materials for scaffolds ultimately to be used in dermal wound healing. This aim established repeatable methods for 3D printing of type I collagen by MPC and the quantifiable modification thereof with proteins BSA and VEGF through systematic analysis and modification of relevant fabrication and post-processing parameters.

The second specific aim investigated the functional applicability of MPC to scaffolds for HUVEC culture and vasculogenesis. In vitro testing was performed to examine how MPC-fabricated 3D type I collagen scaffolds with or without further protein ligand modification can be used to support HUVEC viability, remodeling, and vascular tube formation.

This dissertation encompasses studies that established new additive manufacturing methods for cellular scaffolds in dermal wound healing. Completion of the two specific aims has expanded the capability of MPC to allow 3D fabrication of type I collagen scaffolds with spatial control of local structural and biochemical signals at a sub-cellular scale. Initial studies were performed to assess the effectiveness of these scaffolds patterned with VEGF for the induction of vasculogenesis by spatially defining the presentation of vasculogenic signals. These materials were used in a form as near as possible to their native state in an attempt to present a signaling environment more similar to a native ECM. These studies were directed toward implementation in vasculogenic dermal wound scaffolds, but the methods used are generally applicable to creation of structural and chemical patterning in any TE/RM application.
Acknowledgements

As much as I might like to, I cannot take all the credit for this dissertation. Many, many people have contributed their efforts to help bring this about. I will attempt to name them all here, but have almost certainly missed someone. As such, I will preface by first offering a grateful acknowledgement to anyone and everyone who has aided me along the way.

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I must also thank the other members of my advisory committee. Dr. Jason Shearn has provided a wealth of knowledge on methods and approaches to tissue engineering problems and has helped to keep this work on track and grounded at many times when I may have been tempted to try something just to see if it could be done. Dr. Matt Kofron is an invaluable resource for finding ways to implement a wide variety of imaging and cell biology techniques within the framework of the novel additive manufacturing processes I have been developing. Dr. Lilit Yeghiazarian provided an outside perspective and enlightened me to a wide range of related work that I never would have otherwise come across, but which has greatly expanded the range of applications I can see for my own research.
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>1D/2D/3D</td>
<td>1-, 2-, or 3-Dimensional</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic Acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>(b)BSA</td>
<td>(biotinylated) Bovine Serum Albumin</td>
</tr>
<tr>
<td>BD/CD/DVD</td>
<td>Blu-Ray/Compact/Digital Video Disc</td>
</tr>
<tr>
<td>(b)VEGF</td>
<td>(biotinylated) Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>CAD/CAM</td>
<td>Computer-Aided Design, Computer-Aided Manufacturing</td>
</tr>
<tr>
<td>CNC</td>
<td>Computer Numerical Control</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin Mononucleotide</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>HV</td>
<td>High Voltage</td>
</tr>
<tr>
<td>MEMS</td>
<td>Micro Electro-Mechanical System</td>
</tr>
<tr>
<td>MPC</td>
<td>Multiphoton Crosslinking</td>
</tr>
<tr>
<td>MPP</td>
<td>Multiphoton Polymerization</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>PEG(da)</td>
<td>poly (ethylene glycol) (diacrylate)</td>
</tr>
<tr>
<td>PI</td>
<td>Photoinitiator</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly (methyl methacrylate)</td>
</tr>
<tr>
<td>RB</td>
<td>Rose Bengal</td>
</tr>
<tr>
<td>RGDS</td>
<td>Arginine-Glycine-Aspartic Acid-Serine</td>
</tr>
<tr>
<td>SA-AF594</td>
<td>Streptavidin - AlexaFluor® 594</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SHG</td>
<td>Second Harmonic Generation</td>
</tr>
<tr>
<td>STL</td>
<td>Stereolithography (file format)</td>
</tr>
<tr>
<td>TE/RM</td>
<td>Tissue Engineering and Regenerative Medicine</td>
</tr>
<tr>
<td>Ti:Sa</td>
<td>Titanium:Sapphire</td>
</tr>
<tr>
<td>TMPTA</td>
<td>Trimethylolpropane Triacrylate</td>
</tr>
</tbody>
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Chapter 1

Impetus, Rationale and Objectives

**Impetus for this Research**

Though much has been achieved through the manipulation of cellular genetics,\(^1,2\) the soluble growth factor content of culture media,\(^3,4\) and the macro-scale tuning of scaffold features such as pore size, fiber size and fiber orientation,\(^5,6\) laboratory methods are still unable to recapitulate the spatial distribution of structural and biochemical factors present in native tissues. Mounting evidence suggests that point-by-point 3D control of cellular-scale features for scaffold structures in tissue engineering and regenerative medicine (TE/RM) will greatly improve the efficacy and translational applicability of TE/RM solutions.\(^7\) Extracellular environments feature heterogeneous, but well-defined, three-dimensional networks and gradients which influence cellular behavior and development, giving rise to the complex structures which provide for the bulk function of a tissue.\(^8\)

Multiphoton cross-linking (MPC) is an additive manufacturing technology with single-micron spatial resolution in three dimensions.\(^9,10\) While MPC has been extensively employed for the fabrication of cell-culture devices for tissue engineering and regenerative medicine applications, the materials used have largely been commercial polymers.\(^11–14\) Recent research has shown that MPC can be applied for the fabrication of 3D structures from unmodified soluble proteins.\(^15–18\) To date, little progress has been made translating these fabrication advances into workable cell culture devices. Previous studies employing MPC to crosslink proteins have utilized bovine serum albumin (BSA), a highly soluble and readily-available serum protein which is inexpensive and cytocompatible.\(^15,19,20\) However, BSA is not an extracellular matrix protein. As increasing evidence highlights the degree to which cells can sense and respond to
their environment, the importance of developing a scaffold fabrication technology which can utilize the extracellular structural and signaling proteins cells naturally experience in a tissue environment becomes apparent.

A central challenge for the engineering of 3D constructs for most tissue types is the creation of a vascular supply to deliver oxygen and nutrients throughout the scaffold volume. In addition to general vascular supply, several tissues, such as bone, kidney, and liver, have a well-defined microscale vascular geometry which is essential to establishing the functional units within that tissue. Vascular endothelial growth factor (VEGF) has been demonstrated to guide the localization and tube formation of endothelial cells. Developing the capacity to fabricate VEGF signaling patterns within a scaffold to spatially guide the development of these functional microscale vasculatures in 3D is an important challenge to overcome for tissue engineering and regenerative medicine.

In total, this dissertation represents the summation of efforts to apply a microscale, fully definable 3D fabrication technique to structural and signaling proteins as near as possible to their native form. The studies performed for this dissertation were designed to expand the current capabilities of MPC to allow for the additive manufacturing of cell culture scaffolds from the extracellular matrix protein type I collagen. Further, studies were carried out to use MPC to selectively pattern these scaffolds in 3D with VEGF. Finally, efficacy of these fabrication capabilities for the guidance of vascular formation using human umbilical vein endothelial cells (HUVECs) was assessed.

Specific Aims and Rationale for Dissertation Studies
Specific Aim 1

The first specific aim of this dissertation was to establish repeatable methods for 3D printing of type I collagen by MPC and the quantifiable modification thereof with soluble, bioactive proteins.

Hypothesis 1

Systematic analysis and optimization will produce MPC methods for fabricating type I collagen scaffolds with user-defined 3D structural and biochemical patterning at cellular-scale resolution.

My preliminary studies have shown that photocrosslinking of multiple unmodified proteins using the biocompatible sensitizer FMN is possible and that MPC of BSA can be achieved and optimized using currently available technology. The first specific aim of this dissertation is designed to establish that 3D printing of type I collagen and subsequent functional ligand modifications can also be performed with MPC. This specific aim established quantified working parameters for the additive manufacturing of type I collagen and for 3D patterning of functional protein ligands onto collagen structures.

Study 1.1 - Multiphoton crosslinking for 3D printing of Type I collagen (Chapter 4).

Hypothesis 1.1

Through systematic analysis of average laser power, scanning speed, and collagen concentration, a fabrication window will be established allowing unmodified type I collagen to be used for MPC additive manufacturing.

The objective of this study is the development and assessment of a technique which allows unmodified, acid-soluble type I collagen to be 3D-printed with micron-scale resolution using a chemistry proven to be cytocompatible. A fabrication window for the factors of collagen
concentration, laser power and pixel dwell time was determined. Following the determination of working methods to achieve fabrication, the output capabilities of the technique were investigated.

Assays were performed for positive and negative resolution and aspect ratio, with positive resolution being the smallest features that can be created and negative resolution being the smallest space that can be created between features. The concentration of collagen needed to retain a 3D form was assessed by repeating a series of fabrication exposures on collagen solutions of increasing concentration until structures were consistently present and complete following all washing and dyeing steps. The functional fabrication window for parameters of laser power and pixel dwell time were assessed by exposing a series of fixed-size 3D structures using the available range of settings for these parameters.

The resolution capabilities were measured by exposing the collagen solution to a single linear scan at the slide/solution interface and measuring the mean width of the lines from fluorescent and scanning electron microscope (SEM) images of dyed lines. Negative-space resolution was measured by fabricating pairs of rectangular structures with decreasing space between them. The ability of the resulting material to form 3D structures was assessed by fabricating structures with increasing vertical aspect ratio (AR) to determine the AR limit at which the material can no longer support itself. Results from this study establish that MPC is a viable 3D microfabrication technique for collagen scaffolds and provide working parameters for repeatable production of 3D printed type I collagen structures.

**Study 1.2 Multiphoton crosslinking for 3D modification of collagen I structures (Chapter 5)**
Hypothesis 1.2

Discrete patterns of soluble proteins VEGF and BSA will be fabricated onto 3D, type I collagen structures and the deposited concentration relative to applied laser power and scan speed will be determined through quantitative fluorescent imaging.

The objective for this study was designed to develop and assess a method for creating 3D patterns of bioactive signaling ligands using MPC. Washing and rinsing conditions for applying and removing ligand solutions were optimized and the relationship between fabrication laser power and deposited ligand concentration was quantified. Ligand patterning was performed on type I collagen structures fabricated by the MPC methods developed and validated in study 1.1.

As the MPC chemistry is non-protein specific, biotinylated BSA (bBSA) stood in for biotinylated VEGF (bVEGF) in the first portion of this study for fiscal purposes. The bBSA was visualized by hybridization with Streptavidin-AlexaFluor® 594 conjugate. Washing procedures to remove non-crosslinked protein were optimized. Crosslinking was performed by exposing areas of MPC collagen structures with unexposed areas in between. Washing conditions which were analyzed were detergent concentration, efficacy of pre-exposure protein blocking with unmodified BSA, efficacy of including detergent in bBSA solution during MPC, and optimal washing duration. The outcome measure to be compared was the amount of protein retained in the exposed area compared to the unexposed area.

Following the optimization of these conditions, a further test was performed to relate laser power during exposure to concentration of protein deposited. These exposures were performed at average laser powers of 9.6 mW – 144 mW with an increment of 9.6 mW (2% to 30% by 2% increments of available laser power). Fluorescence-concentration curves developed for SA-AF594 were used to establish the concentration of protein present based on fluorescence
values obtained through imaging under identical conditions. The concentration was then plotted against the laser power and pixel dwell time. This series of tests was performed for both bBSA and bVEGF.

It is known that multiphoton reaction rates are proportional to the square of the incident optical intensity, but the ultimate amount of material that can be deposited is limited by the amount of available material present in the exposed volume during the crosslinking exposure as well as the availability of binding sites on the target scaffold. Completion of this study provides both the methods necessary to perform 3D functional labeling of collagen structures as well as a method to quantifiably control the local concentration of protein molecules. The washing procedures and power/concentration curves will differ based on the specific protein used, but this study provides a blueprint for determining them efficiently and in a repeatable manner. The studies of specific aim 1 will develop generally-applicable technology allowing 3D spatial control of structural and biochemical features of type I collagen scaffolds.

**Specific Aim 2**

The second specific aim if this dissertation was to assess, *in vitro*, how MPC collagen scaffolds with VEGF patterning support HUVEC viability, migration, and induce vascular tube formation.

**Hypothesis 2**

MPC collagen patterned with VEGF will support HUVEC viability similar to non-patterned controls. VEGF-patterned MPC collagen scaffolds will show greater spatial specificity of HUVECs to patterned regions and will induce vascular tube formation.

**Study 2.1 Fibroblast viability and remodeling on MPC collagen scaffolds.**
The objective of this study was to assess the extent to which MPC collagen scaffolds supported cell survival and proliferation and whether the patterning of VEGF onto those structures could spatially guide the localization and vascular tube formation of cultured HUVECs. This study showed the extent of the ability of MPC collagen scaffolds fabricated under the parameters established in study 1.1 to resist degradation during HUVEC culture.

Cells were seeded onto 500 µm x 500 µm x 120 µm porous MPC collagen I scaffolds with 32 µm pores and struts spaced evenly. Initial cell seeding density was 1 x 10⁶ cells mL⁻¹, but was reduced to 1 x 10⁵ mL⁻¹ due to rapid scaffold degradation. The scaffolds were imaged by confocal microscopy at days 0, 3, and 7 for as long as the scaffold structures persisted under cell culture. Transmitted light microscope images were taken daily. Cells were visualized by staining with CellTracker® Blue and scaffolds were visualized using the inherent fluorescence imparted by the FMN photosensitizer used during MPC. Three scaffold conditions were used: 1) Scaffolds patterned with VEGF by MPC using the methods established in study 1.2, 2) scaffolds soaked in the VEGF solution used for patterning but not exposed to crosslinking by MPC, and 3) control scaffolds with no VEGF present. Cell survival, scaffold degradation, cell localization relative to patterning, and tube formation were compared across scaffold conditions.

The combined results of these three studies represent initial steps for development of MPC as a biocompatible scaffold fabrication method which allows the spatial recreation of the structural and biochemical extracellular environment using unmodified proteins.
Chapter 2

Literature Review

Introduction

The work presented in this dissertation was devoted to applying the microscale additive manufacturing technique of MPC to type I collagen, BSA and VEGF as near as possible to their native form. This was done to begin to develop the means to create structural and signaling shapes and patterns for vasculogenic dermal wound healing scaffolds which more closely resemble the native tissue environment than currently available technologies can achieve. While this work was performed in pursuit of solutions for dermal wound healing, the applicability of these sub-cellular scale scaffold structuring and patterning techniques, for vasculogenesis or many other tissue micro-architectures, is broadly applicable to TE/RM research.

Scaffold Materials and Clinical Relevance

Type I collagen, or collagen I, is the most abundant protein in mammalian organisms and is the principle component of the ECM in connective tissues, responsible for conferring tensile strength. Each collagen monomer is a triple helix approximately 300 nm long and 2 nm in diameter. Polymerized collagen molecules form fibrils of varying diameter with monomeric units arranged periodically and aligned to the axis of the fibril. Collagen I is water-insoluble in its final, deposited form. The deposition process is carried out by specialized secretory vesicles called fibropositors which, in conjunction with the natural liquid-crystalline nature of collagen molecules, dictate the alignment of the forming fibril. Many cell types interact with type I collagen fibrils via integrin receptors, allowing them to sense fibril alignment and stress. The alignment, stress, diameter and pore size of collagen fibril networks all significantly affect the behavior and fate of cells during tissue healing and development.
Interactions between cells and the ECM are a primary factor influencing cell fate in native tissues. Recreating the spatial patterns of structural and biochemical signals \textit{in vitro} is a critical obstacle in the long-term goal of engineering functional tissue replacements.\textsuperscript{29} Mounting evidence suggests that the ability of cells to sense and respond to the complexity of the ECM environment \textit{in vivo} requires fabrication methods for \textit{in vitro} systems which can reproduce these patterns at a sub-cellular scale.\textsuperscript{3} Across multiple tissues types, one of the principal sets of patterns and signals which are crucial to recapitulation of tissue function are those which guide and define the formation of a vascular supply to allow nutrient and oxygen delivery for further tissue development.\textsuperscript{7}

Dermal wounds arise from multiple distinct causes and are one of the target clinical problems for engineering of vascular networks within tissue constructs. Currently, the annual domestic cost of treatment for acute and chronic dermal wounds is approximately $25 billion.\textsuperscript{30} Large-area burns and chronic pressure ulcers, the latter often a co-morbidity of diabetic neuropathy of the extremities, are the most prominent wound types which present clinical challenges to healing.\textsuperscript{31} Engineered scaffolds for skin are an important treatment tool for both conditions as neither heal well naturally and the current standard of treatment, split-thickness autologous grafting, essentially leads to a doubling of the original wound area.\textsuperscript{32}

Healing of all but superficial skin wounds results in the formation of scar tissue, which restores a protective barrier, but will not recover the full form or function of healthy tissue. Scar tissue is highly fibrous and can lead to chronic pain on top of cosmetic irregularities and loss of the finer tissue functions, such as hair growth and sweat production.\textsuperscript{33} A variety of commercial engineered skin substitutes have been approved for wound treatment, but as of now the results are still far from regeneration of full skin function.\textsuperscript{34} At best, these products tend to provide a
conduit for closing of large-area wounds and a barrier to help hold off infection while this happens; scar tissue is still formed.\textsuperscript{31} For these reasons, there is a strong push to develop an engineered scaffold solution which will allow healing of healthy skin. Defining a pathway for the early and complete development of a vascular network in such scaffolds is considered a critical step to regenerative healing.\textsuperscript{35}

Vascular endothelial growth factor (VEGF) is a vasculogenic and angiogenic factor which is shown to regulate the formation of vasculature in a concentration-dependent manner in cultured endothelial cell lines.\textsuperscript{36} Sequestration of VEGF and VEGF-derived peptides within cell scaffolds produces enhanced angiogenesis compared with the equivalent concentration of soluble VEGF.\textsuperscript{37} This improved response is largely due to the prolonged release of VEGF when it is sequestered, allowing the vasculogenic/angiogenic stimulus to the cells to be presented over the full time period required for vascular network development, while mitigating the potential inflammatory response of a bulk release.\textsuperscript{38} By utilizing tunable, enzymatically digestible hydrogels, the temporal release profile can be optimized for \textit{in vitro} use.\textsuperscript{39} \textit{In vivo} application of VEGF-laden scaffolds has proven successful for development of mature blood vessels, but has also shown that VEGF contributes to a complex inflammatory response which can impede the desired developmental pathway and lead to unwanted scar tissue formation.\textsuperscript{40}

\textbf{Multiphoton Absorption Phenomena}

Two-photon, or multiphoton, absorption excitation is a nonlinear optical process in which a molecule achieves an excited electron state by simultaneous absorption of two, or more, photons whose summed energy achieves the quantum amount needed for the state transition.\textsuperscript{41} This process is dependent on the photons being absorbed by the target molecule within a certain window of time and space, which leads to the probability of absorption increasing proportional
to the square of incident light intensity.\textsuperscript{42} When using a focused laser beam for excitation, the intensity of light increases from the objective to the focal plane as the beam diameter narrows. The quadratic dependency of multi-photon absorption and excitation provides a mechanism to limit the occurrence of these phenomena along the axis of the beam to the plane where the threshold for activation is reached.\textsuperscript{43} Different molecules have varying levels of sensitivity to these excitations, but the excitation volume can be limited to an ellipsoid-shaped point at the very focus of a diffraction-limited lens.\textsuperscript{44}

This process can be used to limit both fluorescence and chemical reactions spatially within the focal excitation volume. For microscopy, this provides an alternative imaging method to traditional confocal microscopy, as the limiting of fluorescence to the focal plane greatly reduces the noise generated when an entire conical volume above and below the pinhole is excited.\textsuperscript{45} Aside from axial isolation, it has also been shown that multiphoton effects can be modulated in the XY plane of the focal volume by adjusting the laser power, as intensity is not uniform across a beam but increases in intensity toward the center in a Gaussian relationship.\textsuperscript{46} While the laser power is attenuated somewhat by absorption as the beam passes through the depth of the solution, this is generally not a significant factor in multiphoton processes used for imaging or fabrication as the focal length of the lenses only allows the depth to reach 1 mm or less.\textsuperscript{47}

Exceedingly high optical intensities are needed to achieve multiphoton absorption in most materials.\textsuperscript{42} For this reason, ultrafast pulsed lasers are employed which allow peak power in the 100 – 400 kW range but have average power output around 1-3 W, reducing damage to the target materials.\textsuperscript{9} In general, tunable Titanium:Sapphire (Ti:Sa) crystal mode-locked lasers are employed as the excitation source, but multiple studies have also detailed methods utilizing
lower-cost, Q-switched neodymium-doped yttrium aluminum garnet (Nd:YAG) lasers to stimulate multiphoton absorption.\textsuperscript{15,48} The latter options do not have the option of tuning the operating wavelength, but are far more economical.\textsuperscript{49}

**Multiphoton and SHG imaging and image-guided fabrication**

Second-harmonic generation (SHG) is a frequency doubling phenomenon wherein an emission signal of exactly half the wavelength of input light is produced. This process occurs when light is incident on peptide bonds in proteins, and polymeric, fibrous proteins such as type I collagen produce strong SHG signals due to their highly aligned molecular structure.\textsuperscript{50} Computational methods have been devised allowing the direct calculation of fibril size and orientation from SHG images of collagen.\textsuperscript{51} It has been shown that SHG can be used to monitor the formation of collagen fibrils from solution in real time, as the signal coherence needed to produce an SHG image is only attainable when collagen molecules are aligned after polymerization.\textsuperscript{52} Deformation of the collagen matrix in response to cellular activity can also be observed via SHG without further staining or labeling.\textsuperscript{53} This method is especially practical for the visualization of fibrils in tissues and hydrogel scaffolds because it requires no modification or alteration of the fibril structure.\textsuperscript{54} SHG images of collagen fiber networks have been used to create detailed 3D images of extracellular matrix structures from dermal, ovarian and cardiac tissues.\textsuperscript{55} These images were then used as models allowing replication of this ECM geometry using type IV collagen with 95% pattern fidelity. Tests have yet to show how cells respond to these structures.\textsuperscript{56}

Aside from SHG, targeted immune-fluorescent probes have been used to visualize patterns of neural and vascular markers in retinal, cardiac and brain tissue to allow for the recreation of tissue structure morphologies. These studies utilized functional peptide fragments
to reconstruct the native anatomy, and though it was shown that cells aligned to the recreated patterns in hydrogels, no measure of cellular activity beyond location was presented. The combined capacity of multiphoton methods for both high-resolution 3D imaging and fabrication have led to them being used complementarily for extensive work examining the effects of cellular environments for tissue engineering and regenerative medicine purposes. It has been shown that fluorescence imaging can be used to determine the amount of a given protein locally deposited in a gel by MPC, allowing non-destructive, quantified assessment of the resulting 3D spatial distribution. Continuing improvement of optics, detectors and computational processes is allowing these technologies to develop to the point of being able to image, define and recreate structures well below the traditional diffraction limit, down to 100 nm or less. In turn, these techniques are now providing optical detection and modeling of features at a near-molecular scale.

Application of Multiphoton Absorption to Additive Manufacturing

Multiphoton polymerization (MPP) and multiphoton crosslinking (MPC) are additive manufacturing technologies which take advantage of the axial spatial constraints of multiphoton absorption phenomena to pattern 3D geometries within a monomeric solution by initiating chemical reactions. Translating the supporting stage and/or raster-scanning the input laser beam provides means to control crosslinked geometry. This has largely been applied using commercial or novel photopolymers. Poly(ethylene glycol) diacrylate, poly(ε-caprolactone), poly(methyl methacrylate) (PMMA) and Accura™ SI10 have been used extensively for the production of microfluidic and microelectromechanical devices as well as tissue engineering scaffolds. Photoactive, organically-modified ceramics, the ORMOCER® compounds, are hybrid materials which have mechanical properties that can be varied widely.
These have also been used extensively in MPP, including in the production of middle-ear prostheses.\textsuperscript{11,63,66,67}

The substrate for MPP/MPC must be either a material which will itself become activated and react by direct excitation from light of a given wavelength, or will react with another molecule activated by exposure to a given wavelength; either a photoinitiator or photosensitizer.\textsuperscript{68} Most photopolymers, photoinitiators and photosensitizers have excitation wavelengths in the ultraviolet range of the light spectrum. The wavelengths of the lasers used for MPC are in the near-infrared range,\textsuperscript{69} which means that two or more photons must be simultaneously absorbed to achieve activation and polymerization and that single-photon reactions can be eliminated. The probability of simultaneous absorption of multiple photons increases proportional to the square of the light intensity,\textsuperscript{70,71} which increases with the distance from the focusing objective to the focal point, effectively limiting polymerization to a small volume near the focal point of the laser.\textsuperscript{72} Resolution of structures fabricated by MPP/MPC techniques can range from $<100$ nm to 10 $\mu$m or more, depending on the focusing objective used.\textsuperscript{73}

The range of materials has been further expanded by the development and implementation of several photoinitiator and photosensitizer molecules which will operate under a wide variety of solubility conditions.\textsuperscript{74} These include Rose Bengal, Irgacure\textsuperscript{®}, Benzophenone Dimer, and many proprietary chemicals synthesized for specific purposes.\textsuperscript{17,18,72} When excited by light, these molecules will form radicals or ions which will propagate a chain reaction ending with polymerization of the substrate by various chemical mechanisms.\textsuperscript{44} While the specific activation wavelengths and sensitivities of photopolymers and photoinitiators vary, they all
follow general rules relating the sensitivity and reaction rate of the photoactive material to the power, wavelength, pulse rate and exposure time of the laser being applied.\textsuperscript{75}

A complementary method to MPC, laser-induced forward transfer (LIFT), employs an inverted slide coated in a thin metallic layer beneath a gelatinous cell suspension. Focusing a pulsed laser onto the metallic layer creates a jet which propels small volumes of the cell suspension onto a scaffold in a high-resolution 2D patterning manner.\textsuperscript{76,77}

**Multiphoton Crosslinking of Proteins and Cell Scaffolds**

The use of translating stages with 3 axes has allowed MPC to be applied to proteins to create arbitrary, user-defined geometries to not only mimic biological structures but also create cell-influencing shapes purpose-designed for specific laboratory experiments on single cells.\textsuperscript{78} In general, it has been shown that the individual amino acids of cysteine, tyrosine, tryptophan, methionine and histidine all react with photo-excited radicals at varying rates, implying that nearly all proteins containing these residues can be crosslinked with the MPC method.\textsuperscript{79} The patterning of methacrylated cell-adhesive peptide fragment arginine-glycine-aspartic acid-serine (RGDS) into collagenase-sensitive hydrogels has been used to create defined pathways for fibroblast migration through the gel.\textsuperscript{80} This same technique has also been successful in controlling the localization and tube formation in cultured endothelial cells on synthetic hydrogels patterned by MPC.\textsuperscript{81} Cell studies have utilized non-degradable, polymeric matrices fabricated with MPC to examine migration rate as well as traction forces.\textsuperscript{11,82}

MPC has been used successfully to create bioactive patterned scaffolds with micron-scale resolution capable of supporting cell growth and differentiation for engineered tissue,\textsuperscript{68} and has also been shown effective for fabrication of a variety of MEMS devices.\textsuperscript{83} In addition to initiating crosslinking and polymerization, multiphoton techniques have also been used to
spatially define photolysis of hydrogels of various chemistries, creating defined reactive sites to which an applied ligand will then selectively bind.\textsuperscript{84,85} This method has been used to pattern hydrogels with 3D patterns of growth factors and enzymes.\textsuperscript{86,87} Depending on the composition of the hydrogel, this mechanism can also be used to locally modify scaffold elasticity.\textsuperscript{88}

3D fabrication and patterning by MPC has been demonstrated in protein and peptide structures using several different photoinitiator or photosensitizer molecules.\textsuperscript{15,89,90} The technique has been applied to a range proteins, and has even been performed on cytoplasmic proteins within live cells.\textsuperscript{91} Proteins such as bovine serum albumin (BSA),\textsuperscript{15,16,20,92} laminin,\textsuperscript{93} collagen type IV,\textsuperscript{90} fibronectin,\textsuperscript{94} and streptavidin\textsuperscript{58} have been crosslinked under multiphoton conditions. Combinations of soluble proteins have been made, such as BSA and laminin, to provide both an appropriate extracellular component as well as a bulk supportive material which is more suited to processing with MPC.\textsuperscript{93} Gelatin can be acrylated to be made more sensitive to crosslinking and increase speed for MPC.\textsuperscript{68,95} Gelatin has been used to fabricate three-dimensional structures by MPC, though some gelatin structures show low mechanical integrity.\textsuperscript{68,92}

BSA has been the most extensively utilized protein for fabrication by MPC. Structures have been made which change shape under varying pH conditions,\textsuperscript{96} utilize motile bacteria for controlled motion,\textsuperscript{20} support cell growth,\textsuperscript{16} and direct axonal growth.\textsuperscript{97,98} However, BSA has been used mostly for its convenient attributes which make it easily used for multiphoton processing. Albumin, being a serum protein, is readily soluble in aqueous media at physiological pH and is largely benign to most cell types.\textsuperscript{17} For engineered tissue scaffolds, the goal is to create an environment which, as closely as possible, mimics the native ECM and guides cells in the
development of the desired tissue structures and functions. While BSA supports cell survival, it has no inherent structural cues to provide cells on the extracellular environment.

MPC has been applied before to VEGF isoform 165 for patterning of spatial gradients in agarose gel scaffolds, to guide the ingrowth of endothelial cells from primary culture. This study showed that VEGF remains viable following all MPC processing steps. Importantly, the gradient created in these scaffolds was uniform through the gel and specific geometric control of the vascular growth was not attempted.

Soluble collagen type I has been crosslinked by MPC using multiple photoinitiators, however no method has yet been able to produce structures with full 3D form. As such, little analysis was presented into the composition or mechanical integrity of the resulting products. It is important to note that the collagen polymers formed through this crosslinking method are not of the same molecular conformation as native collagen fibrils and the organization of individual collagen molecules is essentially random.

The low pH of acidic collagen I solution precludes the use of most photoinitiators and photosensitizers. One notable exception is flavin mononucleotide (FMN), a biocompatible photosensitizer in the near-infrared which retains its activity under the pH and ionic conditions in which collagen I is soluble. FMN is a phosphorylated form of riboflavin, which has been shown to act as a strong photosensitizer in the presence of proteins and which has a broad absorption spectrum. FMN is a naturally occurring biomolecule in the electron transport chain and, as such, has excellent biocompatibility for tissue engineering and has been demonstrated as a photosensitizer on several proteins.

Flavin-family photosensitizers, riboflavin and FMN, have been used for the crosslinking of collagenous hydrogels in corneal and cardiac tissue engineering, but only on hydrogels in
which fibrils had already been formed and soluble collagen was no longer present. These studies provide reaction mechanisms which give a general understanding of the process by which photo-excited flavins interact with type I collagen, even if the reaction kinetics will be altered due to the different state of the collagen. Collagen hydrogels crosslinked with rose Bengal photoinitiator by standard UV photo-crosslinking methods show improved compressive strength and increased lifespan while maintaining similar biocompatibility after implantation.

The rate of the reaction between the PI and proteins during MPC is dependent on several factors, including optical intensity, exposure time, and reactant concentration. In particular, the dependence of reaction rate on parameters of optical exposure provides means by which local concentration of deposited proteins can be spatially varied, as these factors can be easily adjusted in situ. The reaction kinetics vary widely with different proteins and are nearly impossible to predict analytically, but any protein can theoretically be cross-linked by such a reaction. While the reaction rate constants vary for every protein and photosensitizer, the reaction rate remains quadratically proportional to the optical power of the laser.

For vascularization of dermal wound scaffolds, MPC shows distinct promise. In native skin tissue, the cellular environment is comprised of multiple materials arranged into heterogeneous functional units with micron-scale features. Many techniques, including micro/nano-imprint lithography, electrospinning, and micro-molding are well-established for the fabrication of micron-scale features in tissue scaffolds. However, these lack the ability to produce the 3D spatial heterogeneity seen in native tissue. Other free-form 3D technologies grouped under the labels of bioprinting or bioplotting use more traditional extrusion and deposition methods to pattern cells and support materials to create 3D tissue constructs, but
these do not provide the high resolution in spatially defining the extracellular environment that can be achieved with MPC.\textsuperscript{118-121}

There are many microfabrication techniques which have been applied to make scaffolds for engineered tissues, but greater control over 3D spatial signals at a sub-cellular length scale is still needed to address these factors on the level at which cells are able to sense them. MPC provides both high resolution for 3D fabrication and the ability to be applied to soluble proteins without further chemical modification. While these factors provide great promise for spatially defining and inducing vascularization in wound healing scaffolds, more development of the MPC technique is needed to realize this promise. The dissertation work presented here utilizes MPC to establish methods to produce type I collagen scaffolds and pattern them in 3D with unmodified BSA and VEGF, assessing the spatial capabilities of the fabrication technique as well as the performance of the resulting scaffolds for vasculogenesis in HUVEC culture.
Chapter 3

Preliminary Results

3.1

Preliminary Fabrication Capabilities of a 2-Axis Photolithography System Based on Optical Drive Motors and Laser Diodes

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Introduction.

Photopolymerization methods such as multiphoton polymerization have been used successfully to create bioactive patterned scaffolds with micron-scale resolution capable of supporting cell growth and differentiation for engineered tissue. They have also been shown effective for fabrication of a variety of MEMS devices. Currently, multiphoton polymerization and similar technologies require a bulky and expensive optical system based on a femto- or picosecond pulsed laser and an XYZ arrangement of high-resolution translating stages. Such systems are currently prohibitive in both cost and effort required to assemble, calibrate, and maintain. Consolidating optical components and motors into a smaller, less-complex device may facilitate the manufacture of customized tissue engineered constructs and MEMS devices on-site in more remote locations on an as-needed basis.

The optical drives found in most computers contain all the elements necessary to create a fundamental multi-axis photopolymerization device. Each drive has an optical carriage which is
moved along an axis of travel by a small stepper motor. The data lines on optical media are between 0.5 µm and 2 µm apart, depending on the media type, so the resolution of the stepper motors is on this order. This carriage can contain up to 3 laser diodes focused through the same output lens; a 405 nm diode for Blu-Ray, 650 nm for DVD and 780nm for CD media. Optical writing drives have diodes with continuous wave power ranging from 150 mW for the 780 nm diode up to 1 W for the 405 nm diode.\textsuperscript{122} By leaving the essential elements of these small optical and mechanical translation systems intact, we were able to fabricate a device capable of micron-scale photopolymerization at a small fraction of the size and cost of a standard multiphoton polymerization system.

**Materials and Methods.**

Two DVD-RW drives were repurposed from recycled computers. The frame containing the optical carriage and associated motor and rails for translation were recovered from the drives. On the Y-axis drive, the optical carriage was replaced with a custom-designed microscope slide holder. This slide holder attached to the motor lead screw and moved along the guide rails just as the optical carriage had. The holder allowed a 25 mm square area in the center of a standard microscope slide to be used for the polymerization exposure (Fig. 3.1).
Figure 3.1 – Slide Holder. In the drive serving as the Y-Axis, the optical carriage was replaced with a microscope slide holder.

Except for removal of diffraction gratings over the lens of each diode, the optical system of the second drive was left intact. A frame was designed which allowed the two drives to be mounted perpendicular with the lens of the optical system positioned 3.05 mm above the slide, the industry standard focal distance for optical media drives. This formed a 2-axis translation system for the laser. (Fig. 3.2)

Figure 3.2 – Assembled Device. The two drives were mounted into a custom frame allowing each to serve as an axis of motion while maintaining the focal length of the laser lens.
The motor from each drive was connected to an EasyDriver stepper motor driver set to 1/8 microstepping (Schmalzhaus). Each of the diodes (650 and 780 nm) was connected to an adjustable-current laser diode driver (Aixiz). An Arduino Uno R3 open-source microcontroller was used to control motor motion and diode power (on/off).

The minimum step distance for each motor was determined. This was done by programming the motor to repeatedly move 500 steps forward and back. Both motors had a lead screw pitch of 3.00 mm. The distance moved was measured with Vernier calipers with 20 µm accuracy. This was performed for each motor at 4 different speeds.

Samples for photopolymerization were prepared on a standard microscope slide. A 0.12 mm thick spacer with outside dimensions 25 mm square and a 20 mm central well (Grace Bio-Labs) was used to hold the material under a standard coverglass. Trimethylolpropane Triacrylate (TMPTA, Sigma Aldrich) was used as the monomer with 0.25 wt% H-NU 780 photoinitiator (PI, Spectra Group, LTD). The 780 nm diode was used for polymerization with an operating current of 200 mA.

For these tests, the Arduino microcontroller was programmed manually to trace out the lines in the test pattern. However, it is possible to program the Arduino to accept G-Code from a CAM program based on STL files.

**Preliminary Results.**

The minimum step distance of each motor was determined from a 500-step motion performed repeatedly at different speeds. Speeds of 0.01, 0.05, 0.1 and 0.25 were used. These speeds are native functions for the Arduino library and documentation was not available for how this translated to motor RPM. However, a speed of 0.1 leads to linear translation at approximately 2 mm/s. It was found that the motor from the X-axis had a minimum 1/8 step size.
of 18.07 µm (±0.07 µm) at a speed of 0.05. The motor for the Y-axis had the smallest 1/8 step size of 17.89 µm (±0.07 µm) at a speed of 0.1. The slowest speed from the Y-axis was discounted because the motion, though consistent, was rough and unsuitable for fabrication.

(See Table 3.1)

<table>
<thead>
<tr>
<th>SPEED</th>
<th>X MOTOR</th>
<th>Y MOTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>19.02 ±0.11</td>
<td>10.20 ±0.77</td>
</tr>
<tr>
<td>0.05</td>
<td>18.07 ±0.07</td>
<td>18.61 ±0.10</td>
</tr>
<tr>
<td>0.1</td>
<td>19.17 ±0.06</td>
<td>17.89 ±0.07</td>
</tr>
<tr>
<td>0.25</td>
<td>19.17 ±0.07</td>
<td>18.96 ±0.64</td>
</tr>
</tbody>
</table>

Table 3.1 – Step Increments. This table shows the minimum achieved step increment for both motors with 1/8 microstepping enabled. Data are shown as mean step size ±SD in µm, where n = 12 for all tests.

Polymerization was successful only in scans where the laser passed over a given pattern a minimum of 37 times. Width of lines produced was between 100 µm and 200 µm.

Discussion.

Rapid prototyping allowed for easy construction of the frame and slide holder necessary to convert the chassis from the DVD-RW drives into a multi-axis photopolymerization device. Inexpensive electronics were used to establish a baseline of capability for these components. Stepper motor drivers supporting up to 1/128 microsteps/step are available. In selecting a driver which supported only 1/8 microstepping, it was assumed that the motors in these drives produced many more steps/revolution. However, motor testing showed that they only provided approximately 20 steps/revolution. Fine control of the positioning of the laser focus is provided by an electromagnetic servo loop within the drive’s optical system. For the purposes of this initial device, it was not practical to attempt to control both the lens magnets and the motor in synchronization to determine simple motions.

The 100 µm minimum line width achieved was significantly greater than predicted based on the native focal size of an optical drive laser. However, polymerization is not limited to the
focal spot, and the beam width is larger at the top surface of the solution than at the slide surface where the focus was directed.

The amount of exposure necessary to induce polymerization was also greater than anticipated based on previous experience with this monomer and PI under similar exposure conditions. The power of the 780 nm diode was not directly measured. It was assumed to be near 150 mW as this is standard for writing CD media. The input current to the diode was set at 200 mA because this was the maximum current which would permit continuous operation of the diode. No documentation was available to determine what the optical power output is with this input current, though.

**Conclusion and Future Directions.**

Preliminary results show that this device is capable of micro-scale resolution from both the stepping action of the motors and the focus of the laser. Therefore, the small, all-in-one optical systems from media drives may provide a means for constructing micro-scale fabrication systems with a far smaller footprint and significantly lower cost than the systems currently used for photopolymerization. Aside from reducing space taken up by the system in a laboratory, smaller optical systems and motor drives will potentially allow fabrication devices to be implemented in more remote locations and allow for increased modularity of parts. Future research will focus first on increasing resolution of this system and adapting it for use with more sophisticated laser sources. Beyond those immediate aims, further work will be devoted to adding three-dimensional and multiple material capabilities.
3.2 Assessment of BSA photo-crosslinking reactions with 2-axis lithography system.

Background

Other researchers have demonstrated that the BSA/FMN chemistry was successful for multiphoton crosslinking, but details were incomplete on the methods used. This study was designed to validate that BSA would react with the FMN sensitizer and form crosslinked structures when stimulated by a focused laser operating within the FMN absorption spectrum. The device described in 3.1 was optimized to crosslink this material as a precursor study to begin crosslinking other proteins that had not already been demonstrated in the literature.

Methods

The device described in study 3.1 was modified by replacing the X-axis optical system with one from a BD-RW drive containing a 405 nm laser diode. A Bovine Serum Albumin (BSA) solution was made consisting of 200 mg mL\(^{-1}\) BSA in Phosphate buffered saline (PBS), pH 7.4, with 4.0 mM FMN photosensitizer. This solution was placed in the photolithography device in wells as described in study 3.1. Tests were run to examine the effects of focal height and exposure time on morphology of crosslinked products. Focal height of the BD-RW drive lens was varied by 0.25 mm over a 1.0 mm range. Exposure times were varied from 20 seconds to 5 minutes (20s, 40s, 1m, 2m, 3m, 4m, 5m). The width of crosslinked spots and lines were measured and structural completeness was qualitatively analyzed. Mass-loss tests were done to determine the nature of unintended polymerization in the bulk BSA/FMN solution.

Results

It was determined based on the mass lost that the BSA solution was drying out during long exposure tests, and that ambient light will not initiate crosslinking reactions. Exposure times below 1 minute did not produce crosslinked structures. 1-minute exposure produced incomplete,
porous spots. Complete, circular spots with diameter of 150 µm (± 50 µm SD) were produced at 2 minutes exposure. Increasing exposure times beyond 2 minutes produced larger diameter spots with irregular geometry. In the focal length optimization, the smallest spot size of ~150 µm was produced from a focal height with the lens fully extended toward the slide surface. Lines were produced by stepping the stage in the X-axis at a rate of 1.8 µm/s over a 2.0 mm path. The exposure time per unit area was the same as spot test, but the minimum measured line width was 1.0 mm, indicating that much more diffusive crosslinking occurred when the laser was translated through the solution. (Fig. 3.3)

Figure 3.3 - Transmitted light image of BSA crosslinked using the 2-Axis lithography system.

Conclusions

The BSA solution used was able to be photo-crosslinked with sub-millimeter resolution in 2 dimensions using the designed device. Fabrication time was exceedingly slow, but this study demonstrated settings by which crosslinking is achievable. This device is a suitable test platform for validation of the ability to crosslink other, unproven protein solutions.
3.3 Type I collagen crosslinking with 2-axis lithography system.

Background

Fabrication of soluble type I collagen into desired 3D structural geometries using FMN photosensitizer has never been demonstrated. Many other proteins have been used with this technique, but the acid-soluble nature of type I collagen combined with few compatible photoinitiators or photosensitizers have thus far precluded it from being used.\textsuperscript{14} This study showed that the same photosensitizer used with BSA could successfully crosslink acid-soluble type I collagen, in a variety of geometries, without any prior chemical modification to the protein.

Methods

The device described in study 3.2 was used to crosslink the collagen. Single spots and lines were fabricated from a basic control program as in 3.2, as well. Additionally, the open-source CNC control program GRBL was loaded onto the Arduino Uno microcontroller to allow the device to receive and interpret motion instructions in G-code format. Numerical Code (.nc) files were generated for simple shapes that fit into the 25 mm x 25 mm fabrication area of the device. A type I collagen solution consisting of $5 \text{ mg mL}^{-1}$ bovine skin collagen dissolved in 0.02 mM Acetic Acid (as received from Gibco) was prepared with 4.0 mM FMN. This solution was placed in the photolithography device as described in study 3.1.

Initially, the same spot test from study 3.2 was used to assess optimal crosslinking exposure time. This collagen solution, however, necessitated shorter exposure times. A similar test was performed with exposure times of 1s, 2s, 3s, 5s, 10s and 20s per spot, with the spots spaced 1mm apart. (Fig. 3.4) Lines were scanned across the width of well at the slowest speed
the Arduino control would allow. Multiple passes were performed (n = 1 to n = 12) to determine the minimum needed to produce a crosslinked structure. Minimum exposure times from spot and line tests were used to set maximum speeds for CAD-based tracing of hand-drawn shapes (script letters “A” and “X”). Letters were used to assess how well the technique could be applied with complex shapes requiring acceleration and deceleration of the laser head. All structures were washed in 0.02M AcOH and dyed by soaking in a 1.0 mM Rose Bengal solution for contrast during imaging. Images were captured on a Nikon TS100 light microscope with the field of view calibrated for diameter. The structures were then measured based on this calibration using ImageJ.

Figure 3.4 - Transmitted light image of 5mg mL⁻¹ Type I collagen spot crosslinked using the 2-Axis lithography system. The resulting structure was dyed with Rose Bengal for visualization.
Results

Structures need to remain hydrated to maintain shape. The collagen content proved too low to even be visualized if structures dried out. The dye was also very transient, leaching out of structures after a few minutes in solution. In general, the structures that were formed had very low mechanical integrity and were frequently lost during the soaking steps, even though no agitation was used.

The minimum time required to form a crosslinked spot was 3 seconds. Spots formed in this time were incomplete and irregularly shaped. 5 seconds was the minimum time needed to form complete, circular spots. At 10 seconds, the spots became significantly larger and amorphous. At 20 seconds, there were no individual spots, only a line across the center of the well. The solution dried out quickly and the surface became uneven, leading to somewhat irregularly shaped spots in even the 5-second exposure series.

Collagen spots were larger than BSA spots and somewhat elliptical in shape, with vertical diameter 486 µm (± 50 µm) and horizontal diameter 455 µm (± 109 µm) (n = 6). 4 passes of the laser in the X axis across the width of well at slowest programmable speed were required to leave a solid line. 5 or more passes led to a line that was too wide to be completely viewed in the microscope at one time. Line width after 4 passes was 598 µm (± 62 µm) (n = 6). Letters were complete and visible following crosslinking and initial wash, but were never able to remain intact through 2 subsequent washes required for dyeing. However, no obvious irregularities were visible as a result of laser speed variation.
Conclusions

The lower concentration of collagen likely facilitates much more rapid diffusion of the activated species, leading to increased structure sizes outside the focal spot. The low concentration also makes the structures very difficult to retrieve and analyze following fabrication. However, this study showed that the combination of acid-soluble type I collagen and FMN can be used as a photocrosslinkable biomaterial substrate.

3.4 Multiphoton crosslinking of Bovine Serum Albumin.

Background

Multiple previous studies have reported the ability to crosslink BSA using multiphoton methods.\textsuperscript{15,16,20} This study was a systematic determination of the operating parameters within which the reported chemistry could be activated using the available equipment to achieve repeatable results. The use of a protein and photoinitiators/photosensitizers that were previously reported to be successful was done to minimize the possibility of equipment incompatibility or human error when attempting multiphoton collagen crosslinking, which has not been previously demonstrated.
Figure 3.5 – BSA Crosslinked by MPC. This SEM image shows a 3D structure fabricated from BSA using MPC. The 3D form of the structure collapsed considerably when dried for SEM imaging.

Methods

Multiple variables were assessed for optimization of the crosslinking process of BSA on a Nikon A1R MP microscope using a Nikon 25x, 1.1 NA objective lens. The excitation source was a Coherent Chameleon Vision II Titanium:Sapphire (Ti:Sa) laser. XZ scanning was used to find the slide surface to ensure adhesion of crosslinked structures. Optimal Z-axis step size and laser wavelength were determined. Laser power and pixel dwell were optimized in relation to pixel size for the exposed fabrication “image.” An ideal slide surface was determined. An ideal photosensitizer was determined by performing fabrication with 3 known biocompatible options. X-Y resolution was measured by measuring fabricated lines using ImageJ. Power stability and transmission efficiency of the system were measured. BSA/FMN solution was prepared and placed in wells on slides as in 3.2.
Results

It was determined that 16.7% (± 0.25%) (n = 3) of the initial laser output power was delivered to the sample when the Ti:Sa laser was operated at 750 nm and 17.5% (± 0.25%) (n = 3) was delivered at 800 nm within the output power ranges used for fabrication. Power stability was measured to be quite reliable in short term tests, with standard deviation of ± 3.3 mW at 750 nm (n = 18) and ± 7.6 mW at 800 nm (n = 18). However, observations outside the experiment used for these measurements were in conflict with this stability. Occasional shifts of as much as 100 mW were recorded during multiple tests, but were not frequent enough to predict or quantify.

FMN photosensitizer was successful for fabricating structures in a repeatable manner. Irgacure 2959 initiator did not produce any result. Rose Bengal (RB) initially produced structures comparable with those from FMN, but even at very low RB concentration, crystals precipitated after a short time and caused excessive crosslinking when exposed to the laser. Square, single-layer structures and lines were fabricated using FMN. These were achieved with average laser intensities between 0.06 mW/µm²/s and 0.17 mW/µm²/s. Line widths varied from structure to structure and within structures, but widths as low as 0.75 µm, the theoretical diffraction limit for a 750 nm beam, were achieved.

Layer spacing of 0.5 µm allowed successive layers to adhere but resulted in both excessive crosslinking and cavitation when more than 5 layers were exposed. At 1.0 µm layer spacing, successive layers adhered to form 3D structures and damage occurred if more than 9 layers were exposed. Waiting 30s and 60s between layers did not change the occurrence of damage after layer 9. At any layer spacing beyond 1.0 µm, successive layers did not adhere to
the layer below and amorphous structures appeared scattered all over the slide following fabrication.

Conclusions

3D multiphoton fabrication with micron scale resolution was achieved with BSA and FMN requiring no modification to the protein. The limit of 9 layers was less than desired, preventing the development of complex 3D geometry. However, consultation with other experts in the field confirmed that this is more or less the limit for this combination of protein and photosensitizer without adding other reagents to the solution. This confirms that the methods developed allowed for determination of optimal fabrication conditions with this materials and minimizes the likelihood that future studies with untested materials will be subject to operator error or device limitations.

3.5 Relating multiphoton-excited fluorescence intensity to concentration.

Background

The ultimate goal for the use of MPC is to be able to fabricate structures and pattern them in 3D with desired functional proteins and peptides. In order to quantify the patterns that are created, it is necessary to have a nondestructive method to probe their local ligand concentration. Many labeling methods based on immunofluorescence, avidin/biotin affinity fluorescence and direct fluorophore-protein conjugation exist. If the fluorescence intensity in a structure visualized with such methods can be related to the number of molecules of a given fluorophore present, the local concentration of the ligand can be determined. This will allow the subsequent determination of a relationship between the applied laser parameters during pattern creation and the resulting ligand density, which will, in turn, allow for 3D control of local ligand density.
Methods

BSA conjugated with Texas Red and BSA conjugated with Fluorescein Isothiocyanate (FITC) were each dissolved in PBS 7.4 to a concentration of 1 mg mL\(^{-1}\). Serial dilutions were then prepared from these solutions of 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025 and 0.001 mg mL\(^{-1}\). Each dilution of both solutions was placed in a 120 µm-deep well on a standard glass slide. Using the 1 mg mL\(^{-1}\) solution, a set of imaging parameters was established with a Nikon A1R MP microscope that allowed visualization of fluorescence with less than 1% of pixels being saturated. Multiphoton images were then collected at each concentration (n = 9 for each) using these same imaging parameters for all dilutions of a given solution. Mean fluorescence intensity gray value was plotted against the concentration of the solution and a linear regression was performed.

Results

For the dilutions of BSA tagged with Texas Red, a linear model of \(F = 4017.8C + 61.0\) was obtained with \(R^2 = 0.9882\) (Fig. 3.6, \(F\) – gray value of fluorescence intensity, \(C\) – concentration of tagged protein in mg mL\(^{-1}\)). The 0.001 mg mL\(^{-1}\) solution was not distinguishable from control images of pure PBS 7.4. BSA tagged with FITC has not yet been imaged at concentrations below 0.05 mg mL\(^{-1}\), but for concentration from 0.05 mg mL\(^{-1}\) to 1.0 mg mL\(^{-1}\), the model of \(F = 4025.5C + 149.63\) was obtained with \(R^2 = 0.9878\).
Figure 3.6 – Fluorophore Concentration Curves. This plot shows the relation between measured fluorescence intensity (12-bit, 0-4095) for BSA tagged with Texas Red (Red) and FITC (Green). The fitted models are linear, the plot is in logarithmic form as the concentration of fluorophore was spread across multiple orders of magnitude.

Conclusions

The results of this study show that a strong linear relationship exists between concentration and fluorescent intensity excited by multiphoton absorption. Furthermore it shows that the sensitivity of the detectors on the microscope being used allow the concentration to be quantified across several orders of magnitude.
Chapter 4

Multiphoton Crosslinking for Biocompatible 3D Printing of Type I Collagen

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Abstract

Multiphoton fabrication is a powerful technique for 3D printing of structures at the microscale. Many polymers and proteins have been successfully structured and patterned using this method. Type I collagen comprises a large part of the extracellular matrix for most tissue types and is a widely used cellular scaffold material for tissue engineering. Current methods for creating collagen tissue scaffolds do not allow control of local geometry on a cellular scale. This means the environment experienced by cells may be made up of the native material but unrelated to native cellular-scale structure. In this study, we present a novel method to allow multiphoton crosslinking of type I collagen with flavin mononucleotide photosensitizer. The method detailed allows full 3D printing of crosslinked structures made from unmodified type I collagen and uses only demonstrated biocompatible materials. Resolution of 1 µm for both standing lines and high-aspect ratio gaps between structures is demonstrated and complex 3D structures are fabricated. This study demonstrates a means for 3D printing with one of the most widely used tissue scaffold materials. High-resolution, 3D control of the fabrication of collagen scaffolds will facilitate higher fidelity recreation of the native extracellular environment for engineered tissues.

1. Introduction

Three-dimensional (3D) printing technologies based on multiphoton absorption phenomena have demonstrated impressive new capabilities for fabrication of 3D structures with sub-micron features. With this technique, the multiphoton absorption threshold for the polymerization or crosslinking reaction is reached only within a small volume at the focus of the objective lens – usually below 1 µm. This allows crosslinking to occur at any specified point within a solution. Ultrafast pulsed lasers are used to deliver the high optical intensity needed to initiate these reactions. Movement of the focal point either by directly translating the stage
or by raster-scanning of the input laser beam provides means for controlling the crosslinked geometry.\textsuperscript{19}

For tissue engineering, multiphoton crosslinking (MPC) has shown great promise. In native tissues, the cellular environment is comprised of multiple materials arranged heterogeneously with micron-scale features.\textsuperscript{24,110} A variety of fabrication techniques have been developed to replicate these micron-scale features in tissue engineering scaffolds, including micro/nano-imprint lithography,\textsuperscript{111,112} electrospinning,\textsuperscript{113–115} and micro-molding.\textsuperscript{116,117} However, these lack the ability to produce the spatial heterogeneity of architecture seen in native tissue as well as full 3D geometric control. Likewise, other 3D printing techniques loosely grouped under the labels of bio-printing or bio-plotting have shown the ability to pattern unrestricted 3D geometries but lack the resolution of multiphoton processing.\textsuperscript{118–120} By combining the ability to create sub-micron features with precise 3D geometric control, multiphoton techniques have enabled significant forward steps in tissue replication.\textsuperscript{13,56} Previous studies have shown that type I collagen can be crosslinked by MPC to form single-width, single-layer lines.\textsuperscript{17,90,100} However, ours is the first study to leverage the abilities of MPC to create complex 3D microstructures of the most abundant extracellular matrix (ECM) protein, type I collagen, in an unmodified form.

It has been shown that many proteins, including bovine serum albumin (BSA),\textsuperscript{15,16,20,92} laminin,\textsuperscript{93} collagen type IV,\textsuperscript{90} fibronectin,\textsuperscript{94} and streptavidin,\textsuperscript{58} can also be crosslinked under multiphoton conditions. Gelatin, a decomposed form of collagen, has been modified with acrylate to make it more sensitive to crosslinking and improve processing speed for multiphoton crosslinking.\textsuperscript{68,95} As all proteins are comprised of the same amino acid building blocks, it can be inferred that most, if not all, proteins would be reactive under multiphoton conditions, albeit with varying sensitivity based on their amino acid composition and folding geometry.
BSA has been the most widely used protein for fabrication of structures by MPC.\textsuperscript{16,20,96–98} However, BSA has been used largely out of convenience for multiphoton processability. Albumin is a serum protein which is highly soluble in aqueous media at physiological pH and is largely benign to most cell types.\textsuperscript{17} For tissue engineering scaffolds, though, the goal is to recreate a native ECM to allow for differentiation of a desired tissue type.\textsuperscript{99} While BSA is compatible with survival in most cell types, it is unlikely to further this goal as it has no inherent structural information to provide cells on the extracellular environment. Differentiation is a complex process in which manufactured scaffolds are resorbed and replaced by the cell population, but starting with an environment as close as possible to the native ECM of a target tissue will provide cells better stability and direction from the on-set.\textsuperscript{111}

Type I collagen is the most abundant structural ECM protein in animal tissues. It comprises a principal part of the ECM of bone, tendon, ligament, skin, muscle, teeth and cornea.\textsuperscript{24} For this reason, collagen I hydrogels have been widely used as scaffold materials for many types of engineered tissues. While numerous techniques have been developed to control the pore size and fibril orientation of collagen I hydrogels, complete 3D spatial control of the micro-scale architecture needed to recreate a native ECM has been largely unattainable.\textsuperscript{123} MPC has been used with collagen I, but only to the extent of forming single lines.\textsuperscript{17,90,100} Collagen I is only weakly soluble and the low pH of acidic collagen I solution has precluded the use of most photoinitiators/photosensitizers.\textsuperscript{101} However, collagen I solutions can be concentrated much more after the collagen has been dissolved, with concentrations as high as 375 mg mL\textsuperscript{-1}, achieved by dialysis methods and without premature fibrillization occurring.\textsuperscript{124} In addition, at least one family of photosensitizers, the flavins, including riboflavin and the 5'-phosphorylated flavin
mononucleotide (FMN), are biocompatible and functional at the lower pH of collagen solutions.\textsuperscript{15,102,103}

This paper describes the use of a concentrated collagen solution and FMN photosensitizer for MPC 3D printing of type I collagen structures with micron-scale geometric features. Studies were performed to assess the working ranges of collagen concentration, laser power, and pixel dwell time. Resolution was assessed both for size of fabricated features as well as negative spaces and vertical aspect ratio was analyzed. 3D structures were fabricated to qualitatively demonstrate some of the possibilities enabled with these novel methods. The development of these techniques allows for the fabrication of higher precision cellular-scale features within a scaffold made of a principle ECM protein.

2. Materials and Methods

2.1 Preparation of Materials.

Collagen solutions (5 mg mL\textsuperscript{-1}, 15 mg mL\textsuperscript{-1} and 30 mg mL\textsuperscript{-1}) were prepared from acid-solubilized Collagen I from Bovine skin (Life Technologies, Grand Island, NY, USA), used without further modification. To increase concentration to 15 mg mL\textsuperscript{-1} and 30 mg mL\textsuperscript{-1}, the stock 5 mg mL\textsuperscript{-1} solution was dialyzed against a 50/50 (v/v) mixture of 10,000 MW Poly-(ethylene glycol) (PEG, Sigma-Aldrich) and Acetic Acid (AcOH, 0.02M Sigma-Aldrich). Dialysis was performed with a 400-500 µL, 3.5 kDa MWCO Spectra/Por microFloat-A-Lyzer kit (Spectrum Laboratories, Rancho Dominguez, CA, USA). Riboflavin 5’ Monophosphate (FMN, Sigma-Aldrich, St. Louis, MO, USA) was added at a ratio of 1.0 mM for every 25 mg mL\textsuperscript{-1} of collagen concentration (0.6 and 1.2 mM for 15 and 30 mg mL\textsuperscript{-1}, respectively). Concentrations above 30 mg mL\textsuperscript{-1} were not achievable by dialysis due to minute recoverable volumes. For higher concentration, Semed S type I collagen powder (DSM, Exton, PA, USA) was dissolved (1.0 g, 5 mg mL\textsuperscript{-1}) in
AcOH (0.05 M, 200 mL). This solution was placed in a fume hood and stirred constantly as solvent evaporated. The final measured collagen concentration was 45 mg mL$^{-1}$ ±1.5 (20 mL). FMN was added (1.8 mM, 17.1 mg) and stirred by hand. For the dye, Rose Bengal (0.5 mM, 34.38 mg) (RB, Sigma Aldrich) was dissolved in DI water (68.0 mL) and stirred for 12 hours to ensure thorough dissolution.

2.2 Fabrication Apparatus.

Fabrication was performed on silanized glass microscope slides (Lab Scientific, Highlands, NJ, USA). Wells were formed on the slides using silicone grease (Dow Corning, Midland, MI, USA). Slides were marked with ink to facilitate location of structures post-fabrication. Collagen solution was placed in the wells under a #1 glass coverslip (Fisher Scientific, Waltham, MA).

A Nikon (Melville, NY, USA) A1R MP multiphoton microscope was used for multiphoton fabrication. The excitation source was a Coherent (Santa Clara, CA, USA) Chameleon Vision II Titanium Sapphire laser tuned to 750 nm and producing 140 fs pulses at 80 MHz. A 25X, 1.1 NA Nikon objective with a 2.0 mm working distance was used. To align the bottom of a structure to the slide surface, a 1D live Z-scan was used to find the vertical position where the fluorescent signal from the FMN abruptly dropped. The center position of a structure was set using the XY-translating stage. Galvanometric scanning was used to scan the laser through the fabrication volume in XY, with a piezoelectric drive used for Z-translation. For all multi-layered structures the layer spacing in Z was 1.0 μm. The Nikon Elements control software only allows power modulation by percentages. At 750 nm, the maximum output power is approximately 2.8 W. Measurement at the stage showed that 17% of the output power is delivered to the sample.

2.3 Washing and dyeing fabricated structures.
Following fabrication exposures, the samples were immersed in AcOH, .02M or .05M, depending on the collagen solution, for 4 hours to remove un-crosslinked material. The slide was then removed from the AcOH bath and RB dye (0.5 mM, 100 μL) was added to the well chamber on the slide. The dye was left on the slide for 2 hours, then removed with 3 successive washes in Phosphate Buffered Saline solution pH 7.4 (PBS 7.4, Sigma Aldrich). Finally, the well was filled with PBS 7.4 and a new coverslip was placed for imaging.

2.4 Fluorescence Imaging

The Nikon A1R MP was used to image the structures. The laser was tuned to 800 nm with detection at 575 nm. Laser power and pixel dwell time were varied as needed for different structures to minimize pixel saturation and optimize contrast between structures and background.

2.5 Scanning Electron Microscopy

Collagen structures were prepared for SEM by electroless gold-labeling. The structures were soaked in Mono-Sulfo-N-Hydroxy-Succinimido- Nanogold® Labeling Reagent (Nanoprobes, Yaphank, NY). Three successive washes of 1 hour each were applied. Subsequently, the structures were soaked in GoldEnhance™ EM Plus (Nanoprobes) using three 30-minute washes. Gold-labeled structures were dried through an ethanol series of 25%, 50%, 75% and 100%, then 24 hours of air-drying. SEM was performed on an FEI XL30 Environmental Scanning Electron Microscope. Images were collected in secondary electron mode at 10 kV – 20 kV.

2.6 Collagen concentration comparison

To find the working range of laser power and scanning speed for fabrication, a series of 4 exposures were taken under several conditions. Laser powers used were 48 mW, 96 mW, and 144 mW (10%, 20% and 30% of maximum, respectively). These produce peak focal intensities of 5.75 kW μm⁻², 11.50 kW μm⁻², and 17.25 kW μm⁻², respectively. Pixel dwell times of 4.1 μs pixel⁻¹,
9.5 μs pixel\(^{-1}\), 23.8 μs pixel\(^{-1}\) and 52.8 μs pixel\(^{-1}\) were employed at each power and collagen concentration. The exposure was a 30 layer stack of 256 x 256 pixel images with 0.5 μm pixels, leading to a theoretical crosslinked volume 128 μm x 128 μm x 30 μm. Nikon Elements sets available pixel dwell times to correspond to a given frame rate. If the pixel size or image size are changed, the pixel dwell times are automatically changed in the software. The dwell times used for this experiment were the only available options for images with the utilized parameters, though other dwell times become available if the image parameters are changed.

2.7 Laser power and dwell time analysis

45 mg mL\(^{-1}\) collagen I solution was fabricated using the parameters described in section 2.6. The structures were imaged at a single plane through the approximate vertical center. The final image is a composite of 64 images with 1.0 μm pixels (Fig. 4.2). As multiple scan parameters were varied, these outcome measures were plotted against energy fluence to consolidate all structures to a single input measure. No relationship was determined relating energy fluence to these measures. ImageJ software was used to calculate the area, mean pixel brightness, and brightness standard deviation for each structure. Structure outlines were selected by hand.

<table>
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<tr>
<th>Power [mW]</th>
<th>Pixel Dwell [μs]</th>
<th>5 mg mL(^{-1})</th>
<th>15 mg mL(^{-1})</th>
<th>30 mg mL(^{-1})</th>
<th>45 mg mL(^{-1})</th>
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<td>48</td>
<td>4.1</td>
<td>No Result</td>
<td>No Result</td>
<td>No Result</td>
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<tr>
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<td>9.5</td>
<td>No Result</td>
<td>No Result</td>
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<td>48</td>
<td>23.1</td>
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<td>48</td>
<td>52.1</td>
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<tr>
<td>96</td>
<td>4.1</td>
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<td>96</td>
<td>9.5</td>
<td>No Result</td>
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<td>96</td>
<td>23.1</td>
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<td>96</td>
<td>52.1</td>
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<tr>
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<td>4.1</td>
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<td>Single Layer</td>
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<tr>
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<td>9.5</td>
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<td>144</td>
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<td>Single Layer</td>
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<tr>
<td>144</td>
<td>52.1</td>
<td>No Result</td>
<td>Single Layer</td>
<td>Single Layer</td>
<td>Single Layer</td>
</tr>
</tbody>
</table>

*Conditions yielding successful 3D fabrication are in bold. Results are for 0.5 μm pixel size.*

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2.8 Feature sizes

Single-line exposures were carried out with 1024 pixel line with 0.5 μm pixels, laser power 144mW and pixel dwell of 6.2 μs (Fig. 4.3). This is the shortest available pixel dwell time in this setting. 10 lines were exposed with 30 μm spacing. Lines were washed, dyed and imaged. Pixel size for the collected image was 0.14 μm. The width of the lines was calculated by direct measurement from the best achievable image using ImageJ. Measurements were taken by hand selection. 72 measurements were taken for each line. A one-way ANOVA was used to compare the widths of the 3 imaged lines.

A series of parallel rectangular bars were fabricated at increasing distances from one another. The bars were fabricated as 256 x 32 pixel exposures at 144 mW, 4.1 μs dwell time, 0.5 μm pixels with sixteen layers (Fig. 4.4). Three sets of bars were fabricated at each gap width of 1.0, 2.0, 5.0 and 10.0 μm. Following washing, dyeing and imaging, the gap was measured using ImageJ at 66 points on each pair on a representative image from the center of the stack. The average width between each pair was calculated. One-way ANOVA analysis was used to compare the widths of each set of 3 gaps intended to be the same distance.

To assess achievable aspect ratio, 128x128 pixel exposures were taken at 0.25 μm pixel$^{-1}$. Structures were spaced 32 μm apart (Fig. 4.5). Aspect ratios of 100% - 300% were fabricated by exposing stacks 32, 48, 64, 80 and 96 μm high, respectively. 3 structures were fabricated at each aspect ratio. Only qualitative assessments were made from the images of these structures to assess how well material can withstand post-processing methods.

A woodpile structure was fabricated by exposing 256 x 32 pixel, bar-shaped images with 0.5 μm pixel size at 144 mW and 4.1 μs dwell time (Fig. 4.6). Each bar had 16 z-slices. The lateral
spacing between bars was 12 μm. Bars were rotated by 90 degrees for each successive layer and the layers were overlapped in Z by 3.0 μm to ensure adhesion.

The table structure was fabricated with the “legs” exposed as 128 x 128 pixel scans, 0.25 μm pixels, 144 mW and a 2.7 μs pixel dwell (Fig. 4.7). Each was 32 μm high. These were spaced in a square pattern 96 μm apart on center, with a 5th centered inside the outer 4. The “top” was a 256 x 256 pixel scan with 0.5 μm pixels, 144 mW laser power, and 4.1 μs pixel dwell centered at the same point as the center leg. The top had 10 Z slices and was overlapped 5 μm onto the legs to ensure adhesion.

![Image](image_url)

**Fig. 4.1. Collagen structures attempted at 4 different concentrations.** (a) 5 mg mL⁻¹. A slight shadow is visible in the exposed area. (b,c) 15 mg mL⁻¹ & 30 mg mL⁻¹. Structures are visible in area exposed for fabrication at 144 mW, 4.1 μs and 9.5 μs, but are surrounded by residue and did not retain 3D shape. (d) Structures fabricated from solution of 45 mg mL⁻¹. Clear edge definitions were visible and 3D form was present. (e) Volume rendering of 45 mg mL⁻¹ structures shown in (d). Exposed volume was 128 x 128 x 30 μm for all.
3. Results

3.1 Collagen Concentration.

A series of structures 128 µm square and 30 x 1.0 µm layers tall were attempted in collagen I solutions at 4 different concentrations – 5 mg mL⁻¹, 15 mg mL⁻¹, 30 mg mL⁻¹, and 45 mg mL⁻¹ (Fig. 4.1). 3 different average laser powers, 48 mW, 96 mW, and 144 mW, and 4 pixel dwell times, 4.1 µs pixel⁻¹, 9.5 µs pixel⁻¹, 23.8 µs pixel⁻¹, and 52.8 µs pixel⁻¹, were applied to each concentration. The ability to fabricate structures from type I collagen is dependent on the concentration of the solution (Table 1). At 5 mg mL⁻¹, no structures appear on the slide in the exposed area (Fig. 4.1(a)), only a slight shadow of the exposure in some instances, which was also present on DI water controls. At 15 mg mL⁻¹ and 30 mg mL⁻¹, only a single layer of collagen adheres to the slide where fabricated structures were exposed at 144 mW (Fig. 4.1(b,c)). While crosslinking leaves behind visible structures with higher fluorescent signal, they are irregularly shaped and not robust enough to retain 3D form through the subsequent washing and dyeing procedures. At 45 mg mL⁻¹ concentration, with 96 mW and 144 mW average power, 3D structures can be formed which retain geometry at the micron scale following washes, though some warping often occurs during post-processing (Fig. 4.1(d,e)).

3.2 Laser power and dwell time dependence.

All exposures were 256 x 256 pixels, 0.5 µm pixel width, and 30 layers at 1.0 µm Z-spacing, giving an exposed volume of 128 x 128 x 30 µm. Figure 2B shows the entire range of pixel dwell time and laser power applied to determine the fabrication window. No structures were produced in the 45 mg mL⁻¹ solution at 48 mW laser power, regardless of dwell time. At 96 mW and 4.1 µs dwell time, there was also no result. At 96 mW and 9.5 µs dwell time, the structures were present but the shape was not well retained. Well-defined structures were fabricated at 96
mW with 23.8 µs dwell time and 144 mW with both 4.1 µs and 9.5 µs dwell times (Fig. 4.2(a)). Power/pixel dwell combinations of 96 mW/52.8 µs, 144 mW/23.8 µs and 144 mW/52.8 µs produced damage. ANOVA analysis showed that brightness was significantly different between all 4 successful conditions with p < 0.001.

The fluorescent intensity in dyed structures shows a complex relationship with regard to the applied laser power and pixel dwell time (Fig. 4.2(c)). This is to be expected, as multiphoton excitation is proportional to the square of the applied optical intensity and not total energy. Therefore, 144 mW laser power can produce greater reaction rates than 96 mW even when less total energy is delivered. Unfortunately, the window of working fabrication parameters was too small to attempt to define a relationship between these parameters. It can be inferred that a higher crosslink density is present as brightness increases in the structures, resulting in the retention of more dye. This is supported by the relative area of structures from each fabrication condition. The average areas of the structures show relative increases and decreases inversely with the increases and decreases in brightness (Fig. 4.2(d)). All conditions except 144 mW/9.5 µs resulted in structures swelling beyond the original area. Structures fabricated at 144 mW/9.5 µs contracted slightly, though they were the closest to the exposure area.
Fig 4.2. Fluorescence and structure size compared to fabrication conditions. Exposures were 256 x 256 pixels, 0.5 µm pixel width, and 30 layers at 1.0 µm Z-spacing. (a) Structures fabricated from 45 mg mL⁻¹ solution. Highlighted structure was damaged during processing for imaging and was omitted from calculations. Average laser power and pixel dwell time are indicated for every exposure condition used, even where no structures were formed. Energy fluence of each series with successful fabrication is also indicated. (b) Diagram depicting conditions for all exposures performed. (c) Average fluorescence for structures at each fabrication condition vs. optical energy of fabrication. (d) Average area of structures at each fabrication condition. Vertical line indicates area of initial exposure, 16,384 µm². Increases in fluorescence correspond to decreases in area, indicating both measures are related to collagen crosslink density.

3.3 Feature Sizes.

Lines were fabricated by exposing a single-pass 1024 pixel, 0.5 µm pixel⁻¹ linear scan at 144 mW and 6.2 µs dwell. Collecting images of the lines proved especially difficult as there was a very rapid photo-bleaching effect and the slide was not level enough to permit single-pane
imaging of an entire line. Measurements were taken from a representative image with pixel resolution of 0.14 µm (Fig. 4.3). Line widths of ~1 µm were achieved. Significant differences in width were present between all three measured lines by student’s t-test comparison of each pair (n = 72) (Fig. 4.3(a)). Slight differences in line width are likely due to variation in focal distance relative to the slide surface for each iteration, as a plan-corrected objective was not used. All measured lines were intact with no gaps, but were wavy in appearance after washing. SEM images were taken, but measurement was done by fluorescence to allow relevant feature sizes to be determined using aqueous conditions under which the structures are most likely to be used.

![Fig. 4.3. Single-exposure lines. (a) Line width measured from fluorescent image. Brackets indicate the significance level between average widths of pairs of lines as measured by Student’s T-test. (b) Scanning electron micrograph of single exposure lines. Note that these are from a different sample than (a). (c) Higher magnification of boxed area in (b). Fluorescent images were used for measurement as dehydration and gold labeling potentially altered final width of lines. Line exposures were done at 144 mW average power, 1024 pixels long with 0.5 µm pixels. Pixel dwell time was 6.2 µs. Exact pixel dwell times from volume exposures could not be replicated in the Nikon Elements software.](image)

Pairs of bars were fabricated at increasing distances from one another to determine minimum achievable negative space. The bars were fabricated as 256 x 32 pixel scans at 0.5 µm pixel⁻¹. 16 vertical layers were used at 1.0 µm Z-spacing with 4.1 µs pixel dwell and 144 mW. Spacing was set at 1.0, 2.0, 5.0 and 10.0 µm. All pairs of bars showed clear gaps with no discernible connection between the two (Fig. 4.4). Mean gap spacing between structures as small as 1.37 µm ± 0.49 µm was achieved. The variation between gaps is likely due more to limitations of the positioning apparatus than optical control of crosslinking, as significant differences between structures with the same prescribed gap spacing were present in every group. Even the narrowest
gaps were present throughout the depth of structures 16 µm tall, the only exception the center structure of the 5.0 µm gap series where an air bubble was present during fabrication.

Squares with 32 µm base width were used to assess the achievable vertical aspect ratio for this technique. These structures were exposed as 128 x 128 pixels at 0.25 µm pixel⁻¹. Laser power was 144 mW and pixel dwell was 2.7 µs. 1.0 µm Z-spacing was used. Structures with aspect ratios as high as 250% were fabricated and recovered through washing (Fig. 4.5). In these structures, warping was present after processing which likely stems from the removal of the coverslip. Exact exposure conditions cannot be scaled up or down because changing image parameters changes available pixel dwell times in the Nikon software.

3.4 3D Geometry

Complex geometric shapes were fabricated and retained their shape following washing and dyeing. Multilayered 3D woodpile structures with connected pores were created. Each individual member was exposed as a 256 x 32 pixel scan, 0.5 µm pixel⁻¹, 16 vertical layers at 1.0 µm Z-spacing, 4.1 µs pixel dwell and 144 mW. Pore sizes of 12 µm were retained in (Fig. 4.6). This was the smallest size attempted due to limitations in the software affecting the size and placement of exposures – structures with smaller pores could not be aligned orthogonally in successive layers. Given that 1-2 µm gaps were demonstrated in Fig. 4.4, much smaller pores can likely be formed as these structures were fabricated using the same exposure conditions.
Fig. 4.4. Measured gaps between parallel bar-shaped structures. Bars were fabricated as 256 x 32 pixel scans at 0.5 μm pixel width. 16 vertical layers were used at 1.0 μm Z-spacing with 4.1 μs pixel dwell and 144 mW. (a) Average width of gaps shown ±SD. The prescribed gap width is shown at left for comparison. Significant differences at a level of at least p < 0.05 were present between all 3 structures at every gap. Gaps were clear in every structure with no apparent connections between the parallel bars, except in the center 5.0 μm structure where an air bubble was present during fabrication. (b,c) Volume renderings of the bars generated from the multiphoton image stack. Pair shown in (b) marked with * in (a). (d) SEM showing structures fabricated under the same conditions but not used for measurement.
Fig. 4.5 Assessment of achievable aspect ratio. Structures were 128 x 128 pixels at 0.25 µm pixel width (32 µm). Laser power was 144 mW and pixel dwell was 2.7 µs. 1.0 µm Z-spacing was used for all. (a) Aspect ratio as high as 250% of base width was achieved. The base of 1 structure attempted at 300% remained on the slide, but all 3 were lost during the processing steps. (b) Alternate view.

A table-like structure was fabricated comprising a 128 µm² x 10 µm thick membrane supported by 5 concentrically spaced, cubic pillars 32 µm on each side (Fig. 4.7). The top membrane structure was fabricated as a 256 square pixel image (0.5 µm/pixel) at 4.1 µs/pixel dwell time. The pillars were 128 square pixels (0.25 µm/pixel) at 2.7 µs/pixel dwell time. 144 mW and 1.0 µm Z-spacing was used for both. Some warping was present in the structure from the subsequent washing steps, giving it a slight lean. Aside from the warping, the entire structure appears to have contracted somewhat when compared to a 3D isosurface of the original exposure geometry (Fig. 4.7(a,c)). This is more pronounced in the lower pillars than in the upper membrane.

4. Discussion

4.1 Collagen Composition

It is worth noting that the collagen structures formed from MPC will not have the same molecular organization as collagen fibrils formed either in vivo or in vitro by the pH-induced gelation of acid-soluble collagen. Collagen crosslinked by MPC will align more or less randomly, whereas the molecules within a collagen fibril align in a periodic pattern along the axis of the
It is currently unclear how this may affect cells being cultured on a scaffold of this material. In similar studies with modified gelatin as a substrate, cells adhered well to the scaffold and differentiated into desired lineages. However, in these studies the scaffolds were only required to provide a support structure for the cells. Further studies will be needed to determine how well this photo-crosslinked collagen can impart spatial and mechanical cues to cells.

**Fig. 4.6. Porous Woodpile structure.** Each individual member fabricated as 256 x 32 pixel scan, 0.5 µm pixel width, 16 vertical layers at 1.0 µm Z-spacing and 4.1 µs pixel dwell and 144 mW. (a) The structure in red (top) is a volume rendering generated from the mutiphoton image stack of the original structure. In green (bottom), an isosurface representation of the geometry as designed. (b) Light microscope image of woodpile structure. (c) Side view of the structure. Some warping of the structure is evident, likely from washing and dyeing.

4.2 *Image-based characterization.*

Characterization of the crosslinked collagen structures by means of imaging proved to be difficult. Scanning electron microscopy (SEM) was performed for qualitative imaging, but structures needed to be labeled with gold to survive the drying process. At 45 mg mL⁻¹, the collagen solution is still only 5% collagen by mass. This was not enough to leave a 3D structure after drying for SEM without the gold label. Only small, amorphous patches of collagen were seen on the slide. While collagen hydrogels can be dried, imaged and quantitatively measured well with SEM, their fibrillar composition likely imparts both better imaging contrast and greater mechanical
rigidity during drying. The gold labeling imparted enough solid mass to the structures to retain form through the drying process, but altered the original geometry enough that measurements taken from these images would be unreliable.

**Fig. 4.7.** (a-d) various views of table structure consisting of 10 μm-thick membrane on 32 μm cubic “legs.” Membrane top was 256 x 256 pixels, 0.5 μm pixel width, and 10 Z-layers. Legs were 128 x 128 pixels at 0.25 μm pixel width. Laser power was 144 mW and pixel dwell was 2.7 μs. 1.0 μm Z-spacing was used for both. The structure shown in red is a volume rendered from the multiphoton stack, the green is an isosurface of the original fabrication geometry. Some warping and shrinking relative to the original geometry is evident. (e) SEM image of a table structure with dimensions exactly ½ of the structure in (a-d) (Z-spacing and pixel dwell were the same, pixel count was reduced by ½). Features were much better preserved at this scale during preparation for SEM, though overall shrinking was still evident.

4.3 Comparison to earlier techniques.

The feature sizes achieved with multiphoton fabrication of collagen I in this study are comparable to those reported by others for BSA. Sub-micron resolution as low as 200 nm was demonstrated for faint 2D BSA lines, but in 3D freeform structures the smallest features were 800 nm. A higher NA objective capable of a smaller focal volume was also used in that study than was available for our work, which may have contributed to slightly smaller achievable features. The lack of plan-corrected optics made ascending voxel assays impractical, as the constant variation of
the focal position relative to the slide which comes from beam scanning would hinder meaningful interpretation. The ambiguity in observed line width, ~200nm, in our experiments is likely of little practical consequence to the application of this technique for cellular scaffolds.

Overall, the capabilities for MPC of type I collagen reported here are on par with those reported for BSA. For tissue engineering, the aim is to replicate, as closely as possible, a native ECM to allow differentiation of the desired tissue type. While BSA can support cell survival and growth, the development of a technique which allows type I collagen to be used with similar resolution provides a potential scaffold material which is already inherently part of the native ECM and provides more realistic structural cues to cells.

The speed of this technique is of importance as the ultimate goal of using it for the creation of engineered tissue constructs will require that something of an implantable size be able to be fabricated in reasonable time. In the fastest settings under which crosslinking was achieved, a 16,384 µm² area can be crosslinked in 268.7 ms. Expanding this to a cubic millimeter, using 1.0 µm layers, would give a fabrication time of about 5 hours/mm³. The fastest reported crosslinking method of a protein-based biomaterial by MPC is about 5-fold faster. This was achieved using methacrylamide-modified gelatin with a commercial initiator, Irgacure 2959. Using unmodified collagen and FMN, the reaction speed cannot be increased significantly. To speed this process up, use of a lower NA objective would allow thicker Z-sections. Parallel processing using multiple beams would also allow for reduction in fabrication time while allowing retention of the same resolution. This latter method has been used previously for other materials.

4.4 Significance and Future Implications.

This work is the first to demonstrate a fabrication approach allowing full 3D control of micron-scale geometry in type I collagen. The lines and gaps fabricated demonstrated that the
resolution of this method is on the cellular scale. While the smallest pore structures fabricated were 12 μm, given that 1-2 μm gaps were achieved in section 2.3, much smaller pores can likely be formed. Previously demonstrated methods which modify scaffolds selectively with various bio-active ligands mean that this technique has the potential to be used in the making of highly heterogeneous cell-specific micro-environments.\textsuperscript{13,58,108} By combining these two capabilities, MPC scaffold fabrication and ligand patterning, greater tissue-type specificity may be achieved for the biological cues imparted to cells cultured in engineered tissue constructs. Future studies will demonstrate methods which allow these type I collagen microstructures to be selectively modified with functional signaling molecules.

Moving forward, a thorough understanding of the relationship between laser exposure parameters, crosslinking density and construct elasticity will lead to the ability to locally tune the mechanical properties of the collagen scaffold. In combination with the ability to use multiphoton methods to 3D print scaffolds and selectively pattern bioactive ligands, this will allow for the contiguous fabrication of structures with varying mechanical and biochemical properties, providing a means to more readily recreate cellular niches within tissues and interfaces between multiple tissue types.

5. Conclusion

The results of this work demonstrate that unmodified type I collagen can be fabricated in three dimensions with micron-scale resolution. In addition, the described fabrication method uses only materials previously demonstrated to have high biocompatibility. This method extends the use of 3D free-form fabrication to one of the most commonly used biomaterials for tissue engineering scaffolds. This will allow tissue engineers the ability to directly dictate the geometry of a scaffold at the cellular scale.
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Chapter 5

VEGF patterning of 3D-printed type I collagen cell culture scaffolds

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Abstract

Spatial definition of the chemical and mechanical cues in cell scaffolds are important factors influencing the fate of tissue development. In native tissues, these spatial aspects are controlled by cells working in concert to create a complex, heterogeneous environment that defines the tissue’s functional units. Though much has been achieved through manipulating cellular genetics, soluble growth factors in culture media, and the macro-scale tuning of scaffold features, laboratory methods are still unable to recapitulate the environment of structural and biochemical factors present in native tissues. Multiphoton crosslinking (MPC) is a powerful tool for addressing these shortcomings. MPC can be applied to crosslink soluble proteins with single-micron resolution. Recently, we have applied this method to produce 3D structures from type I collagen with fully definable, cellular-scale features. One of the primary needs for engineering tissues of any type is the establishment of a vascular network. In the current study, methods have been developed to allow type I collagen structures to be patterned in 3D with vascular endothelial growth factor (VEGF). This paper describes the development of these methods and initial studies examining the effects of the VEGF-patterned collagen structures on human umbilical vein endothelial cells (HUVECs) in culture.

1. Introduction

Multiphoton crosslinking (MPC) is a microscale additive manufacturing, or 3D printing, method which employs the nonlinear effect of multiphoton absorption by a photo-reactive species to limit crosslinking to small volume at the focus of an objective. Translating this focus through a solution, either by raster scanning of the input beam or translation of mechanical stage, allows for full 3D control of the crosslinked geometry. Unexposed material can then be washed away using the appropriate solvent. The amino acids tyrosine, tryptophan, cysteine,
methionine, and histidine possess inherent reactivity, which allows them to crosslink when exposed to optical excitation in the presence of an appropriate photosensitizer. Flavin mononucleotide (FMN) is a photosensitizer with broad-spectrum activity and excellent biocompatibility.

We have recently developed methods to use MPC for the fabrication of complex 3D structures made of type I collagen using FMN as a sensitizer. Type I collagen is a widely used scaffold material for tissue engineering. It is also nearly ubiquitous biologically, being found in nearly every mammalian tissue type. However, a 3D structure is only part of what is needed to create a successful scaffold material that can guide the development or regeneration of the complex form and function of a tissue. Cells in functional tissues are also presented with a dynamic and heterogeneous environment of biochemical cues from soluble signaling factors. Replication of this environment in three dimensions is a difficult task, but the same principles employed to create collagen scaffolds through MPC can be applied. The inherent reactivity of multiple amino acid residues means that nearly all proteins are able to be crosslinked using MPC.

One of the fundamental challenges for the engineering of any tissue type is the establishment of a vascular supply that can deliver oxygen and nutrients and remove cellular waste products throughout the entire volume. Furthermore, many tissue types, such as bone, kidney and liver, have a very specific microscale vascular arrangement required to establish functional units within the tissue. The ability to create signaling patterns within a scaffold that can guide the spatial development of these functional vascular geometries, as well as potentially many other tissue structures, is an important technological challenge for tissue engineering and regenerative medicine.
Vascular endothelial growth factor (VEGF) is a platelet-derived growth factor (PDGF)-family signaling protein which has the capacity to induce angiogenesis and vasculogenesis in cell and tissue culture.\textsuperscript{35} When VEGF is included as a soluble factor in culture media, it has been shown to induce the formation of tubular networks in endothelial cells.\textsuperscript{36,38} Additionally, endothelial cells cultured on immobilized patterns of VEGF will align to the location of the growth factor.\textsuperscript{81} Literature shows that a wide range of VEGF concentrations have been used to induce angiogenic and vasculogenic behavior in endothelial cells.\textsuperscript{37,39}

For these reasons, VEGF is an ideal soluble signaling factor to use with MPC for the patterning of culture scaffolds to spatially control the formation of vascular networks. When using type I collagen as a scaffold material, MPC can also be used to control the geometry of the scaffold at the cellular scale, allowing mechanical cues to play a part in guiding angiogenesis and vasculogenesis.\textsuperscript{23,80} Multiple methods have been shown to pattern VEGF successfully for the ability control the geometry of endothelial cell networks in culture.\textsuperscript{37,81} While these are useful for many studies in cell behavior and biochemical function, the engineering of a tissue construct substantial enough to be a viable implant will require utilization of material which is not only biocompatible but which, ideally, is also a native, resorbable ECM component with inherent cell guidance capabilities. However, VEGF also has a natural affinity to type I collagen that will necessitate the development of washing conditions to adequately remove protein from unexposed regions of the patterned scaffold.

Using the ultrafast, pulsed lasers needed to induce multiphoton absorption phenomena provides a relatively narrow fabrication window between the threshold crosslinking power and the threshold for damage.\textsuperscript{46} It will be necessary to determine a set of parameters within this window that produce patterns of the desired soluble factor within the required concentration
range. Multiphoton-excited chemistry is nonlinear and the reactions between protein molecules involve significant steric considerations.\textsuperscript{109} Therefore, direct measurement of the deposited protein concentration is a more practical method for determining the relationships between fabrication parameters and protein deposition than trying to do so analytically.

It has been shown that the concentration of a fluorescent probe is linearly proportional to brightness measured in a fluorescent image within the range between dark and bright level saturations.\textsuperscript{86} This provides a means for assessing the concentration of patterned molecules in three dimensions in a non-destructive manner, which will allow direct measurement of the concentration of protein deposited onto type I collagen structures.

In this study, we describe methods to perform patterning of soluble factors onto 3D collagen scaffolds fabricated by MPC and assess the effects of exposure parameters on the concentration of proteins being deposited. VEGF is the signaling factor intended for use in this series of studies. However, as the method is generally applicable to many soluble proteins, we demonstrate many of the methods using bovine serum albumin (BSA), as well. We have also performed initial studies examining the effects of the VEGF-patterned collagen structures on human umbilical vein endothelial cells (HUVECs) in culture. This paper describes a preliminary set of experiments assessing the effectiveness of MPC for the functional structural and biochemical spatial fabrication and implementation of cell culture scaffolds.

2. Materials and Methods

2.1 Preparation of materials for MPC

The collagen solution for fabrication was prepared using Semed S type I collagen powder (DSM, Exton, PA, USA). The lyophilized powder was dissolved at 5 mg mL\textsuperscript{-1} in Acetic Acid (0.05 M, Sigma-Aldrich, St. Louis, MO). This solution was placed in a fume hood and stirred
constantly as solvent evaporated until the volume was reduced by a factor of 12 for a final collagen I concentration of 60 mg mL\(^{-1}\) (± 1.8 mg mL\(^{-1}\)). Flavin mononucleotide (FMN) photosensitizer was added and stirred into the collagen solution by hand (8.0 mM, Sigma-Aldrich).

Biotinylated vascular endothelial growth factor, recombinant human isoform 165, (bVEGF, G&P Biosciences, Santa Clara, CA) was dissolved in phosphate buffered saline (PBS, Sigma-Aldrich), pH 7.4, also containing 4.0 mM FMN. The bVEGF solution was separated into 200 µL aliquots and frozen at -20°C until needed (0.01 mg mL\(^{-1}\)). Biotinylated bovine serum albumin (bBSA, Thermo Scientific, Waltham, MA) was dissolved in PBS with 4.0 mM FMN and stored at 4°C (2.0 mg mL\(^{-1}\)). Desired working dilutions were prepared as needed. Streptavidin conjugated with AlexFluor® 594 (SA-AF594, Jackson ImmunoResearch, West Grove, PA) was dissolved in PBS and stored in concentrated form at 4°C until needed (1.6 mg mL\(^{-1}\)), then dissolved to the desired working concentration at the time of use (0.0001 – 0.1 mg mL\(^{-1}\)).

2.2 MPC fabrication system

All MPC fabrication was performed on a Nikon (Melville, NY, USA) A1R MP multiphoton microscope. The excitation source was a Coherent (Santa Clara, CA, USA) Chameleon Vision II Titanium Sapphire laser producing 140 fs pulses at 80 MHz. A 25X, 1.1 NA Nikon objective with a 2.0 mm working distance was used.

All collagen structures were fabricated on silanized glass microscope slides (Lab Scientific, Highlands, NJ, USA). Slides were marked with fluorescent ink to facilitate location of structures post-fabrication. Collagen solution was placed under a #1 glass coverslip (Fisher Scientific, Waltham, MA). 1/32” thick, 3 mm diameter silicon rubber spacers (McMaster-Carr,
Aurora, OH) were used to separate the slide from the coverslip while allowing solvent to reach the collagen solution without removing the coverslip post-fabrication.

To align crosslinked structures or patterns in the Z-plane, a 1D live Z-scan was used to find the vertical position where the fluorescent signal from the FMN in the protein solutions abruptly dropped at the slide surface. The desired axial position or range was then set from this zero plane. The center position of a fabrication region was set using the XY-translating stage. Galvanometric scanning was used to scan the laser through the fabrication volume in XY, with a piezoelectric drive used for Z-translation. For all multi-layered structures the layer spacing in Z was 1.0 μm. To avoid exposing fabricated structures to any unnecessary irradiation, the laser was used to create a mark in the fluorescent ink on the slide. The distance from this mark to the location of fabricated structures was recorded, allowing navigation back to the structures without directly imaging them by first finding the mark in the ink.

2.3 Fabrication of 3D collagen structures

The method used for MPC of 3D collagen structures has been described in detail previously. For this study, all collagen structures were fabricated at an average laser power of 144 mW, emission wavelength of 750 nm, and 4.1 μs pixel dwell. The pixel size was set to 0.5 μm and the layer spacing in the Z-direction was 1.0 μm. Multiple structure geometries were used for the various experiments described, but these exposure parameters were kept constant.

2.4 Imaging and image processing

Images were collected using the Nikon A1R MP multiphoton system also used for fabrication of structures. All images were collected as single- or multi-channel, 12-bit grayscale images. Visualization and image processing were performed using ImageJ. Low-contrast images were occasionally auto-scaled by the Nikon software to improve visualization. This does not
affect the image processing as the original gray-scale values are retained, but the images
produced may appear brighter or darker as the high or low saturation values are shifted. The
3DViewer plugin for ImageJ was employed to visualize and process image stacks.

2.5 Optimization of washing and labeling conditions of protein-patterned structures

Collagen structures were fabricated using two geometries to assess ideal conditions for
removing un-crosslinked soluble protein following patterning and labeling with SA-AF594 (Fig.
5.1). Optimization was performed using bBSA as the soluble patterned factor. The first geometry
was a series of parallel bars 16 µm x 16 µm x 128 µm with a 16 µm gap in between. 5 single-
plane, 16 µm x 16 µm exposures with a 16 µm gap were used to selectively modify these
structures with bBSA. These patterning exposures were performed at 144 mW with 4.1 µs pixel
dwell and 0.5 µm pixels. The second geometry was a square structure 128 µm x 128 µm x 16
µm. 32 µm x 32 µm single-plane exposures were used to crosslink a checkerboard pattern of
bBSA on these structures at 144 mW with 0.25 µm pixels and 2.7 µs pixel dwell time.

Three variables in bBSA dissolution and wash condition were assayed to determine
optimal washing procedure to expose the crosslinked protein pattern – inclusion of Tween 20
(Sigma-Aldrich) in the protein solution during patterning, duration of post-exposure wash in
PBS/Tween 20 (24h, 48h, and 72h), and concentration of Tween 20 for washing (0.5%, 1.0%
and 2.0%). Optimization of labeling using 0.04 mg mL⁻¹ SA-AF594 was performed by washing
in PBS/Tween 20 for 1h, 3h, and 6h, then hybridizing bBSA-patterned structures with SA-
AF594 followed by another 24h washing in PBS/Tween 20. All structures were then imaged
using the Nikon A1R MP using identical settings (780 nm, 38.4 mW, 2.2 µs pixel dwell, and
0.34 µm pixel⁻¹). Collagen structures with no bBSA added were also washed in SA-AF594 to
correct for the direct adhesion of the SA-AF594 to the collagen.
2.6 Quantification of soluble protein patterning on collagen structures

Quantification of bBSA and bVEGF concentrations following patterning onto collagen structures was performed using multiphoton fluorescence imaging in order to spatially assess concentrations without destroying the structures. First, serial dilutions of SA-AF594 were prepared in PBS (0.0001 – 2.0 mg mL\(^{-1}\)). Three different imaging settings were created using the A1R MP microscope which allowed imaging of a series of concentrations from the lowest detectable level to near full saturation level. These settings were a) 22 mW laser power, 75 HV NDD gain, b) 25 mW and 90 HV NDD gain, and c) 8 mW and 110 HV NDD gain. For each setting, the wavelength was 780nm, the pixel dwell time was 4.1 µs and the pixel size was 0.34 µm. Each dilution was imaged 10 times in non-overlapping regions to avoid any double exposures that could lead to photobleaching. A linear model was fitted to these data relating concentration of SA-AF594 to measured fluorescence under a given condition.

Once these relationships were determined for soluble SA-AF594, they were applied to bBSA and bVEGF labeled with SA-AF594. The molar ratio of biotin/protein was used to calculate the amount of target protein present when a given concentration of SA-AF594 was measured. To relate exposure conditions of average laser power and pixel dwell time (effectively, scan speed) on the amount of bBSA and bVEGF deposited, a fabrication series was performed which allowed the entire working range of these factors to be employed and imaged under identical conditions.

Series of sixteen 3D collagen structures 128 µm x 128 µm x 32 µm were fabricated. These were then patterned with bBSA or bVEGF with exposures of 128 µm x 16 µm spaced 16 µm in the Y direction, using 0.5 µm pixels. These exposures were in a Z-plane 24 µm above the slide surface. Each individual structure was exposed at a single laser power from 9.6 mW to 144
mW at 9.6 mW intervals (2% to 30%, at 2% intervals, of maximum available laser power). Within each structure, the pixel dwell was varied for each individual exposure. For the bVEGF structures, these were 4.1 µs, 9.5 µs and 23.8 µs. For the bBSA structures, the previous three dwell times were used as well as 52.8 µs (Fig. 5.4). Following these exposures, all structures were washed, labeled with SA-AF594 and imaged using identical conditions. Imaging was performed such that each structure was exposed only once to minimize any effect from photobleaching. The previously determined relationship between fluorescence and SA-AF594 concentration was used to determine the concentration of bBSA and bVEGF patterned onto the structures, as well as the background amount remaining in unexposed areas.

2.7 HUVEC culture on VEGF-patterned 3D collagen structures

Collagen structures for cell culture were woodpile-type scaffolds with a fully connected pore network. Porosity was 50% by volume (Fig. 5.8). The woodpile structure consisted of beams 32 µm x 32 µm x 512 µm spaced 32 µm apart. They consisted of 4 layers, with each subsequent layer perpendicular to the layer below. Interconnected VEGF patterns were created by exposing the walls of 4 vertical and 4 horizontal channels within the woodpile structure. Exposure conditions were 86.4 mW average laser power and 4.1 µs pixel dwell. Scans were designed to overlap the walls of each channel by 5 µm in each direction (Fig. 5.7). Three experimental conditions were employed to determine the effect of VEGF patterning on the localization of the HUVECs and the induction of tube formation – structures with an MPC pattern of VEGF, structures soaked in the VEGF solution used for MPC but not exposed, and control structures with no VEGF.

HUVECs were cultured in Media 200 with low-serum growth supplement and 1% streptomycin/amphotericin (Life Technologies, Carlsbad, CA). Media was changed every 48
hours and cells were passaged every 6-8 days as confluence was reached. For visualization, cells were labeled with CellTracker® Blue CMF₂HC (Life Technologies) at 25 µM.

To culture cells on the MPC collagen structures, wells were created from high-vacuum grease on the slides holding the structures to contain cell media. Structures were sterilized by being placed into an open dish containing sterile PBS and then placed under UV light for 3 hours. Cells were applied to the structures at concentrations of 1x10⁶ and 1x10⁵ cells mL⁻¹. Representative structures were imaged using the A1R MP prior to addition of cells. Once cells were added, structures were imaged within the first 24 hours (day 0), then at days 3 and 7 using the same settings. The structures and cells were imaged using single-photon confocal imaging with the FMN in the collagen structure visualized at 525nm and the CellTracker® Blue visualized at 450 nm. Light microscope images were taken daily. For confocal imaging, coverslips were added to the vacuum grease well and removed immediately afterward.

Figure 5.1 - Results of varied hybridization time of Streptavidin-AlexaFluor® 594 onto type I collagen structures patterned with soluble biotinylated BSA. (a-c) 1, 3, and 6 hours hybridization time, respectively. Differences in brightness of patterns in these images are due to auto-scaling by the imaging software and are not necessarily indicative of the true brightness value. No significant differences in mean brightness of patterned areas was found between the three hybridization times, indicating that available biotin tags were saturated by 1 hour. The presence of agglomerates increases noticeably with hybridization time, as did the brightness in the non-patterned areas.
3. Results

3.1 Optimized washing and labeling conditions

3D MPC-fabricated collagen structures were patterned with bBSA, also by MPC, to determine the wash conditions that would produce the clearest distinction between the patterned areas and protein retained in the unexposed region. Labeling of the bBSA using SA-AF594 was used to visualize the patterns. For these washing experiments, the concentration of patterned bBSA was not determined, the measured gray values of patterned and non-patterned regions were directly compared for contrast.

Structures washed without Tween20 showed reversal of intended pattern fluorescence, with exposed areas being darker than unexposed areas (data not shown). No significant differences were found between structures at any of the employed Tween20 concentrations or wash times, so the shortest time/lowest concentration combination 1h and 0.5% Tween20 was selected for simplicity for future experiments. For the determination of SA-AF594 hybridization time, the contrast decreased as hybridization time increased and the variance in fluorescence in the patterned regions increased (Fig. 5.1). The mean fluorescence did not change significantly, so the shortest hybridization time was selected for further work as the quantification of protein would not be affected (Fig. 5.1(a)). In the control structures with no bBSA washed in 0.04 mg mL$^{-1}$ SA-AF594, the structures were not distinguishable from background, indicating very low direct retention of the SA-AF594 by the collagen material.

Working out the labeling and washing conditions required significant trial and error. Initially, labeling was only attempted at the surface, assuming the collagen structures would be too densely crosslinked to allow soluble factor permeation. However, after aligning the laser to the surface proved exceedingly difficult, as the structures naturally warp somewhat from the
original, exposed geometry, it was found that both factors permeated quite readily throughout the volume of the crosslinked collagen and patterning mid-substance was fully feasible.

**Figure 5.2**—Measured fluorescence from soluble Streptavidin-AlexaFluor® 594 conjugate through a series of concentrations. The dashed purple line indicates the lowest dark value for image detection. The two lowest concentrations (green points) were not distinguishable from the dark value of the imaging detectors. Red points represent ±SD at each concentration.
Figure 5.3 – Results of quantitative patterning of bBSA onto type I collagen structures. (a) Deposited concentration of bBSA measured from fluorescent images of hybridized SA-AF594. Variation was observed in the amount which passively bound to unexposed background portions of structures. This plot shows the deposited concentration normalized to background. (b) Plot of the maximum measured concentration of bBSA deposited from each starting concentration. (c) Plot of the maximum measured concentration of bBSA deposited from each starting concentration normalized to the background, giving the maximum contrast achievable at each concentration.
Figure 5.4 – Quantitative patterning of bBSA onto type I collagen structures using 1.0 mg mL$^{-1}$ protein. (a) 2-photon composite image of all patterned structures. The power indicated below each structure is the power with which all patterned areas on that structure were exposed. The pixel dwell times used for exposure increased from top to bottom on each structure. 4.1 µs, 9.5 µs, 23.8 µs, 52.8 µs were employed. (b) Plot of the measured concentration of bBSA deposited at each exposed area by average laser power and pixel dwell time.
3.2 Quantification of soluble protein patterning on collagen structures

Of the three imaging settings employed, the best overlap between the functional range of the imaging and the concentration of protein on the structures was found at 22 mW laser power and 75 HV NDD gain. This configuration produced the following function relating concentration of SA-AF594 to measured fluorescence in the resulting images (Fig. 5.2),

\[ F = 880,140C_{SA} - 161.19 \]  

Eq. 5.1

where \( F \) is the fluorescence gray value in the original, 12-bit images and \( C_{SA} \) is the known imaged concentration of SA-AF594 in mg mL\(^{-1}\). This relation is valid across a concentration range from 0.00040 to 0.0049 mg mL\(^{-1}\) SA-AF594. Solving for concentration, this gives

\[ C_{SA} = \frac{F - 161.19}{880140} \]  

Eq. 5.2

The mass ratio of bBSA : SA-AF594 and bVEGF : SA-AF594, respectively, were 1:7.16 and 1:7.04. The concentration of bBSA and bVEGF can then be calculated from fluorescence images using the following relations,

\[ C_{BSA} = \frac{F - 161.19}{880140} \times \left( \frac{1}{7.16} \right) \]  

Eq. 5.3

\[ C_{VEGF} = \frac{F - 161.19}{880140} \times \left( \frac{1}{7.04} \right) \]  

Eq. 5.4

Using these relations, the concentrations of bBSA and bVEGF crosslinked onto 3D collagen structures was then determined across the functional range of fabrication conditions to determine relationships with average laser power, pixel dwell time, and applied soluble protein concentration (protein conc. bBSA only, Fig. 5.3-5.6).

BSA was applied to the structures with three different starting concentrations; 1.0 mg mL\(^{-1}\), 0.1 mg mL\(^{-1}\), and 0.01 mg mL\(^{-1}\). Only the 1.0 mg mL\(^{-1}\) series showed significant differences in deposited bBSA concentration relative to varying pixel dwell times (Fig. 5.3, 5.4).
When normalized for the amount retained on the unexposed background, the deposited concentrations showed linear relationships to laser power when pixel dwell was consistent over the range of values measured (Fig. 5.3(a)). There was also a linear response of the maximum amount that was deposited at each of the three starting concentrations, both in directly measured concentration and concentration normalized for the background amount (Fig. 5.3(b,c)).

VEGF was only utilized at a soluble concentration of 0.01 mg mL$^{-1}$ (Fig. 5.5). Patterning of bVEGF onto the collagen structures produced a more clearly defined response to the laser power and pixel dwell variables at this concentration than bBSA. However, damage occurred to the collagen structures much more readily when patterning bVEGF. The processing window for VEGF was too narrow to derive a descriptive relationship between input parameters and deposited concentration, but a clear pattern was evident and the conditions producing the maximum contrast between patterning and background were able to be identified (Fig. 5.6).

### 3.3 HUVEC culture on VEGF-patterned 3D collagen structures

In order to assess whether the patterning methods described allow for the creation of a functionally significant difference in VEGF concentration between patterned and unexposed regions, HUVECs were cultured on porous 3D structures. Initially, cells were plated onto the structures at a density of 1x10$^6$ cells mL$^{-1}$ ($n = 6$) and left over night for imaging the following morning, approximately 15 hours later. These comprised 4 VEGF-patterned structures and 2 control structures with no VEGF. All 4 of these structures were gone by the time the first imaging was attempted (Fig. 5.8).
Figure 5.5 - Quantitative patterning of bVEGF onto type I collagen structures using 0.01 mg mL$^{-1}$ protein. (a) Plots showing the concentration of bVEGF measured relative to pixel dwell time for each laser power used for exposure. Pixel dwell values were 4.1 µs, 9.5 µs, and 23.8 µs. The 0 point on each plot is the concentration measured in regions of that structure that were not exposed during patterning (b) Method of applying different pixel dwell to each structure. (c) Surface plot of the measured concentration of bBSA deposited at each exposed area by average laser power and pixel dwell time to show relations to both variables simultaneously.
Figure 5.6 - Quantitative patterning of bVEGF onto type I collagen structures using 0.01 mg mL⁻¹ protein. (a) 2-photon composite image of all patterned structures. The power indicated above each structure is the power with which all patterned areas on that structure were exposed. This image clearly shows the points at which damage started to occur, resulting in loss of all fluorescence. (b) Measured concentration of patterned region at each condition normalized to background to correct for fluctuations in the background amount.
Figure 5.7 – Scaffolds with VEGF patterning for HUVEC culture. Gaps and beams are 32 µm x 32 µm x 512 µm. The VEGF pattern was applied using 86.4 mW laser power with 4.1 µs pixel dwell in the geometry shown in red. VEGF pattern was applied 5 µm into collagen I structure in all directions. (a) XY view of scaffold with VEGF pattern imposed. (b) XZ view of scaffold with VEGF pattern imposed.
In order to slow the degradation and follow the progress of the cells, cells were plated at 1x10^5 cells mL^-1 for all further experiments. Of the 4 remaining structures, 2 were again patterned with VEGF, 1 was soaked and not patterned, and 1 was left as an unmodified control (Fig. 5.9). One of the patterned structures was an incomplete remnant from early fabrication testing which had been labeled with SA-AF594 for imaging following bVEGF patterning and then kept in storage for several months. Even at the reduced cell density, where only about 15 cells initially adhered directly to each structure, the fresh patterned and soaked structures were both lost within 24 hours. However, confocal imaging was performed prior to this loss. These images showed significant degradation at only 3 hours after cell addition compared to images taken prior (Fig. 5.10). Images of cells on the slides where these structures were located showed the flattened, elongated morphology typical of HUVECs.

The control structure and the incomplete patterned structure persisted in culture for 8 and 9 days, respectively. Cells on these structures did not proliferate, nor did they form networks or adopt a typical HUVEC morphology. The collagen structures were also degraded and eventually lost. As the duration of cell culture increased, the CellTracker® began to wear off the cells. This necessitated increasing the laser power and detector gain to image the cells. In the un-patterned structure, this reached a level where the FMN in the scaffold was also visible in the blue channel, somewhat obscuring the cells in resulting images (Fig. 5.9(c)).
Figure 5.8 – Cells cultured on collagen I scaffolds at 1x10⁶ mL⁻¹. Images taken 24 hours after adding cells to scaffolds. No intact structures were present on slides. Red squares represent footprint of scaffold structures which were on the slide when cells were added. All but (B) are in original location of scaffold. (a,b) Non-patterned control structures. In B, remnant of scaffold structure was found, but not in its original location. (c,d) Slides with scaffolds that had been patterned with VEGF.

Structure remnants were found in other locations on the slide after the structures disappeared from their original location. The structures that were lost were likely not completely degraded, but simply broken down enough at their base that they were no longer attached to the slide and were lost either during media change or transport. Also, the breakdown of the scaffolds visible in images indicates that degradation of the scaffolds occurred by extracellular protease mechanism and not by endocytosis, as it occurred at locations not directly adjacent to cells in many instances.
Figure 5.9 – Volume images generated from confocal stacks of 3D cell scaffolds. (a-c) Control scaffold with no VEGF incorporated. The intense blue in C is likely not from CellTracker but the broad-spectrum fluorescence of the FMN, as intensity of CellTracker diminished over the 7 day culture, higher laser power and gain were needed to image the cells. (d-f) Scaffold that was patterned with VEGF and also labeled with SA-AF594 for earlier imaging experiments. This scaffold did not break down nearly as quickly as any other that incorporated VEGF, likely due to the application of SA-AF594 and the extended time between VEGF patterning and cell application. The long blue structures in (d) and (f) were pieces of auto-fluorescent fiber that adhered to the structure.

5. Discussion

The collagen material employed for 3D printing of structures is readily degraded by cells and does not produce significant cytotoxic effects. It is possible to increase the concentration of collagen in solution well beyond what was employed for this study. Increasing the collagen concentration may increase the time needed for cells to degrade the structures and also inhibit the permeation of soluble factors throughout the structure. This could allow for greater contrast between patterned and unexposed areas and provide better means to spatially control the effects induced by these factors.
**Figure 5.10** – **Images of scaffolds** before VEGF patterning/cell addition and 3 hours after the addition of cells. Cell density was 1x10^5 mL⁻¹. (a-d) Structure patterned with VEGF. (e-h) Structure soaked in VEGF solution, but not exposed by the laser. In both structures, significant degradation of the scaffold is seen after 3 hours of cell culture.
While the rapid degradation and loss of many of the structures prohibited much quantitative analysis of the effects of the variables employed, there are some implications that can be used for guiding future work. The reduction of cell density greatly slowed the degradation of control structures when no VEGF was present. Initially, the density of $1 \times 10^6$ was chosen because this would allow for approximately 1 cell to every axial pore in the structure, which in the context of the structure of itself would be an appropriate seeding condition. However, this led to a structure that was completely surrounded by a dense mass of cells.

It is also worth noting that there was no observed difference in the rate at which scaffolds patterned with VEGF and those merely soaked in the same VEGF solution were degraded and lost, with the exception of the partially compromised structure. It is likely that the contrast achieved through patterning was not sufficient to create a differential effect, given that the patterned portion of the structure was less than 20% of the total structure volume and no observed effects were concentrated in those regions. Increasing wash time for VEGF-patterned structures did not improve contrast, it only reduced overall amount of VEGF regardless of exposure status (data not shown).

Porosity of the scaffold structures was lower than would be considered ideal and was necessitated by the relatively low mechanical strength of the randomly-crosslinked collagen material. Increasing the concentration of the collagen in solution could also help to produce scaffolds that allow for much greater porosity by volume. As collagen concentration in solution is increased, the molecular alignment also increases.\textsuperscript{118,127} A secondary effect of increasing concentration may be that the presumably random molecular arrangement of collagen crosslinked by MPC can be better aligned to present more consistent spatial signals to cells.
The window of working exposure parameters under any given fabrication condition was too narrow to determine a reliable relationship between laser power or pixel dwell and deposited concentration. The amount deposited under a variety of conditions when concentration of protein was consistent was sufficiently reliable to predict within 100 ng mL\(^{-1}\) what the deposited concentration would be. For most applications, this would likely be a sufficient level of accuracy. A broad range of VEGF concentrations have been employed for various outcomes\(^{36,37}\) and the 100 – 400 ng mL\(^{-1}\) we observed would be at the low end of that spectrum. However, given the rate at which these scaffolds were degraded, it may be that much less is needed when the protein is covalently bound to the culture scaffold as opposed to free in solution. Other methods have also shown much better contrast when patterning VEGF and other biomolecules into hydrogels\(^{13,81,108}\). In those studies, synthetic hydrogels were used that have a much lower natural affinity for VEGF. Our intention is to use type I collagen to maintain an extracellular environment more closely resembling a native ECM. For this reason, future studies will focus on means by which the attachment of soluble VEGF to the MPC collagen I scaffolds can be prevented by modifying the solution used in patterning.

Breakdown of the collagen scaffolds by the HUVECs was not directly measured in a biochemical manner. However, Basu et al measured collagenase degradation of similar materials, which indicated that the breakdown could occur fairly rapidly, with 60 µm x 40 µm x 5 µm type II collagen gels degraded by collagenase in under 20 minutes\(^{90}\). It is unclear how this corresponds to our observations, as the collagen type and concentration were very different. It is also possible that loss of structures may have been through detachment at their base and not total degradation. For the culture of HUVECs alone, a scaffold that degrades much more slowly will be needed, as the cells will not produce a sufficient structure on their own to maintain the desired
three-dimensional form. As more complex co-culture experiments including cell types such as fibroblasts which are much more active in producing extracellular matrix proteins are attempted, careful tuning of degradation will be need to ensure that the rate of regeneration is equal.

A better method of applying cells to structures is also needed. Cells in culture surrounding the structure will not respond to the desired spatial and biochemical cues imparted by the structure. However, these cells will make up the vast majority of the cells in culture and will have significant influences on the fate of cells on the structure and the structure itself, especially when extracellular protease processes are as active as they appear to be in these studies. There are methods for selectively applying cells which may be employed to achieve this.\textsuperscript{76} Isolating cells cultured on the scaffolds from the effects of similar cells in different conditions immediately adjacent will give a much clearer picture of the influence the designed scaffold elements have on intended cellular outcomes.

6. Conclusion

The studies described in this paper demonstrate that type I collagen scaffolds fabricated using the MPC additive manufacturing process can be quantifiably patterned in three dimensions with soluble bioactive factors. Our results demonstrate that visual patterns are readily producible, but a differential great enough to spatially isolate the cellular effects of these factors is difficult to achieve given that, at most, an increase of only 3.5 times the background adhesion concentration was achieved with directed MPC patterning. The scaffold material supported cell survival with no observed cytotoxic activity and was readily degraded by extracellular digestive processes. Future work will focus on means by which the 3D patterning method facilitated by MPC can be improved to better spatially guide and define cellular activity to produce more complex, cooperative biological functions in engineered tissue constructs.
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Chapter 6

Discussion, Recommendations, and Conclusion

Discussion

This dissertation describes strategic efforts to develop and apply multiphoton crosslinking, a microscale additive manufacturing technology, for both structural and signaling proteins to create tissue scaffolds which induce and guide vasculogenesis. The first studies performed were designed to expand the capabilities of MPC to allow for fabrication of cell culture scaffolds from the ECM protein type I collagen. Further studies were carried out to establish methods to use MPC to selectively pattern these collagen I scaffolds in 3D with VEGF and BSA. Finally, the techniques developed were used to fabricate 3D cell culture scaffolds and assess their efficacy for the guidance of vasculogenesis using HUVECs. While results of these final studies were mixed, the techniques developed are still preliminary and the total of these results provide promise that further development of this technology is possible and has strong potential for achieving the goal of spatially guided vasculogenesis.

Chapters 3 and 4 - MPC Additive Manufacturing of Type I Collagen

The preliminary studies in chapter 3 and the development of MPC methods in chapter 4 have demonstrated that it is possible to use MPC as a method for additive manufacturing of unmodified type I collagen. These were the first studies to demonstrate complex 3D structures from type I collagen at a cellular length scale. Given the abundance of collagen I in the ECM of a multitude of tissues, as well as the increasing awareness that the cellular and sub-cellular scale spatial arrangements of ECM fibers have a profound impact on cell fate, this development provides a useful new tool for the fabrication of microstructures for cell culture. While this method has been demonstrated with multiple other proteins, collagen I is the largest portion of the ECM in most tissues.
The resolution capabilities for MPC of type I collagen described in chapter 4 are equivalent to those reported by other researchers for BSA.\textsuperscript{15,16} The aim in utilizing this technology for TE/RM is to imitate, as near as possible, a native extracellular environment to direct differentiation of the desired tissue physiology. BSA is fully cytocompatible and can support cell survival and growth, but the development of a technique which allows type I collagen to be used with similar resolution offers a scaffold material that is an inherent part of the native ECM and, ideally, can provide more realistic structural cues to cells.

The ability of cells to sense their environment and respond to it is still being realized and their sensitivity to signals is well beyond what we are able to replicate, even with this fabrication method. Preliminary observation suggests the 3D structures fabricated by MPC are not particularly robust, and the aspect ratio required to create self-supporting structures leads to structures which have much lower porosity, \textasciitilde50\%, than would be ideal for cell culture scaffolds. The bulk of the scaffold itself seems to inhibit much of the cell-cell signaling which is also important in tissues, particularly for endothelial cells. Increasing the collagen concentration is likely the most effective way to achieve increased stiffness and strength. To attempt to bridge this gap, further studies into the mechanical properties of this crosslinked collagen material in relation to the concentration of the starting collagen solution as well as the crosslinking parameters, should be performed. Ideally, much like the creation of gradients of chemical signaling cues, the stiffness of a scaffold could be varied spatially.

The arbitrary arrangement of collagen molecules within the MPC-fabricated structures will also need to be addressed. Native collagen fibrils are made up of individual collagen monomers which are all aligned to the axis of the fibril. While MPC can create 1 \(\mu\)m lines of collagen, the molecules within those lines will all be randomly distributed. This has been
confirmed, to an extent, by some of our unpublished studies. SHG can produce very clear images of collagen fibrils in tissues and hydrogels. However, we were unable to detect an SHG signal when imaging MPC collagen structures, even though several tissue samples produced clear signals under the same imaging settings. In addition, when the collagen solution used for MPC was increased in pH, a fibrillar network was formed. This shows that the collagen in solution had likely not degraded, and that the lack of SHG signal was most likely due to the random arrangement of collagen. The strength of signal produced in SHG imaging is directly proportional to the alignment of collagen molecules in the imaged sample. A random arrangement causes the signals to negatively interfere and effectively cancel one another out.

Other researchers have shown that the alignment of collagen I fibrils increases when the solution is polymerized by increasing the pH due to the molecular crowding within the solution.124 In this work, the concentration of collagen had been increased to 375 mg mL\(^{-1}\) using dialysis. The same group showed that directional organization of the collagen matrix is necessary for fibroblasts to in turn produce more organized matrix, providing strong motivation to further pursue this factor.127 The liquid crystallinity of soluble collagen I monomers has been well documented. This is another feature which could potentially be utilized in order to control the alignment of collagen monomers during MPC to improve coherence within the fabricated constructs.26

Dissolving collagen to higher concentrations has also been reported utilizing a solvent system consisting of a 50:50 mixture of ethanol and 20X PBS.128 We attempted to utilize this method, but found that the surface tension of this mixture was extremely low, making it very difficult to contain during fabrication processes. The high ethanol content also led to rapid solvent evaporation and formation of salt crystals from the PBS. The method described in
Chapter 4 for increasing collagen concentration by solvent evaporation is effective only to a point. The highest concentration which can be achieved by this method is about 60 mg mL\(^{-1}\). At this concentration, the solution becomes too viscous for a magnetic stir bar to spin and it will dry out irregularly. Concentrations higher than this were not employed for this study as the goal for increasing collagen concentration was primarily to achieve the mechanical strength needed to maintain a 3D structure, but moving forward other methods will be incorporated.

**Chapter 5 - VEGF patterning of 3D-printed type I collagen cell culture scaffolds**

Chapter 5 demonstrated methods by which the collagen scaffolds developed in Chapters 3 and 4 can be modified with VEGF, as well as demonstrating general methods using BSA to lay a groundwork for utilizing a multitude of other signaling factors. One of the primary difficulties with this work was that the collagen material will naturally bind a certain amount of the patterned factor without any laser exposure. This was expected, but caused multiple problems, the first being that it is impossible to create an area of the scaffold completely devoid of a functional amount of the patterned protein. Second, the range of concentrations which can be deposited is limited to the difference between this baseline amount and the saturation point of the scaffold under crosslinking conditions, or the point at which damage occurs. The narrow window of processing parameters under which VEGF and BSA were able to be deposited made it impractical to apply a model to the relationship between the deposited concentration and those parameters. In these studies, this range was only 2.5 – 3 fold at the highest level of achievable contrast. At least one similar study using VEGF has shown that this is enough of a contrast to create significant differences in endothelial cell tube formation.\(^8\) However, the intent of developing these techniques is to be able to recreate the native spatial gradients of signaling factors found in tissues, and this method currently falls short of providing that ability.
There are a multitude of potential mechanisms by which bioactive factors such as VEGF can be delivered to a scaffold. However, no laboratory method can yet match the temporospatial precision with which native cells can do this by secretory mechanisms as it is very difficult to introduce molecules on a point-by-point basis within free space.\textsuperscript{4} Cells are capable of generating and depositing the desired molecules on the spot, something we currently cannot replicate. However, laser-trapping and guidance combined with customized vesicles may be used in conjunction with MPC to offer greater control over where the desired factors are placed on a scaffold.

While there are other materials that already show much more advanced functionality for tissue scaffolds in terms of the established techniques that can be used to direct desired cellular outcomes, this material is still worth developing. As has been addressed previously, it is becoming more and more apparent that, if the goal of generating therapeutic tissue replacements in the laboratory is to be achieved, the cellular microenvironment must be replicated at a level which is largely unattainable with current technology. MPC provides, in this author’s opinion, the best means for recreating cellular and sub-cellular scale features of the ECM, not only in the geometric spatial sense, but also from the same materials. More research, then, is needed into how to utilize MPC in a way which allows these protein materials to behave as closely as possible to their \textit{in vivo} state.

**Recommendations for Future Research**

As the MPC technology developed here is fairly new, there are a wide range of possible applications which are yet to be addressed. Moving forward, work in this area will be focused on means by which the 3D patterning method facilitated by MPC can be improved to better spatially guide and define cellular activity to produce more complex, cooperative biological
functions in engineered tissue constructs. Much of this work will focus on the mechanical and chemical resilience of the material to resist premature degradation as cells develop. Implementation of means to increase the concentration of collagen I in the crosslinking solution will be a large part of this effort. Analysis of the MPC collagen I material by transmission electron microscopy can provide information about how the molecular arrangement within these scaffolds compares to native collagen fibrils. This is important information to gather for further improvement of cellular response to the material.

Greater understanding of the reaction dynamics as proteins are being crosslinked by MPC is also needed to provide better quantitative control of patterning soluble factors. Given the myriad steric effects which come into play in these reactions, computational molecular modeling is the only means to form reasonable predictive models for the rates at which these reactions can occur and the spatial limits to deposition. Similarly, development of protein solutions that include agents to prevent or reduce unwanted attachment of protein will be needed. In unpublished preliminary work, inclusion of Tween 20 detergent in the crosslinking solution actually increased the amount of protein, both VEGF and BSA, which was retained in unexposed regions. Modeling simulations may also be needed to understand these interactions and formulate solutions which can prevent them.

The time needed to fabricate scaffolds using MPC must be decreased significantly, as well. The largest structures described in chapter 5 required about three hours each to fabricate, and they were still at least an order of magnitude smaller in volume than what would be needed for implantation for in vivo studies. This is purely a limit of the reaction rate that can be achieved in the collagen solution; the mechanical portion of the system is easily capable of much higher speed. In order to preserve the ability to continue to use unmodified proteins for scaffold
fabrication and patterning, this should mostly be attempted through modifications to the systems used for MPC, beginning with the ability to use several laser beams and scan systems to fabricate multiple regions or structures simultaneously. Given the already considerable expense of systems with the current capability, a large part of this research will be studies into devices which can reduce the complexity of components to produce a simplified, purpose-built system as opposed to the adapted imaging systems largely used now. The work presented in chapter 3 lays a groundwork for these studies. Optical drives contain many of the elements needed, and the methods used to assemble and control them can be employed to develop simplified MPC systems that still retain the requisite functionality.

Improved processes for introducing cells to the MPC scaffolds are also needed. Cells which are in the culture dish surrounding the structure, but not actually on it, will not react to the spatial and biochemical cues in the structure. However, this population makes up the vast majority of the cells in culture. The fate of cells on the structure, and of the structure itself, can be largely dictated by extracellular processes, such as those which appear to be active in the chapter 5 studies. There are methods for selectively applying cells which may be employed to achieve this.\textsuperscript{77,129} Isolating cells cultured on the scaffolds from the effects of similar cells in different conditions immediately adjacent will give a much clearer picture of the influence the designed scaffold elements have on intended cellular outcomes.

A complementary area in which future research must be focused is the spatial evaluation of the structures and signals in native tissues and their influence on cell and tissue function. As multiphoton fabrication and imaging methods develop cooperatively, the means to visualize and recreate structures and patterns with sub-micron resolution will be refined to an increasingly high functional degree. However, these methods will be of little use for TE/RM without clear
knowledge of the effects of those features on desired cell and tissue developmental outcomes. A fair body of work into the quantification of spatial distributions of various factors during development exists already. In order to translate these results better into fabrication parameters for TE/RM outcomes, a systematic approach must be adopted to quantitatively analyze how these factors directly influence the desired functions of cells. This is an emerging process which is being made possible by relatively new imaging and fabrication technology, such as MPC. All told, the development of the techniques contained within the scope of this dissertation begins to open up a wide field of possibilities for micro-scale spatial control of cellular environments for TE/RM and developmental biology research.

Conclusion

This work has demonstrated that type I collagen can be fabricated in three dimensions with micron-scale resolution without any chemical modification to the protein structure. The fabrication method developed in this study uses only materials previously demonstrated to have high biocompatibility. This method expands the use of MPC microscale additive manufacturing to one of the most commonly used biomaterials for tissue engineering scaffolds.

Further, type I collagen scaffolds fabricated with MPC can be quantifiably patterned in three dimensions with soluble bioactive factors. The results presented here demonstrate that clearly discernable and reproducible, though somewhat limited, patterns are readily achievable, but the spatial dictation of signaling with a differential great enough to locally isolate cellular outcomes is difficult. MPC-fabricated collagen scaffolds supported cell survival with no observed cytotoxic activity and were readily degraded by extracellular digestive processes. Not many conclusions can yet be drawn about the suitability of scaffolds fabricated by MPC for application in the engineering of vascularized tissues. MPC collagen I scaffolds supported
HUVEC survival adequately under the employed conditions, but the fabrication methods will need to be improved in relation to achieving greater control over VEGF deposition and reducing the rate of degradation in order to be able to measure the desired cellular outcomes related to vasculogenesis.


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